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

Doctoral Thesis – set of publications – 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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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the seventh most prevalent cancer worldwide. Significant risk factors in the development of HNSCC are tobacco smoking and alcohol consumption. However, the exact impact of human papillomavirus (HPV) on the survival prognosis of patients with HNSCC, particularly those with laryngeal squamous cell carcinoma (LSCC) and hypopharyngeal squamous cell carcinoma (HPSCC), remains somewhat unclear.

This research aimed to examine the prevalence of HPV infection (HPV DNA, E6/E7 mRNA) among individuals diagnosed with oropharyngeal squamous cell carcinoma (OPSCC), HPSCC, and LSCC, and to understand the role of HPV infection in tumour formation and patient survival by evaluating the immunohistochemical (IHC) expression of tumour suppressor proteins (p16 and p53) and HPV16 E6 and E7 oncoproteins.

The first part of the research involved a retrospective study of 247 patients with confirmed OPSCC. The primary outcomes assessed in this study were overall survival (OS) and disease-specific survival (DSS), in addition to histopathological analysis. The results of the Kaplan-Meier survival analysis indicated better survival outcomes for female patients, younger individuals without unhealthy habits (smoking and alcohol abuse), those who underwent surgery and received radiotherapy, and those with lower tumour grade and disease stage. The Cox regression analysis revealed a reduced risk of early death in patients with lower tumour grade, no regional metastases (N0), and without unhealthy habits, as well as in patients who underwent surgery and received radiotherapy. Most tumours were localised in the palatine tonsils and the base of the tongue, but the localisation did not show a correlation with mean survival time or survival outcomes. Significantly lower OS and DSS rates were observed in patients with involvement of the pharyngeal wall and tonsils compared to tumours localised in the soft palate. The histological variant of the tumour did not appear to significantly impact OS and DSS, while the chosen therapeutic approaches had a significant effect on survival outcomes.

The second part of the research encompassed the IHC (p16, p53, HPV16 E6/E7 proteins) and virological (HPV DNA, E6/E7 mRNA) investigation of 106 tumour samples from patients with HNSCC (34 OPSCC, 41 LSCC, 31 HPSCC), as well as clinical assessment of these patients.

To evaluate and compare several molecular biology methods for detecting HPV in nucleic acid material obtained from formalin-fixed paraffin-embedded (FFPE) samples. assessment of the 31 FFPE tumour samples from patients with HPSCC was performed. The

two real-time PCR methods, Anyplex II HPV28 and Sacace HPV High-Risk Screen Real-TM Quant, exhibited strong agreement. A moderate positive correlation was identified between the semiquantitative results obtained from Anyplex II HPV28 and the quantitative results obtained from Sacace. Used nucleic acid extraction kits are good and reliable for extracting qualitative material for further molecular investigation. Real-time PCR methods that target smaller DNA amplicons are effective and dependable techniques for detecting HPV genetic material in FFPE samples.

Further assessment of 106 HNSCC samples revealed that HPV16 was the most prevalent high-risk (HR-) HPV type found. The prevalence of HPV16 was 26/34 (76.47%), 22/41 (53.66%), and 20/31 (64.52%) in OPSCC, LSCC, and HPSCC accordingly. HPV16 E6/E7 mRNA was detected in 15/26 (57.7%) of the OPSCC samples, 2/22 (9%) of the LSCC samples, and 0/20 of the HPSCC HPV16-positive samples. Overexpression of HPV16 E6 protein was immunohistochemically confirmed in 44/106 (41.5%) of the HNSCC samples, and overexpression of HPV16 E7 protein – in 39/106 (36.8%) of the HNSCC samples.

The presence of HPV DNA, both low-risk (LR-) and HR-HPV types, was linked to improved 5-year OS and DSS rates in patients with OPSCC and LSCC. The IHC overexpression of HPV16 E6 protein and p16 protein was associated with better survival outcomes, as observed in both univariate analysis for OPSCC and multivariate analysis for OPSCC and HPSCC. Additionally, the overexpression of p53 was linked to improved survival specifically in OPSCC.

This research has provided crucial insights into our understanding of HPV prevalence and significance in HNSCCs. However, additional studies are necessary to investigate the role of HPV infection (HR- and LR-) in non-oropharyngeal HNSCC and its prognostic value in survival of these patients. Moreover, more studies are needed to evaluate the potential use of IHC for HPV16 E6 protein expression as a prognostic marker in OPSCC and HPSCC.

Keywords: oropharynx; larynx; hypopharynx; squamous cell carcinoma; HPV; PCR; immunohistochemistry; p16; p53; E6/E7 viral oncoproteins; survival analysis.

Anotācija

Cilvēka papilomas vīrusa nozīme galvas un kakla vēžu attīstībā un prognozē

Galvas un kakla plakanšūnu karcinoma (GKPK) ir septītā visizplatītākā vēža forma pasaulē. Nozīmīgi riska faktori GKPK attīstībā ir tabakas smēķēšana un pārmērīga alkohola lietošana. Tomēr precīza cilvēka papilomas vīrusa (CPV) ietekme uz GKPK pacientu dzīvildzi, īpaši pacientiem ar balsenes plakanšūnu karcinomu (BPK) un *hypopharynx* plakanšūnu karcinomu (HPPK), joprojām nav pilnībā skaidra.

Šī pētījuma mērķis bija izpētīt CPV infekcijas prevalenci (HPV DNS, E6/E7 mRNS) pacientiem ar diagnosticētām *oropharynx* plakanšūnu karcinomu (OPPK), HPPK un BPK un saprast CPV infekcijas lomu šo audzēju attīstībā un pacientu dzīvildzē, izvērtējot audzēju supresoru proteīnu (p16 un p53) un CPV16 E6 un E7 onkoproteīnu imūnhistoķīmisko ekspresiju.

Promocija darba pirmajā daļā tika iekļauts retrospektīvs pētījums par 247 pacientiem ar histoloģiski apstiprinātu OPPK. Šajā pētījumā galvenais mērķis bija izvērtēt kopējo un slimības specifisko dzīvildzi, kā arī veikt histopatoloģisku audzēju analīzi. Kaplana-Meijera dzīvildzes analīzes rezultāti norādīja uz labākiem izdzīvošanas rezultātiem sieviešu dzimtes pacientiem, gados jaunākiem pacientiem bez kaitīgiem ieradumiem (smēķēšana un pārmērīga alkohola lietošana), tiem, kuriem bija veikta operācija un kuri saņēma staru terapiju, kā arī pacientiem ar mazāku primāro audzēju un zemāku slimības stadiju. *Cox* regresijas analīze atklāja samazinātu agrīnas nāves risku pacientiem ar zemāku T pakāpi, bez reģionālām metastāzēm (N0) un bez kaitīgiem ieradumiem, kā arī tiem, kuriem bija veikta operācija un kuri saņēma staru terapiju. Lielākā daļa pētījumā iekļauto OPPK pacientu bija ar aukslēju mandeļu vai mēles pamatnes audzējiem, taču audzēja lokalizācija nekorelēja ar vidējo izdzīvošanas laiku vai izdzīvošanas rezultātiem. Būtiski sliktāka kopējā un slimības specifiskā dzīvildze bija pacientiem ar rīkles sienas un aukslēju mandeļu vēžiem salīdzinājumā ar audzējiem, kas lokalizēti mīkstajās aukslējās. Audzēja histoloģiskais variants ievērojami neietekmēja kopējo un slimības specifisko dzīvildzi, savukārt izvēlētajiem ārstēšanas veidiem bija ievērojama ietekme uz dzīvildzi.

Promocijas darba otrajā daļā tika veikta 106 GKPK paraugu (34 OPPK, 41 BPK, 31 HPPK) imūnhistoķīmiskā (p16, p53, HPV16 E6/E7 proteīni) un molekulāri bioloģiskā (CPV DNS un E6/E7 mRNS) izmeklēšana, kā arī veikta šo pacientu klīniskā izvērtēšana un analīze.

Lai izvērtētu un salīdzinātu dažādas molekulāri bioloģiskās izmeklēšanas metodes CPV noteikšanai no parafīnā ieguldīto audu iegūtajā nukleīnskābju materiālā, tika izmeklēts 31 formalīnā fiksēts parafīnā ieguldīts HPPK paraugs. Divas reālā laika polimerāzes ķēdes

reakcijas (PĶR) metodes, *Anyplex II HPV28* un *Sacace HPV High-Risk Screen Real-TM Quant*, demonstrēja statistiski stipru rezultātu saskaņu. Tika konstatēta mērena pozitīva korelācija starp *Anyplex II HPV28* semikvantitatīvajiem rezultātiem un *Sacace* kvantitatīvajiem rezultātiem. Pielietotie nukleīnskābju ekstrakcijas komplekti ir viegli izmantojami un uzticami, lai izgūtu kvalitatīvu materiālu no parafīnā ieguldītiem audiem turpmākai molekulārai izmeklēšanai. Reālā laika PĶR testi, kas mērķēti uz īsāku DNS fragmentu noteikšanu, ir efektīvi un uzticami CPV ģenētiskā materiāla atklāšanā parafīnā ieguldīto audu paraugos.

Tālākā 106 GKPK paraugu izmeklēšana atklāja, ka CPV16 ir visizplatītākais augsta riska CPV tips šajos paraugos. CPV16 izplatība bija attiecīgi 26/34 (76,47 %), 22/41 (53,66 %) un 20/31 (64,52 %) OPPK, BPK un HPPK gadījumā. CPV16 E6/E7 mRNS tika konstatētas 15/26 (57,7 %) OPPK, 2/22 (9 %) BPK un 0/20 HPPK CPV16-pozitīvajos paraugos. CPV16 E6 proteīna pozitivitāte tika imūnhistoķīmiski apstiprināta 44/106 (41,5 %) GKPK paraugos, savukārt HPV16 E7 proteīna pozitivitāte bija novērojama 39/106 (36,8 %) GKPK paraugos.

CPV DNS klātbūtne (augsta un zema riska CPV veidi) audzējā bija saistīta ar labākiem piecu gadu kopējās un slimības specifiskās dzīvildzes rādītājiem pacientiem ar OPPK un BPK. CPV16 E6 proteīna un p16 proteīna imūnhistoķīmiskā pozitivitāte bija saistīta ar labākiem izdzīvošanas rezultātiem gan Kaplana-Meijera analīzē OPPK gadījumā, gan *Cox* analīzē OPPK un HPPK gadījumos. Turklāt p53 pozitivitāte bija saistīta ar labāku dzīvildzi tieši OPPK gadījumā.

Šis pētījums ir sniedzis svarīgu informāciju par CPV prevalenci un nozīmi GKPK gadījumā, tomēr ir nepieciešami papildu pētījumi, lai padziļināti izpētītu CPV (augsta un zema riska) lomu neorofaringeālu GKPK attīstībā un šo pacientu dzīvildzē. Papildus ir nepieciešami pētījumi, lai izvērtētu CPV16 E6 proteīna imūnhistoķīmiskās ekspresijas prognostisko vērtību, īpaši OPPK un HPPK gadījumos.

Atslēgvārdi: *oropharynx*; balsene; *hypopharynx*; plakanšūnu karcinoma; CPV; PĶR; imūnhistoķīmija; p16; p53; E6/E7 onkoproteīni; dzīvildzes analīze.

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Abbreviations used in the Thesis

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

Introduction

Squamous cell carcinoma is the most common malignant tumour in the head and neck region (Sung et al., 2021). 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 OPSCC, 98,412 new cases of LSCC, and 84,254 new cases of HPSCC were registered in 2020 (Sung et al., 2021). HNSCC is more frequently diagnosed in men, usually over the age of 50 (Miranda-Filho & Bray, 2020). The 5-year survival rate for advanced tumours is approximately 50% (Lo Nigro et al., 2017).

Significant risk factors in the development of HNSCC are tobacco smoking and alcohol consumption, both of which have a synergistic effect (Kuper et al., 2002; Hashibe et al., 2007, 2009). In cases of HNSCC caused by smoking and alcohol consumption, p53 gene mutations frequently occur, which play a role in cell cycle regulation (Carlos de Vicente et al., 2004). Mutations in the p16 tumour suppressor gene also occur, resulting in the loss of the tumour suppressor p16 (Beck et al., 2017; Schade et al., 2019; Deneka et al., 2022). p16, as a CDK inhibitor, binds to the CDK4/CDK6 complex, suppressing pRb phosphorylation. Phosphorylated pRb dissociates from the E2F transcription factor, which promotes the transcription of genes crucial for the G1 phase-to-S phase transition of the cell cycle. Disruption of p16 function leads to dysregulation of the cell cycle, resulting in uncontrolled cell proliferation (J. Li et al., 2011; Rayess et al., 2012; Kotake et al., 2015; Senga & Grose, 2021). Although there has been an overall decrease in HNSCC incidence in the past 20 years, primarily due to a decrease in the number of smokers, 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, HR-HPV types, especially HPV-16, 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 (Gillison et al., 2000; Mork et al., 2001; Ernster et al., 2007; Andrews et al., 2009; Chaturvedi et al., 2011; Gillison et al., 2015).

There are more than 220 known types of HPV (*Human Reference Clones – Hpvcenter*, n.d.). Depending on their ability to induce malignancy, HPV can be classified into high-risk and low-risk types. LR-HPV types comprise the majority of HPV types identified and are generally not associated with the development of cancer and usually cause benign warts (Egawa & Doorbar, 2017; Kombe Kombe et al., 2021). The most common LR-HPV variants in cases of HPV infection in the head and neck region are HPV-6 and -11 (Muñoz et al., 2003; de Martel

et al., 2017). On the other hand, HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 are considered high-risk due to their association with cancer. HR-HPV infection has long been recognised as a causal factor for anogenital cancers and has more recently been acknowledged as a causal factor for certain head and neck cancers. While HR-HPV infection, particularly HPV-16, is strongly linked to the development of OPSCC with HPV prevalence being as high as 70% (Dayyani et al., 2010; 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 exhibit distinct characteristics compared to HPV-negative cancers, covering various aspects such as molecular mechanisms of transformation, tumour progression, epidemiology, and most importantly, patient survival. The presence of HPV in squamous cell carcinomas has been identified as a prognostic factor for survival, particularly in HPV-associated OPSCCs, which are associated with a reduced risk of death and recurrence (Mallen-St Clair et al., 2016). Hobs et al. have determined in their study that HPV is strongly associated with tonsil cancer, moderately associated with oropharyngeal cancer, and weakly associated with oral cancer (Hobbs et al., 2006). HPV-16 seropositivity is linked to an increased 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 association between HPV status and survival outcomes has not been definitively established for other types of HNSCCs such as LSCCs and HPSCCs (Sánchez Barrueco et al., 2017; Ahmadi et al., 2018; Dahm et al., 2018; H. Wang et al., 2019; Panuganti et al., 2021; Wu et al., 2021; Burbure et al., 2021; S.-P. Yang et al., 2022; Sharkey Ochoa et al., 2022). HPV-positive head and neck cancers also display distinct molecular signatures, including degradation of wild-type p53, absence of mutations in the p53 gene, decreased expression of pRb, and subsequent increased expression of p16. These molecular differences can aid in distinguishing HPV-associated cancers, facilitating treatment adjustments and serving as prognostic markers (Mallen-St Clair et al., 2016).

The oncogenic potential of HPV relies primarily on two of its early proteins, namely E6 and E7. These viral proteins interact with crucial cell cycle regulators, known as tumour suppressors, in the infected epithelial cells, leading to uncontrolled cell proliferation. Considering the essential role of HPV oncoprotein expression in carcinogenesis and causality, their expression levels can potentially serve as prognostic markers. Some researchers propose that HPV-related head and neck cancers exhibit better prognosis due to a more vigorous and specific immune response against tumour cells expressing HPV antigens, including E6 and E7. Several studies have demonstrated that T cells derived from patients with OPSCC display

increased proliferation and synthesis of inflammatory cytokines upon recognition of HPV16 E6 and E7 oncoproteins. Moreover, T cells from patients with HPV-related head and neck cancer exhibit enhanced responses to E7 epitopes (Hoffmann et al., 2006; Wansom et al., 2010; Heusinkveld et al., 2012; Sharkey Ochoa et al., 2022).

One of the viral oncoproteins, E6, plays a crucial role in promoting the degradation of p53 through E6-associated ubiquitin ligase, leading to the disruption of cell cycle checkpoints, evasion of apoptosis, and inactivation of p21, a target of p53. This in turn, prevents cells from entering the S phase and induces cell cycle arrest in the G1 phase (Pal & Kundu, 2019; Johnson et al., 2020). In non-HPV-associated cases of head and neck cancers, mutations in the p53-encoding gene are commonly observed, resulting in the loss of p53 function or even the acquisition of functions that facilitate invasion, metastasis, and cancer cell proliferation (Nathan et al., 2022). Studies have demonstrated that patients with HNSCCs positive for HPV and lacking p53 expression (due to p53 degradation by E6) exhibit 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 E2F transcription factor and subsequent stimulation of cell cycle progression into the S phase (Boyer et al., 1996; Berezutskaya & Bagchi, 1997; Bodily & Laimins, 2011; Pal & Kundu, 2019). Another consequence of E7-mediated pRb degradation is the upregulation of p16, a potent tumour suppressor. The detection of p16 overexpression has become a molecular hallmark for identifying HPV-associated OPSCCs and has been shown to have a positive impact on patient survival in these cases. However, such an association has not been firmly established for non-oropharyngeal subsites of HNSCCs (Bishop et al., 2015; Du et al., 2019). Several studies have reported either a lack of p16 expression, even in the presence of HPV mRNA, or similar levels of p16 expression regardless of HPV status in these non-oropharyngeal HNSCCs (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). Additionally, the viral E6 and E7 genes are typically the only ones retained and expressed, indicating the crucial role played by these proteins in HPV-associated carcinogenesis (Scheffner & Whitaker, 2003).

Significant results are related to the detection of HPV DNA in tumour tissues and the determination of HPV infection markers in blood serum. 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; Weinberger et al., 2006; 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 (Gillison et al., 2000; Weinberger et al., 2006; Kumar et al., 2007, 2008). Due to the better prognosis of HPV-positive OPSCC compared to HPV-negative OPSCC, treatment de-escalation has been proposed, which essentially involves reducing the radiation and chemotherapy doses to mitigate treatment-related toxicity and long-term morbidity (Attner et al., 2012; Golusinski et al., 2021; Rosenberg & Vokes, 2021). Therefore, by determining the HPV status (and its transcriptional activity) of HNSCC, specifying the risk factors, and considering the stage of the disease, treatment de-escalation can be introduced by reducing the doses of radiation therapy and chemotherapy, as well as including surgery as a third treatment modality, which is itself a de-escalation strategy.

In Latvia, the routine practice of detecting p16 in OPSCC was introduced only recently. However, the determination of HPV status is not a standard procedure for patients with HNSCC, even though standardised methods and procedures are widely implemented in developed countries. There are also no unified guidelines for HNSCC treatment in Latvia. By identifying the aforementioned morphological and molecular virological markers, patients could be classified, allowing for the use of appropriate therapy.

Aim of the Thesis

The aim of this research is to investigate the prevalence of HPV infection (HPV DNA and E6/E7 mRNA) in patients with oropharyngeal, hypopharyngeal, and laryngeal squamous cell carcinomas, as well as its significance in tumour development and survival of the patients with the additional assessment of the immunohistochemical 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 virological findings to investigate the role of HPVs in the development of oropharyngeal, hypopharyngeal, and laryngeal squamous cell carcinoma, and their impact on survival.

2. Determine the presence of HPV's DNA (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 the transcriptional activity of HR-HPV in HR-HPV+ HNSCC by detecting E6/E7 mRNA.
5. Analyse the immunohistochemical expression of tumour suppressor proteins p16 and p53, as well as HPV E6/E7 oncoproteins, in tissues from patients with histologically confirmed squamous cell carcinoma of the oropharynx, hypopharynx, and larynx.

Hypotheses of the Thesis

- HPV infection plays a role in the development of oropharyngeal, hypopharyngeal, and laryngeal squamous cell carcinoma.
- HPV status in patients with oropharyngeal, hypopharyngeal, and laryngeal squamous cell carcinoma is an independent prognostic factor.
- p16, p53, HR-HPV E6/E7 oncoproteins have prognostic value and impact survival.
- There are associations between p16, p53, HPV status (HPV DNA, E6/E7 mRNA, HR-HPV E6/E7 oncoprotein immunoexpression), and survival in patients with oropharyngeal, hypopharyngeal, and laryngeal squamous cell carcinomas.

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:

1. Retrospective study of 247 patients with OPSCC
2. Prospective study of 106 patients with HNSCC (34 OPSCC, 41 LSCC, 31 HPSCC)

The retrospective study was conducted at the Rīga East University Hospital, Oncology Centre of Latvia; 247 patients diagnosed with OPSCC were included, staged following the TNM classification of the Union for International Cancer Control (6th edition) for oropharyngeal carcinoma. The study period ranged from 1st January 2000 to 31st December 2010. The diagnosis of OPSCC was confirmed through histological examination, and the patients' data was obtained from the Hospital Archive and the Centre for Disease Prevention and Control.

The collected data were analysed to determine the 3-year and 5-year OS and DSS rates for all patients and included patient survival status, death date (if applicable), age at the time of the diagnosis, sex, T status, N status, M status, disease stage, hazardous habits (smoking, alcohol abuse), therapy modality (radiotherapy, surgery, chemotherapy, symptomatic therapy, and combinations of aforementioned), primary tumour location, and histopathological variant of the tumour. Chemotherapy treatment consisted of single-agent regimens involving cetuximab or platinum medication (cisplatin).

The second part included a complex analysis of 106 patients with HNSCC. Diagnosis was made upon histological examination and confirmed at the Latvian Oncology Centre; time frame 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. Data about patients' age, sex, TNM stages, hazardous habits (smoking, alcohol abuse), and received therapy was obtained. Survival data of the patients was obtained from the Centre for Disease Prevention and Control. Several morphological methods were used in the research – immunohistochemistry and immunofluorescence. Finally, different molecular biology methods were used – DNA and RNA extraction from fresh frozen tissue and FFPE samples, conventional and real-time PCRs for detection of viral DNA and RNA products. The study was conducted at the Rīga East University Hospital, Oncology Centre of Latvia. In addition to fresh tumour samples obtained during surgery or biopsy, FFPE blocks along with the histopathology reports were collected from the Pathology Centre of Rīga East 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, Rīga 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 3-year and 5-year OS and DSS 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.

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

FFPE samples of OPSCC were obtained from various major subsites, 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.

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 Rīga Stradiņš University (Decisions No. 3/24.09.2015.) and conducted according to the Declaration of Helsinki.

2.2.1 Patients' characteristics

The sex, age, TNM status, differentiation grade (G) of the tumour, smoking and drinking habits at the time of presentation, 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 DNA extraction

Fresh frozen or FFPE tissues form cancer were used to extract DNA material for further investigation.

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) in accordance with the manufacturer's protocol. To avoid cross-contamination, separate sterile blades were used for each specimen.

The quality and quantity of DNA were estimated spectrometrically (Nanodrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). All samples had acceptable 260/280 nm ratios suggestive of high purity. Beta- (β-) globin PCR with appropriate

primers was used to determine the quality of isolated DNA (Vandamme et al., 1995). Only β-globin positive samples were used for further analysis.

2.2.3 RNA Extraction

Fresh frozen or FFPE tissues from cancer tissues were processed for total RNA extraction. Only specimens positive for HR-HPV DNA were submitted to RNA extraction.

Standard RNA extraction with TRIzol LS Reagent from Thermo Fisher Scientific was accomplished 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. Each sample was sectioned separately with a new sterile blade.

A spectrophotometric analysis was used to assess the concentration and quality of the extracted RNA. All samples had acceptable 260/280 nm ratios suggestive of high purity.

2.2.4 Screening of the samples by MY09/11 and GP5+/6+ consensus primers

All extracted DNA samples were submitted for testing by the PCR with consensus primers MY9/MY11 and GP5+/6+ to detect wide range of HR-HPV and LR-HPV types (Şahiner et al., 2014; Shikova et al., 2009). 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 (Table 2.3). Positive and negative controls were included in each reaction.

Table 2.3

Oligonucleotide primers used for HPV DNA detection

Primers	Sequence (5'-3')	Amplicon (bp)
β-globin primers		
GS 268	ACACAACTGTGTTCACTAGC	200
GS 269	TGGTCTCCTTAAACCTGTCTTG	
Consensus primers		
MY09	CGTCC(AC)A(AG)(AG)GGA(T)ACTGATC	450
MY11	GC(AC)CAGGG(AT)CATAA(CT)AATGG	
GP5+	TTTGTTACTGTGGTAGATACTAC	150
GP6+	GAAAAATAAACTGTAAATCATATTC	
Type-specific primers		
16.L1-1	TGCTAGTGCTTATGCAGCAA	152
16.L1-2	ATTTACTGCAACATTGGTAC	
18.1	AAGGATGCTGCACCGGCTGA	216
18.2	CACGCACACGCTTGGCAGGT	

2.2.5 HPV genotyping using HPV16 and HPV18 type-specific primers

Type-specific primers for HPV-16 and 18 were used in the PCR reaction (Table 2.3). Amplification using HPV16 specific primers produces 152 bp long amplicons and using HPV18 specific primers – 216 bp amplicons (Shikova et al., 2009). Results were visualised by electrophoresis in 1.7% agarose gel. Positive and negative controls were included in each reaction. All available specimens were analysed using these primers.

2.2.6 HPV genotyping using Anyplex II HPV28

Anyplex II HPV28 multiplex real-time PCR was performed as recommended by the manufacturer (Seegene, South Korea). 5 µl specimen DNA were added in each of two sets (wells) with a 20-µl PCR reaction mix. Set A consists of primer mix for 14 HR-HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and set B consists of primer mix for 5 possibly HR-HPV types (HPV26, 53, 69, 73, and 82) and 9 LR-HPV types (HPV6, 11, 40, 42, 43, 44, 54, 61, and 70). Both primer sets were designed for the HPV L1 gene and produced 100 and 200 bp amplicon correspondingly.

Melting curves are obtained at 30, 40, and 50 cycles allowing the semiquantitative specimen analysis and differentiating between high (+++), medium (++), or low (+) viral load, and has internal positive and negative controls. The kit has DNA quality control by detecting the β-globin gene. 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.

2.2.7 HPV detection by Sacace HPV High-Risk Screen Real-TM Quant

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). The kit contains quantitative standards with the known concentration of HPV DNA, used for calculation of viral load. Only specimens positive in consensus PCR or PCR with HPV16/18 L1 primers were analysed.

2.2.8 HPV16 E6/E7 mRNA detection

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 high-risk HPV types including 16, 18, 31, 33, and 45. The kit incorporates an

intrinsic sample control to evaluate the quality of the specimen, and samples with positive intrinsic controls were deemed valid. Only samples positive for HR-HPV were utilised for the detection of E6/E7 mRNA.

2.2.9 IHC evaluation of the specimens

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), underwent a standard preparation process and were then incubated overnight at 4°C with specific primary antibodies. The antibodies used were as follows: 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) (J. Yang et al., 2016; Stiasny et al., 2016; Meng et al., 2018), and a monoclonal mouse anti-HPV16 E7 antibody (Santa Cruz Biotechnology, Inc., diluted 1:50, sc-6981). 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, as proposed by Hong et al. (Hong et al., 2013). The evaluation of p53 immunostaining was performed semiquantitatively, considering a sample to be p53-positive (p53+) if it met the criteria described by Halec et al. (Halec et al., 2013). p53 overexpression (upregulation) 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−; downregulation).

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, encompassing

both the tumour and the surface epithelium of the regions of interest. Two ways of interpretation were used for assessment:

- Depending on proportion of immunopositive cells, the levels of the E6 and E7 immunoexpression were graded as negative – 0, weak – with ≤ 10 %, moderate – 11–50 %, and strong – > 50 %, respectively (publication in MDPI Viruses). The levels were asserted in tumour cells and in epithelium cells separately.
- 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). The levels of expression were asserted in whole specimen (tumour and epithelial regions combined).

2.2.10 Immunofluorescence

To better visualize the distribution and localisation of the HR-HPV16 E7 oncoprotein within the cellular context, fluorescence-based immunodetection was employed for the tumour tissue specimens. The sections were subjected to immunoreaction using a mouse monoclonal anti-HPV16 E7 antibody (Santa Cruz Biotechnology, Inc., diluted 1:50, sc-6981) overnight at 4°C. Following this, the sections were washed with PBS and incubated with a secondary antibody, goat anti-mouse IgG-FITC (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, diluted 1:300, sc-2010). Subsequently, the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Invitrogen, Renfrew, UK, diluted 1:3,000) and mounted in Prolong Gold with DAPI (Thermo Fisher Scientific). The imaging process was carried out using an Eclipse Ti-E confocal microscope (Nikon, Tokyo, Japan).

3 Statistical Analysis

3.1 The first part (retrospective)

All statistical analyses were performed using the IBM SPSS. Statistical analysis was conducted to assess the correlation between the mentioned covariates and survival outcomes, as well as the mean overall survival time after diagnosis. To determine the statistical significance of differences between analysed groups, Pearson's chi-squared test or Fisher's exact test (depending on group size) was employed, with a significance level of $p < 0.05$. Cramer's V was used to measure the association between two nominal variables. To determine the significance of differences between nominal variables and mean survival time after diagnosis, an analysis was conducted using either the Kruskal-Wallis test or the Mann-Whitney test, depending on the number of groups involved.

The statistical data was also analysed using the Kaplan-Meier survival analysis. 3- and 5-year OS and DSS were estimated. Differences in unadjusted survival rates were evaluated through the log-rank test, $p < 0.05$ was deemed as significant. To estimate the hazard ratio, the Cox regression method was employed. Various covariates such as age, sex, T status, N status, hazardous habits (smoking, alcohol abuse), therapy modalities (radiotherapy, surgery, chemotherapy, symptomatic therapy, and combinations thereof), primary tumour location, and histopathological variant of the tumour were included in the survival model.

3.2 The second part (prospective)

All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The Anderson–Darling, D'Agostino and Pearson, and Shapiro–Wilk normality tests were applied to assess numerical data distribution. The comparison of means between different groups of numerical variables was performed using one-way ANOVA. For data with non-Gaussian distribution, Kruskal–Wallis or Friedman's test (for paired groups) followed by the two-stage step-up method of Benjamini, Krieger, and Yekutieli as false discovery rate controlling test were used. To compare numerical values between two groups, the Mann-Whitney test or Wilcoxon test (for pared groups) were applied. Relations between analysed groups were investigated using nonparametric Spearman's correlation analysis (Mukaka, 2012; Akoglu, 2018). *p* values less than 0.05 ($p < 0.05$) were considered statistically significant.

Seegene results were assessed semiquantitatively and coded as follows: 1 – negative; 2 – for +; 3 – for ++; and 4 – for +++. Viral load (copies/10^5 cells) from the Sacace assay was expressed in $\log_{10}$ and submitted to the statistical analysis. For negative samples, $\log_{10}$ random

values of 0 (viral load of approximately 1 copies/10^5 cells) as a mean and SD of 0.1 were assigned (generated with GraphPad Prism random number generator).

Cohen's κ test was used to assess agreement between the HPV detection methods with 1 –indicating perfect agreement; 1 to 0.81 – very good agreement; 0.80 to 0.61 – good agreement; and 0.60 to 0.21 – moderate to a poor agreement.

A nonlinear regression model was used to graphically assess the relationship between the viral load and the semiquantitative results of Anyplex II HPV28 assay.

A univariate survival analysis was performed using the Kaplan–Meier method; OS and DSS were assessed. A multivariate survival analysis was performed using the Cox regression method. *p* values less than 0.05 ($p < 0.05$) were considered statistically significant.

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 distribution of subsites within the cohort included palatine tonsils (n = 110, 44.52 %), base of 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, with only a small proportion presenting with stage I (n = 3, 1.22 %) and stage II (n = 19, 7.72 %). Most patients were male (n = 227, 91.90 %), with a median age of 60.20 years (range 27–85). The study investigated various factors related to patient survival and disease characteristics.

The analysis revealed that 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. Interestingly, DSS in female patients was significantly better than in males. The patients were also divided into three age groups (younger than 55 years, 55 to 64 years, and older than 65 years), and it was found that there were significantly more deceased patients in the older age subgroup. However, no correlation was observed between age group and survival. Kaplan-Meier estimates (Table 4.1) showed a decrease in survival with increasing age, but the differences in OS and DSS were not statistically significant when all three age groups were considered. Pairwise comparisons showed statistically significant differences in survival between patients younger than 55 years and 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 (Table 4.1) 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 higher T stage, indicating a moderate correlation between outcome and tumour size. Kaplan-Meier (Table 4.1) analysis demonstrated better OS and DSS for patients with smaller tumours (T1-2) compared to those with bigger tumours (T3-4).

Table 4.1

Kaplan-Meier analysis of potential prognostic factors for OS, DSS

Variable	3-year Kaplan-Meier estimate % (95 % CI)		5-year Kaplan-Meier estimate % (95 % CI)	
	OS	**DSS**	**OS**	**DSS**
Age, years (n; %):				
• <55 (62; 25.10)	25.8 % (14.8–36.8)	24.1 % (13.1–35.1)	22.6 % (12.2–33.0)	20.7 % (10.3–31.1)
• 55-64 (105; 42.51)	21.6 % (13.6–29.6)	19.6 % (11.8–27.4)	15.7 % (8.6–22.8)	14,4 % (7.3–21.5)
• ≥65 (80; 32.39)	14.1 % (6.5–21.7)	12.3 % (4.3–20.3)	7.7 % (1.8–13.6)	7.7 % (1.2–14.2)
	p = 0.092	p = 0.108	p = 0.092	p = 0.108
Sex:				
• Male	19.8 % (14.5–25.1)	19 % (12.7–23.3)	14 % (9.5–18.5)	12,8 % (8.3–17.7)
• Female	30 % (10.0–50.0)	30 % (10.0–50.0)	25 % (6.0–44.0)	25 % (6.0–44.0)
	p = 0.06	p = 0.0486	p = 0.06	p = 0.0486
Disease stage:				
• I	100 % (–)	100 % (–)	100 % (–)	100 % (–)
• II	36.8 % (15.0–58.6)	37.5 % (13.8–61.2)	31.6 % (10.6–52.6)	31.3 % (8.6–54.0)
• III	21.7 % (11.3–32.1)	23.6 % (12.4–34.8)	13.3 % (4.7–21.9)	14.5 % (5.1–23.9)
• IV	16.3 % (10.6–22.0)	13.0 % (7.5–18.5)	11.1 % (6.2–16.0)	10.3 % (5.4–15.2)
	p = 0.0058	p = 0.0058	p = 0.0058	p = 0.0058
T stage:				
• T1	42.9 % (21.7–64.1)	37.5 % (13.8–61.2)	42.9 % (21.7–64.1)	37.5 % (13.8–61.2)
• T2	34.5 % (22.3–46.7)	35.8 % (22.9–48.7)	22.4 % (11.6–33.2)	22.6 % (11.4–33.8)
• T3	16.4 % (8.0–24.8)	16.4 % (7.6–25.2)	9.6 % (2.9–16.3)	10.4 % (3.1–17.7)
• T4	11.4 % (4.7–18.1)	8.5 % (2.4–14.6)	6.8 % (1.5–12.1)	6.1 % (1.0–11.2)
	p < 0.0001	p < 0.001	p < 0.0001	p < 0.001
N status:				
• N0	27.6 % (17.6–37.6)	27.9 % (17.3–38.5)	21.1 % (11.9–30.3)	22.1 % (12.3–31.9)
• N+	19 % (12.9–25.1)	16.8 % (10.7–22.9)	12.3 % (7.2–17.4)	10.7 % (5.8–15.6)
	p = 0.11	p = 0.11	p = 0.11	p = 0.11
Primary tumour location:				
• Palatine tonsil	18.5 % (11.2–25.8)	16.8 % (9.5–24.1)	12 % (5.9–18.1)	9.9 % (4.0–15.8)
• Base of the tongue	24.3 % (14.5–34.1)	22.7 % (12.5–32.9)	17.6 % (9.0–26.2)	18.2 % (9.0–27.4)
• Pharyngeal wall	15 % (4.0–26.0)	13.5 % (2.5–24.5)	7.5 % (0–15.7)	8.1 % (0–16.9)
• Soft palate	40 % (18.4–61.6)	43.8 % (19.5–68.1)	35 % (14.0–56.0)	37.5 % (13.8–61.2)
	p = 0.003	p = 0.003	p = 0.003	p = 0.003
Alcohol abuse and smoking:				
• Neither	34 % (20.5–47.5)	31.8 % (18.1–45.5)	23.4 % (11.2–35.6)	25 % (12.3–37.7)
• 1 factor	22.7 % (14.9–30.5)	20.4 % (12.4–28.4)	16.4 % (9.5–23.3)	14.3 % (7.4–21.2)
• Both	11.4 % (4.0–18.8)	10.9 % (3.3–18.5)	7.1 % (1.0–13.2)	6.3 % (0.4–12.2)
	p = 0.002	p = 0.008	p = 0.002	p = 0.008
Treatment (n):				
• RT (175)	14 % (8.7–19.3)	12.6 % (7.5–17.7)	7.6 % (3.7–11.5)	7.5 % (3.4–11.6)
• OP (7)	42.9 % (6.2–79.6)	40 % (0–82.9)	42.9 % (6.2–79.6)	40 % (0–82.9)
• RT+OP (39)	52.6 % (36.7–68.5)	54.8 % (37.4–72.2)	42.1 % (26.4–57.8)	41.9 % (24.5–59.3)
• RT+ChT (Cetuximab) +/− OP (17)	23.5 % (3.3–43.7)	25 % (3.8–46.2)	17.6 % (0–35.6)	18.8 % (0–38.0)
• RT+ChT (Cisplatin) +/− OP (3)	33.3 % (0–86.6)	33.3 % (0–86.6)	33.3 % (0–86.6)	33.3 % (0–86.6)
• Symptomatic (6)	0 %	0 %	0 %	0 %
	p < 0.001	p < 0.001	p < 0.001	p < 0.001

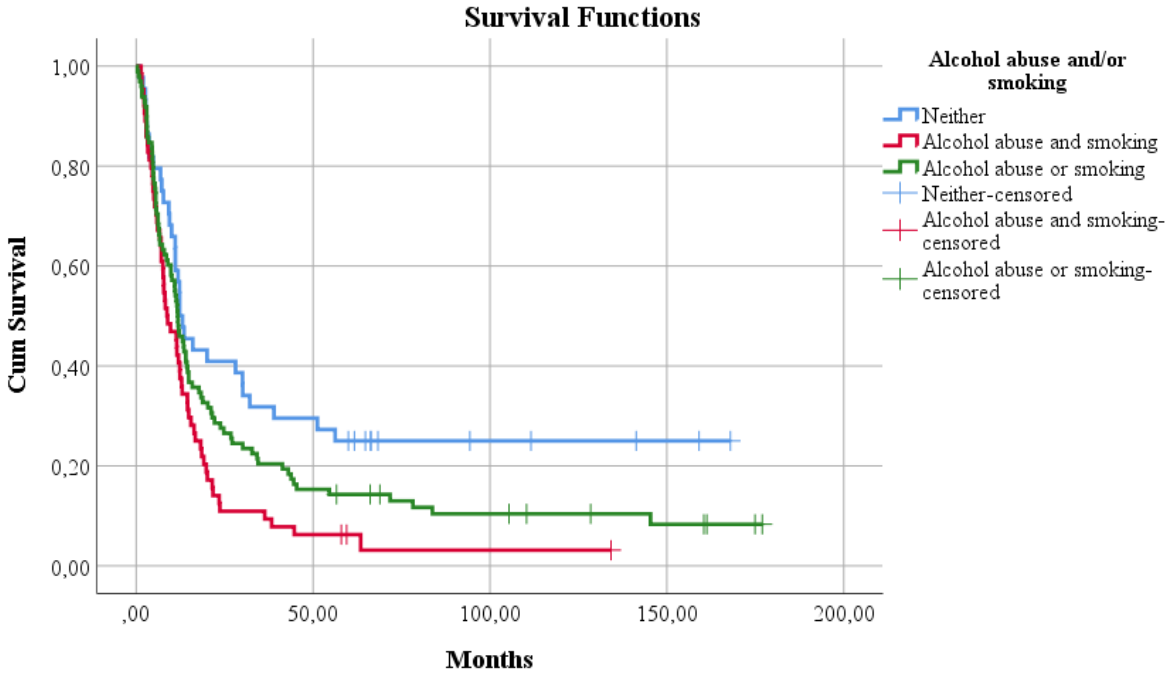

Figure 4.1 **Kaplan-Meier DSS plot according to hazardous habits**

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 higher 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 (Table 4.1, Figure 4.1).

The study did not find an impact of tumour location on mean survival time or survival. Patients with pharyngeal wall and tonsillar tumours had the worst OS (p = 0.03) and DSS (p = 0.026) estimates, while patients with tumours of the soft palate had better outcomes. Histological analysis revealed that most tumours were keratinizing squamous cell carcinoma (KSCC; 70.85 %), while a smaller proportion were nonkeratinizing squamous cell carcinoma (NKSCC; 19.43 %), undifferentiated carcinomas (1.21 %), or adenosquamous carcinoma (0.4 %). The specific histological variant of the tumour did not significantly affect OS or DSS.

The tissue samples of KSCC showed large polygonal squamous cells with distinct cell borders and the presence of keratin formation. The tumours exhibited a range of grades, from well-differentiated to poorly differentiated, with varying degrees of keratinization (Figures 4.2, 4.3, 4.4).

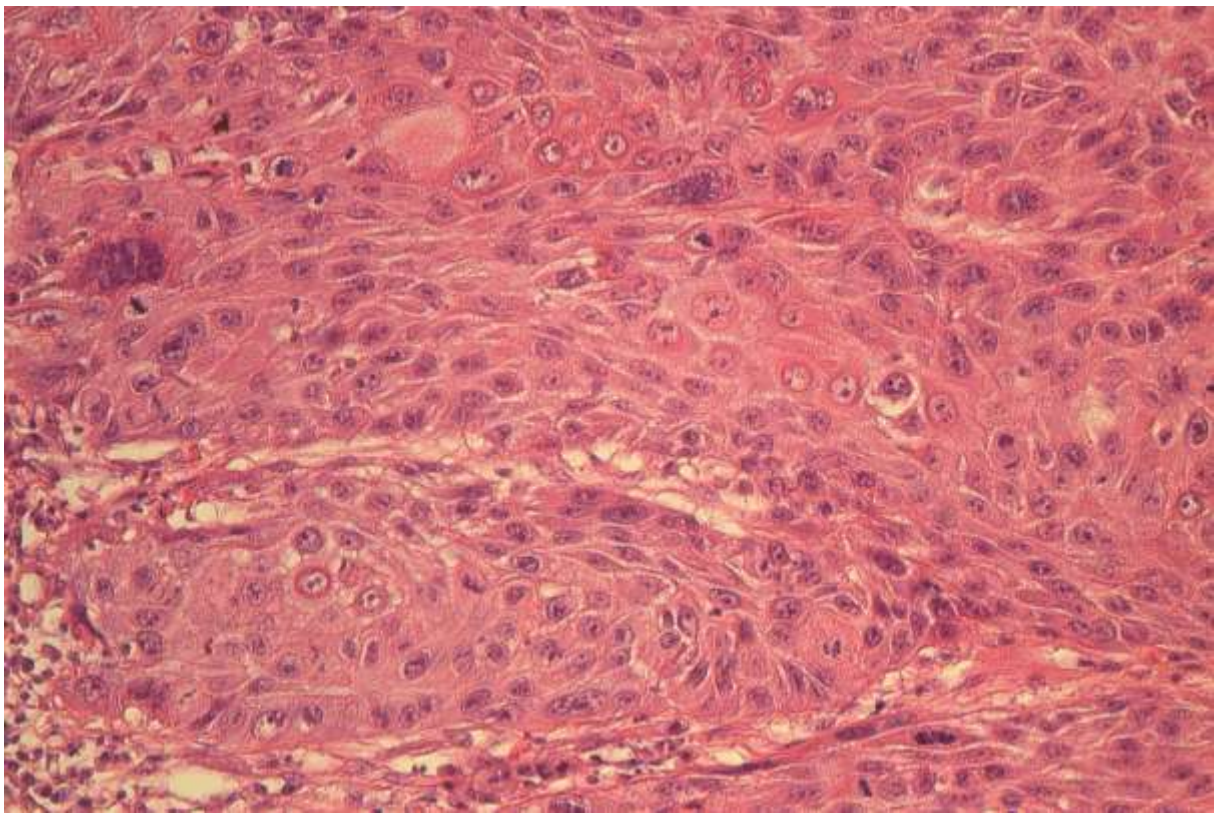

Figure 4.2 **Soft palate region. KSCC (verrucous type) showing folded and thickened neoplastic epithelium comprised of large polygonal cells with distinct cell borders and varying degree of eosinophilia. Nuclei are pleomorphic**

H&E, original magnification ×200.

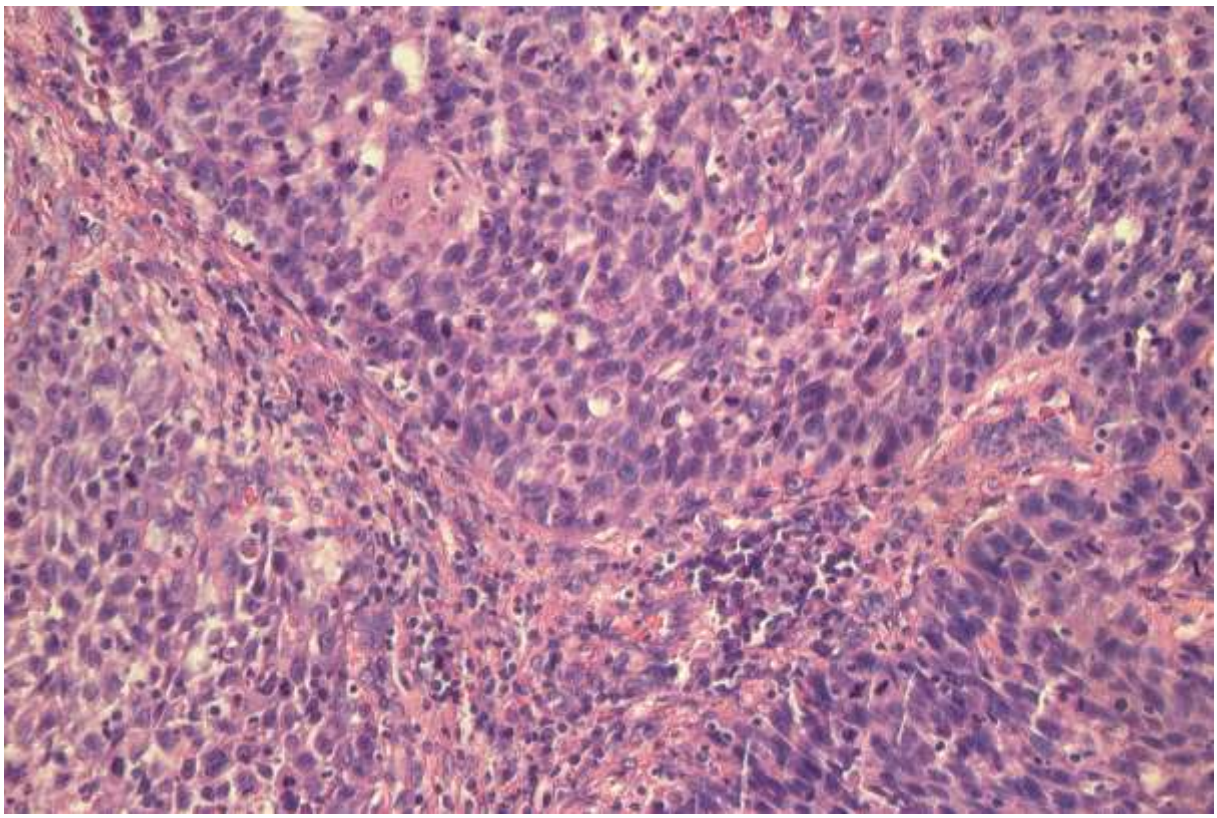

Figure 4.3 **Base of the tongue. NKSCC. Densely packed mitotically active epithelial cells forming the pushing and infiltrating masses of carcinoma**

H&E, original magnification ×200.

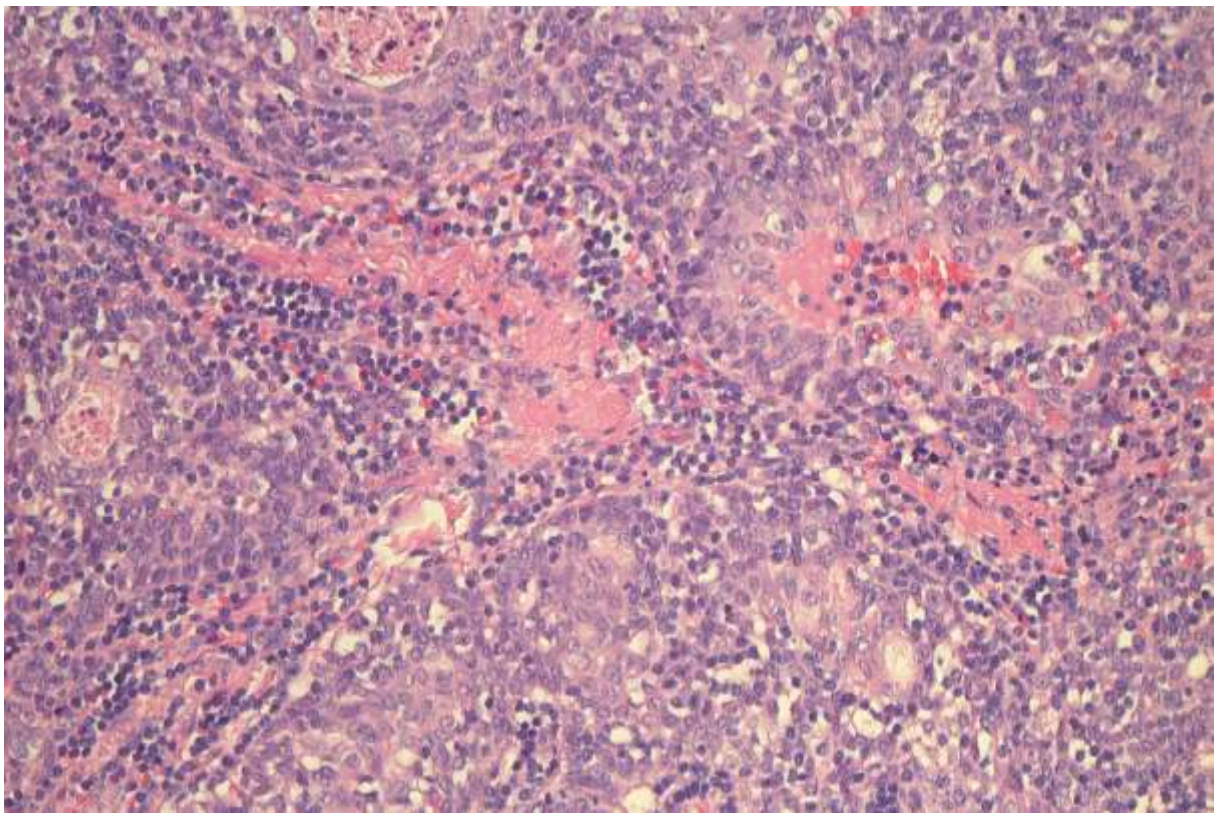

Figure 4.4 **Palatine tonsil. NKSCC. Nests of tumour cells with ill defined borders and necrosis**

H&E, original magnification ×200.

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, displaying nuclear pleomorphism (Figures 4.2, 4.5). Infiltrative nests of tumour cells were commonly found within the stroma, which exhibited prominent desmoplasia.

On the other hand, NKSCC tumours formed nests, sheets, and cords with well-defined borders. These tumours were characterised by relatively monomorphic, densely packed basaloid cells with ovoid and spindle-shaped morphology and indistinct cell borders. The mitotically active tumour cells displayed 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 (Figure 4.6).

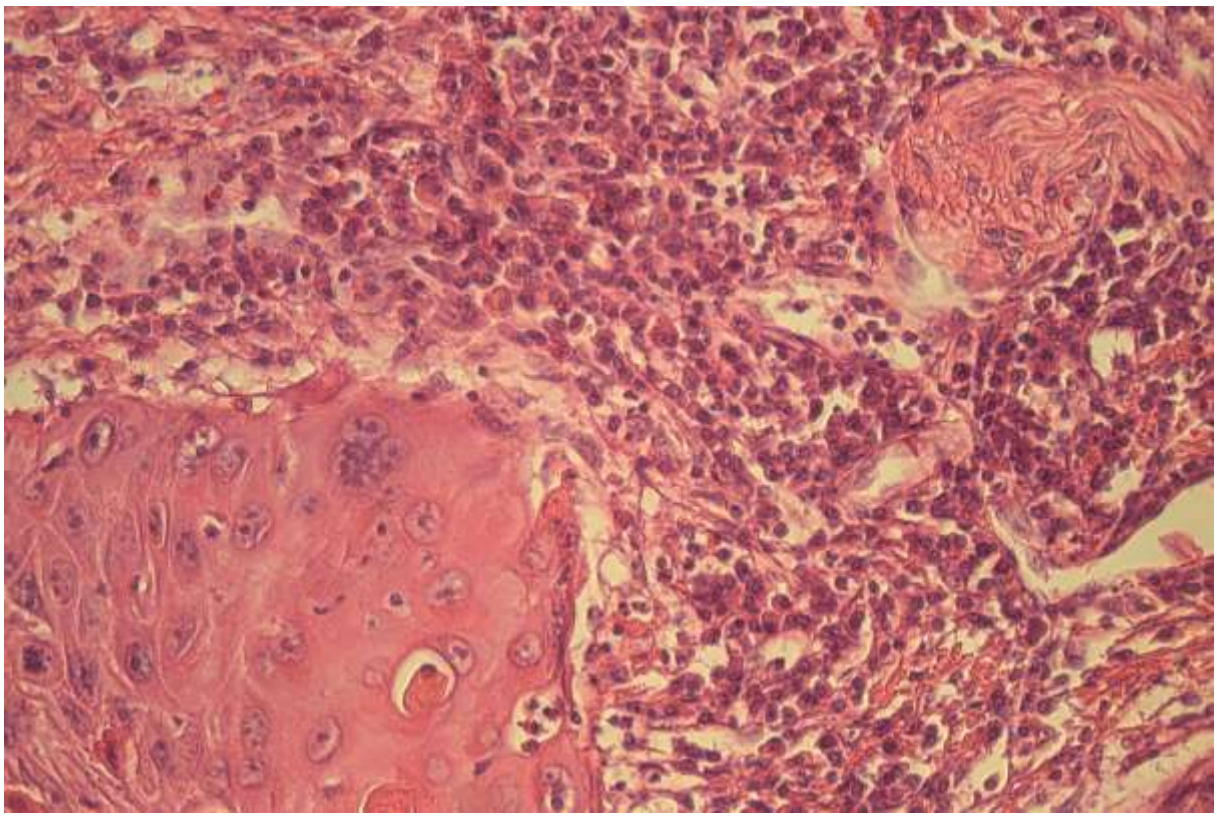

Figure 4.5. **Soft palate region. KSCC. Tumour cells demonstrate nuclear pleomorphism, mitotic and apoptotic features. Some tumour cells contact the nerve bundle.**

H&E, original magnification ×250.

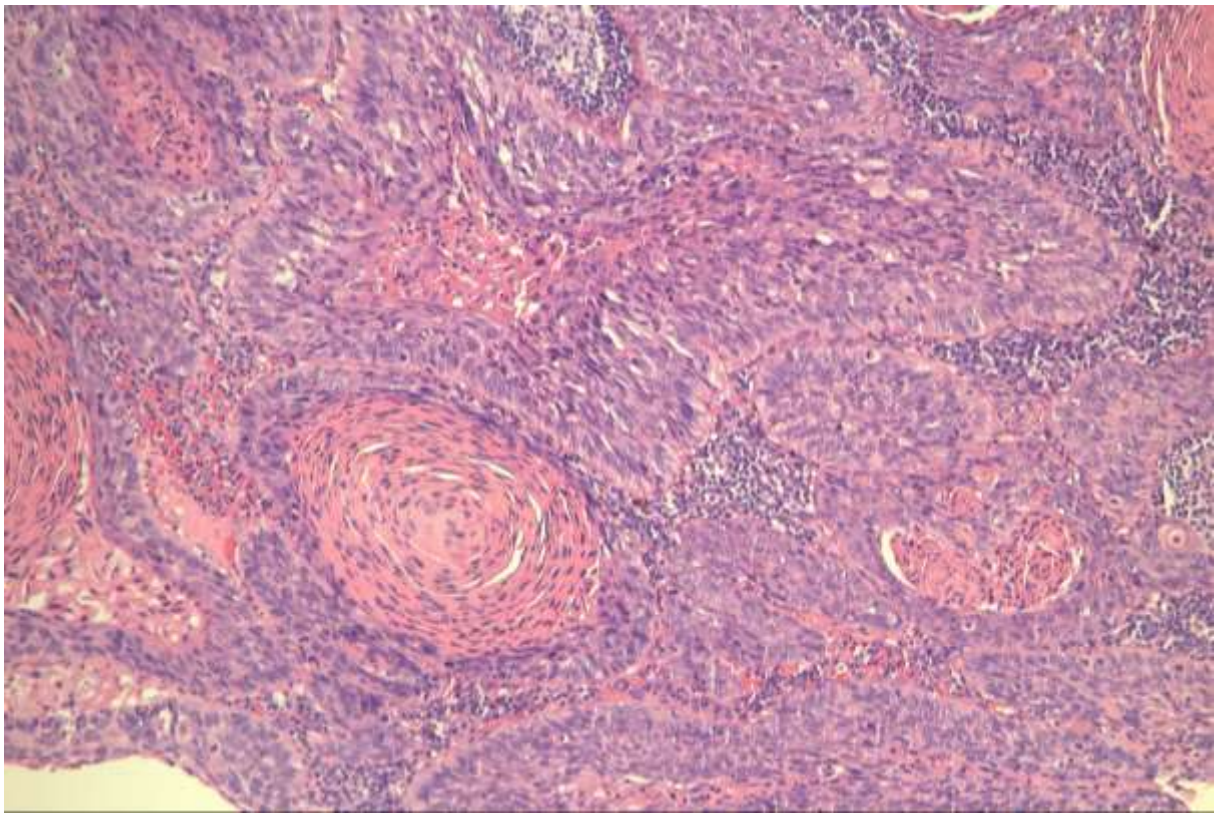

Figure 4.6 **Base of the tongue. NKSCC. Nests and cords of tumour cells with basaloid features, peripheral palisading, intraluminal necrosis, keratocysts**

H&E, original magnification ×100.

To better understand the aggressive behaviour of the tumour, we considered factors such as perineural spread, lymphovascular invasion, and muscular invasion, as we recognised that histological grade based on keratinization alone may not consistently predict clinical outcomes. Our findings revealed that perineural invasion and lymphovascular invasion were commonly observed in squamous cell carcinomas, which correlated with decreased survival rates (Figure 4.5). Furthermore, as the tumour masses invaded deeper, we observed that the malignant cells infiltrated the underlying skeletal muscle tissue, forming islands and cords (Figure 4.7).

A strong correlation between survival and therapy was observed, indicating that the type of treatment received had a significant impact on patient survival. However, there was no correlation between applied therapy and mean survival time. Significant differences in OS and DSS were found among different therapeutic modalities (Table 4.1). 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 that patients who underwent surgery had higher survival rates, while those in the RT group (excluding symptomatic treatment group) had the lowest survival rates (OS an DSS).

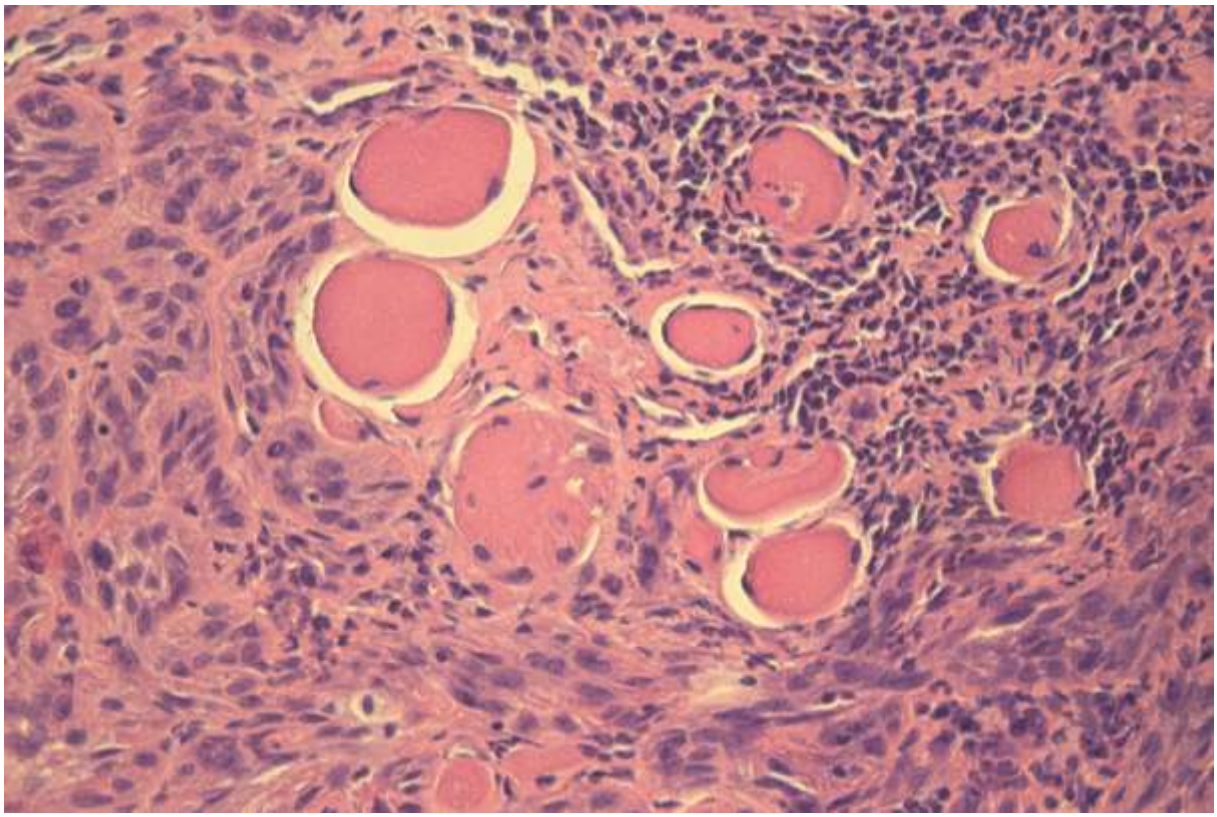

Figure 4.7 **Posterior pharyngeal wall. Poorly differentiated squamous cell carcinoma. Tumour nests and nodules reveal muscular invasion; lymphoplasmacytic infiltration**
H&E, original magnification ×250.

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 (Table 4.2).

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 surgical intervention ($p < 0.0001$). Nevertheless, pairwise comparisons of different types of surgical procedures did not show any significant differences in OS ($p = 0.29$) or DS ($p = 0.11$).

The Cox regression method was applied in two stages in this study. Firstly, all factors were analysed without distinguishing subgroups (univariate analysis, Table 4.3), and then subgroups of each factor were assessed (multivariate analysis, Table 4.4). The analysis revealed that T stage, N status, and sex had a statistically significant or probable impact on mortality after the detection of the disease (Table 4.3). Specifically, for T stage, the risk of death increased by 39 % for each increase in stage, while for N status, the risk of death increased by 51 % when changing from N0 to N+ status. Additionally, the risk of death was 70 % higher for females compared to males. Other factors did not show a statistically significant impact on the risk of early death. The Cox regression plot for cumulative survival indicated that 50 % of patients died within 12 months after the diagnosis of cancer (Figure 4.8).

Table 4.2

Breakdown of patients by type of operation and outcome of the disease

<table>
<tr><th rowspan="2">Type of operation</th><th rowspan="2">N of patients (incidence, %)*</th><th rowspan="2">Outcome of the disease (therapy)</th><th rowspan="2">N of patients (incidence, %)</th><th colspan="4">Statistical analysis between groups</th></tr>
<tr><td colspan="2">All groups</td><td colspan="2">Only operations</td></tr>
<tr><td rowspan="2">Primary Tu excision</td><td rowspan="2">10 (4.05; 19.61)</td><td>Positive (survived)</td><td>3 (30.00)</td><td colspan="2" rowspan="2">All groups</td><td colspan="2" rowspan="2">Only operations</td></tr>
<tr><td>Negative (deceased)</td><td>7 (70.00)</td></tr>
<tr><td rowspan="2">Neck dissection</td><td rowspan="2">28 (11.34; 54.90)</td><td>Positive (survived)</td><td>5 (18.52)</td><td rowspan="6">P_χ</td><td rowspan="6">V_1</td><td rowspan="6">P_χ</td><td rowspan="6">V_1</td></tr>
<tr><td>Negative (deceased)</td><td>22 (81.48)</td></tr>
<tr><td rowspan="2">Both</td><td rowspan="2">13 (5.26; 25.49)</td><td>Positive (survived)</td><td>6 (46.15)</td></tr>
<tr><td>Negative (deceased)</td><td>7 (53.85)</td></tr>
<tr><td rowspan="2">None</td><td rowspan="2">196 (79.35; -)</td><td>Positive (survived)</td><td>12 (6.25)</td></tr>
<tr><td>Negative (deceased)</td><td>180 (93.75)</td><td>7.11×10^{-6}</td><td>0.33</td><td>0.19</td><td>0.26</td></tr>
</table>

* The incidence among all patients and the incidence only between operations.

Table 4.3

Cox proportional hazard, univariate analysis

Variable	B	P	Exp(B)	CI 95 % Exp(B)
Sex	0.53	4.88×10^{-2}	1.70	1 … 2.88
Age groups	0.14	0.14	1.15	0.95 … 1.4
Alcohol abuse and/or smoking	0.02	0.83	1.02	0.85 … 1.22
T	0.33	2.40×10^{-5}	1.39	1.2 … 1.63
N status (N0 vs. N+)	0.41	1.35×10^{-2}	1.51	1.09 … 2.09
Therapy	-0.10	0.14	0.90	0.79 … 1.03
Primary tumour location	-0.08	0.29	0.92	0.79 … 1.07
Histological variant	0.07	0.36	1.07	0.92 … 1.25
Variable	**B**	**P**	**Exp(B)**	**CI 95 % Exp(B)**
Sex	0.53	4.88×10^{-2}	1.70	1 … 2.88
Age groups	0.14	0.14	1.15	0.95 … 1.4
Alcohol abuse and/or smoking	0.02	0.83	1.02	0.85 … 1.22
T	0.33	2.40×10^{-5}	1.39	1.2 … 1.63
N status (N0 vs. N+)	0.41	1.35×10^{-2}	1.51	1.09 … 2.09
Therapy	-0.10	0.14	0.90	0.79 … 1.03
Primary tumour location	-0.08	0.29	0.92	0.79 … 1.07
Histological variant	0.07	0.36	1.07	0.92 … 1.25

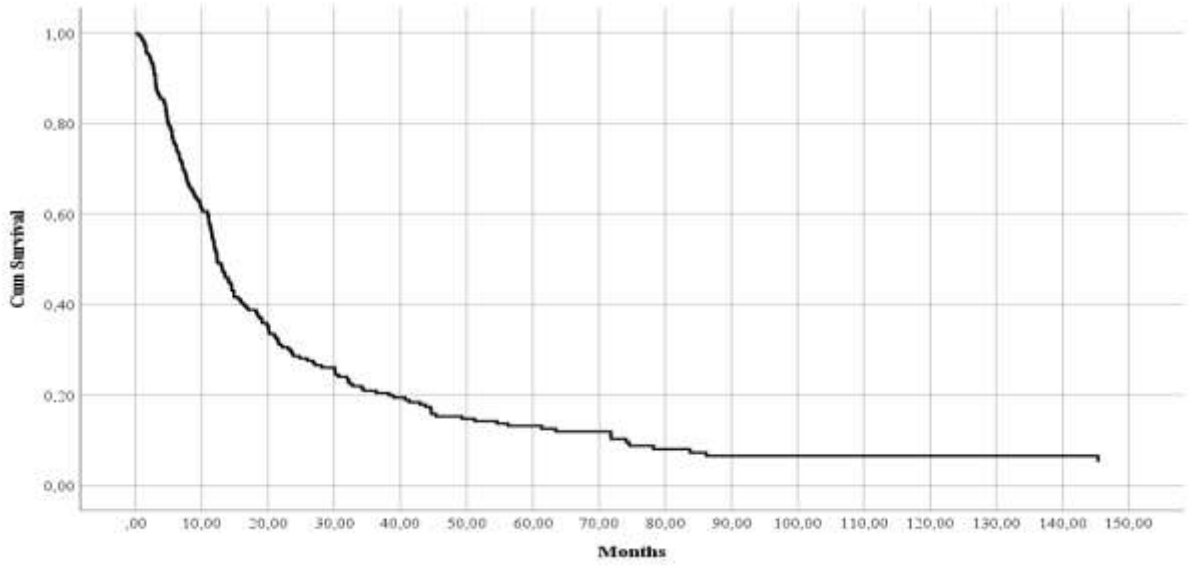

Figure 4.8 **Cox regression plot for cumulative survival (overall) according to sex, age group, T, N status, alcohol abuse and/or smoking, therapy, primary tumour location, histological variant**

When accounting for nine factors and analysing the hazard ratios between subgroups (Table 4.4), it was found that 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.

Table 4.4

Cox proportional hazard model, multivariate analysis

Variables		*p*	Exp(B) or hazard ratios*	CI 95% Exp(B)	Hazard ratios comparing to other groups^
Name	**Groups**				
Sex (female > male)		0.11	0.63	0.36 to 1.11	
Age group		0.15			
	< 55 years old	0.10	0.70	0.46 to 1.06	
	55 – 64 years old	0.08	0.74	0.52 to 1.04	
	> 64 years old		(1.00)		
Alcohol abuse and/or smoking		**0.06**			
	None	0.43	0.84	0.55 to 1.29	
	1 of aforementioned	**0.051**	1.42	1 to 2.01	
	Both		(1.00)		
T		3.51×10^{-2}			
	1	0.13	0.60	0.31 to 1.17	1.06
	2	$\mathbf{6.72 \times 10^{-3}}$	**0.57**	0.37 to 0.85	
	3	0.51	0.89	0.62 to 1.26	1.57
	4		(1.00)		1.77
N status (N0 > N+)		$\mathbf{1.58 \times 10^{-2}}$	**0.66**	0.47 to 0.93	
Therapy		0.09			
	RT	0.42	0.67	0.26 to 1.75	2.02
	OP	0.20	0.42	0.11 to 1.56	1.27
	RT + OP	$\mathbf{4.67 \times 10^{-2}}$	**0.33**	0.11 to 0.98	
	RT + ChT (Cetuximab) +/−OP	0.70	0.80	0.26 to 2.44	2.41
	RT + ChT (Platinum) +/− OP	0.44	0.51	0.09 to 2.82	1.54
	Symptomatic		(1.00)		3.00
Primary tumour location		0.55			
	Palatine tonsil	0.19	1.48	0.82 to 2.64	
	Base of the tongue	0.37	1.32	0.72 to 2.4	
	Pharyngeal wall	0.20	1.52	0.79 to 2.92	
	Soft palate		(1.00)		
Histological variant		0.73			
	KSCC	0.78	0.93	0.54 to 1.59	
	NKSCC	0.90	0.96	0.52 to 1.78	
	Carcinoma, undifferentiated (Epit)	0.35	1.84	0.51 to 6.67	1.91
	Squamous cell carcinoma, BCN (unspecified)		(1.00)		1.04

*calculated using the last group as a reference

^ calculated for significant groups (bold) against others, taking a significant group as a reference

The study also found 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 chemotherapy with cetuximab (Figure 4.9).

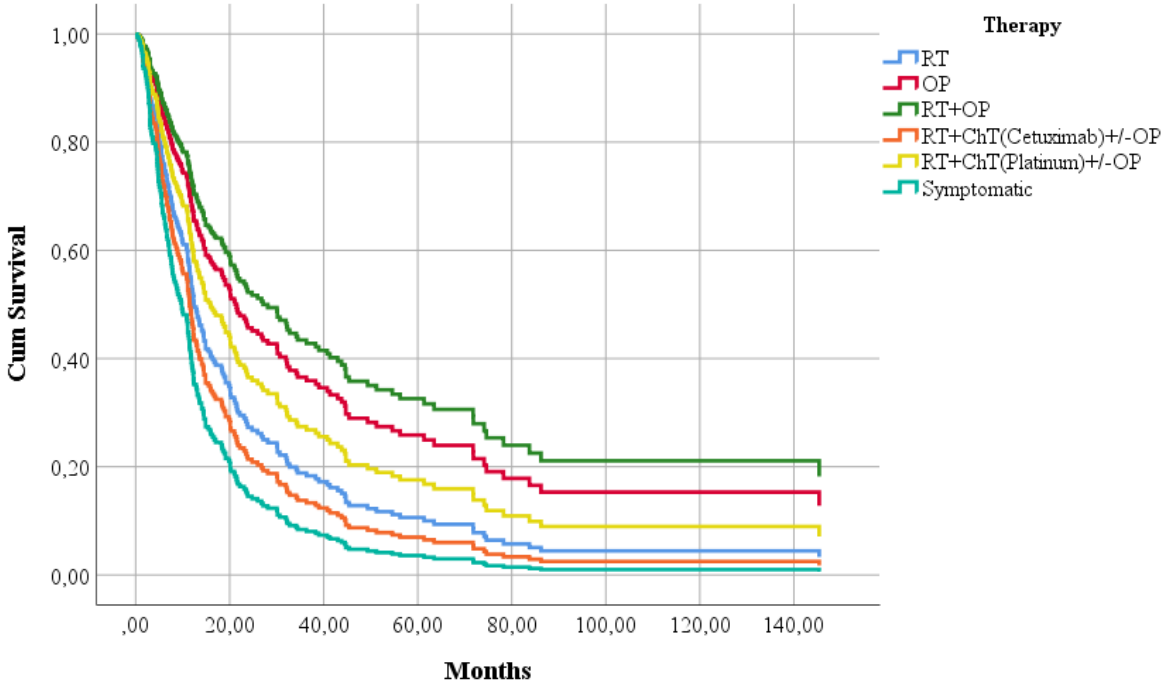

Figure 4.9 **Cox regression plot for cumulative survival. Covariates – sex, age group, T stage, N status, alcohol abuse and/or smoking, therapy, primary tumour location, histological variant. Plot for therapy**

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 prospective part

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

DNA extraction from FFPE tissue blocks using *blackPREP FFPE DNA Kit* was 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 Anyplex II HPV28 assay was 14/31 (45.2 %). In one case, there was 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 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, $p = 0.156$). 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 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, $p < 0.001$).

4.2.3 Comparison of the results of HPV viral loads

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 4.10.

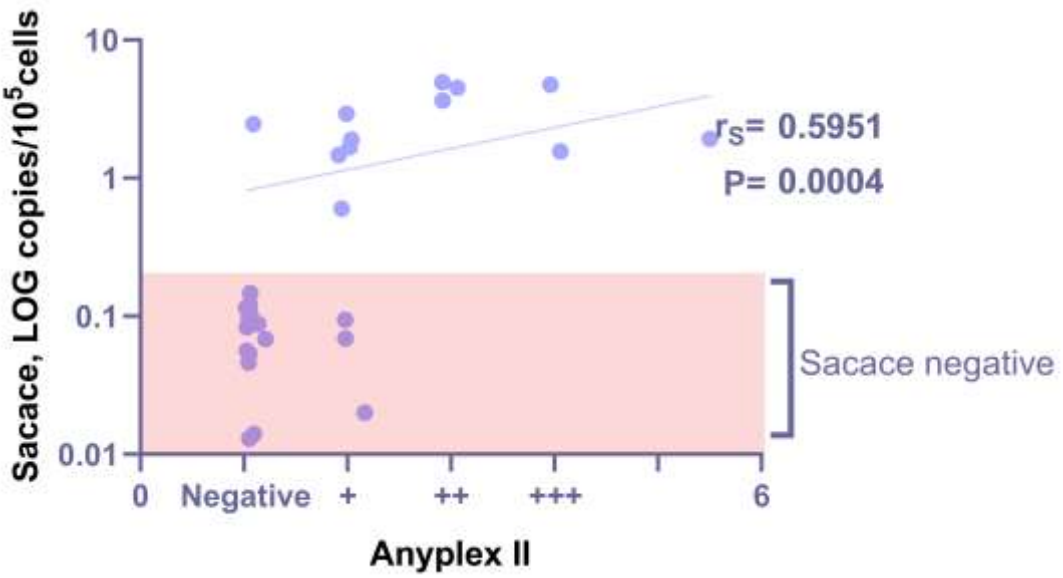

Figure 4.10 **Correlation of two real-time PCR assays**

4.3 The second publication of prospective part

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

The PCR analysis using consensus primers MY09/11 and GP5+/6+ to detect HPV DNA in tumour samples was conducted. 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

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+ samples, which include 61 samples that gave valid results with consensus PCRs and 2 additional samples with HPV16 L1 PCR, were applicable for further analysis.

4.3.3 HPV genotyping using Anyplex II HPV28 real-time PCR

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. All samples were positive for β-globin (internal control). Out of the 63 samples, 28 samples were HPV-negative when assessed by the Anyplex II HPV28 multiplex real-time PCR. 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. When the HPV+ samples were stratified by location, 19 LSCC and 13 HPSCC samples were found to be HPV16+, 2 LSCC samples demonstrated HPV16 and HPV31 coinfection, and 1 HPSCC sample demonstrated HPV16 and HPV56 coinfection.

Interestingly, 7 tumour tissue samples (1 LSCC and 6 HPSCC) that were confirmed as HPV16+ using HPV16 L1 primers' PCR were 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 out of 41 (53.7 %) for LSCC and 20 out of 31 (64.5 %) for HPSCC. All HPV16+ HPSCC samples were stage III or IV tumours. Figure 4.11A shows the distribution of HPV16+ samples stratified according to location and disease stage.

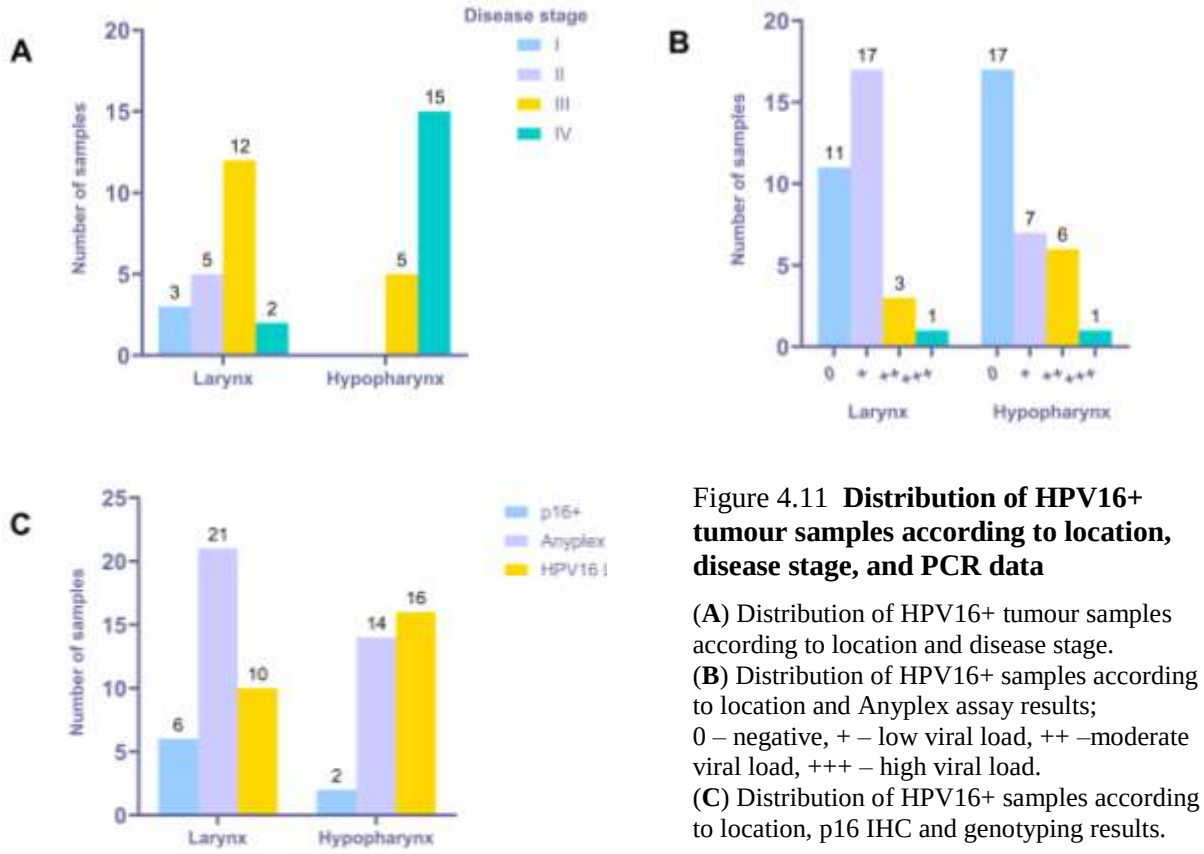

Figure 4.11 **Distribution of HPV16+ tumour samples according to location, disease stage, and PCR data**

(**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.

Among the HPV16+ samples, 21 samples showed low viral load, 9 samples showed moderate viral load, and 2 samples showed high viral load when detected using the Anyplex II assay. Three samples (1 HPSCC and 2 LSCC) showed multiple HR-HPV infections. The details of viral loads are summarised in Figure 4.11B.

4.3.4 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 out of 72 (9.7 %) were p16+/HPV+, 1 out of 72 was p16+/HPV−, 8 out of 72 (11.1 %) were p16−/HPV−, and 56 out of 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 4.11C).

4.3.5 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 out of 22 cases. The immunoreactive structures were observed within the tumour mass and the surface epithelium of the region of interest, 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 out of 22 cases, and two of them also showed strong positivity in the dysplastic epithelium. (Figure 4.12; Figure 4.20A, B).

Most LSCC samples (12 out of 22) showed low expression of E6 oncoprotein in the tumour mass (Figure 4.20A). 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 4.20B).

In most specimens, positive staining in the invasive front was noticed, commonly presented as the decoration of the suprabasal cells (Figure 4.13).

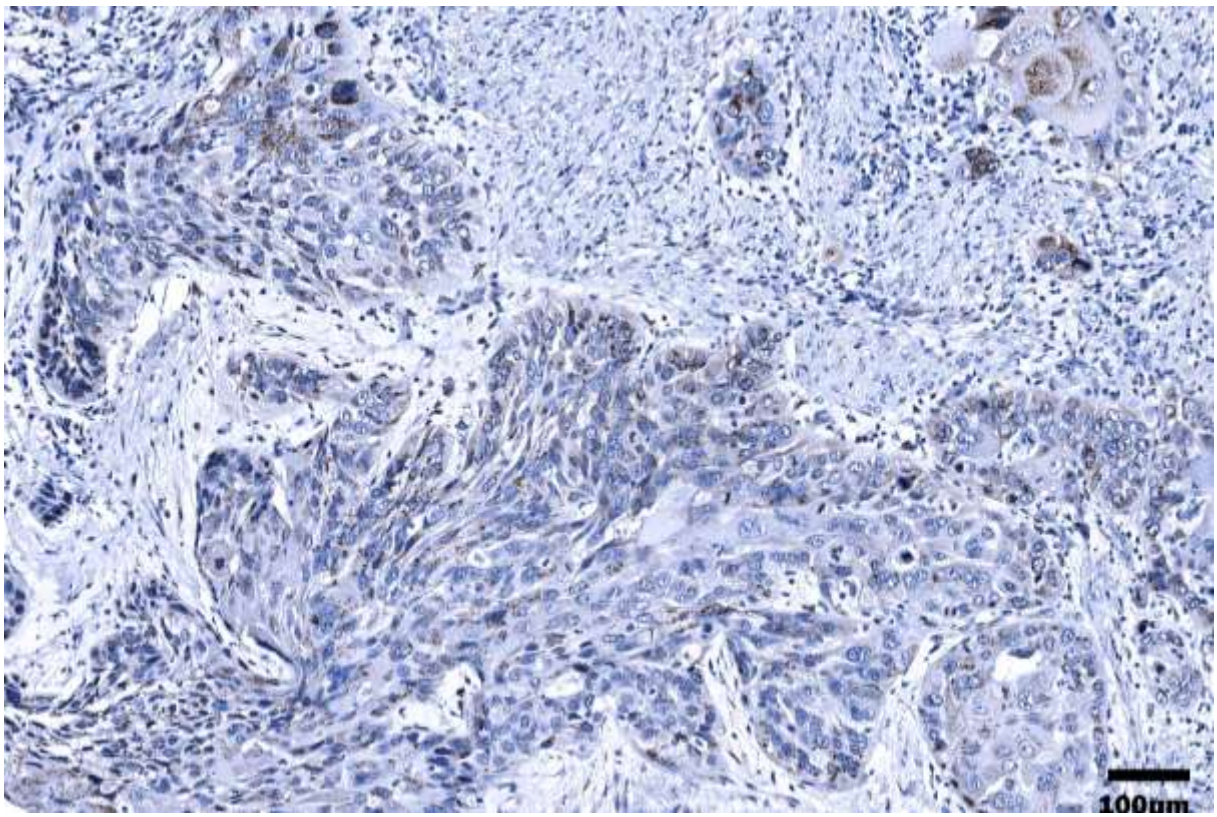

Figure 4.12 **IHC detection of HPV16 E6 oncoprotein, LSCC, tumour cords and nests comprised of diffusely distributed E6 protein-positive cells interspersed by the E6 oncoprotein-negative cells**

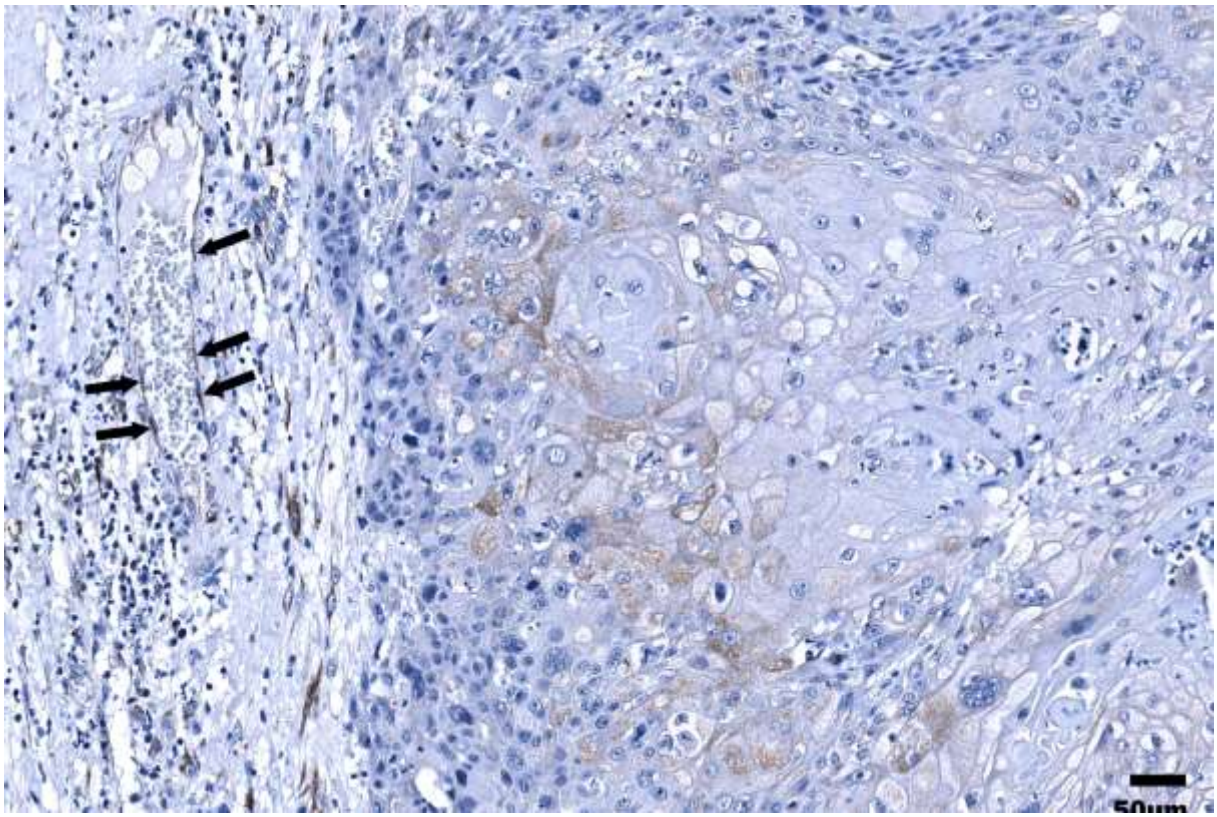

Figure 4.13 **IHC detection of HPV16 E6 oncoprotein, LSCC, HPV16 E6 positivity in suprabasal, more differentiated, tumour cells, E6-positive endothelial cells (black arrows)**

HPV16 E6 viral protein expression was also frequently observed in the endothelial cells of small blood vessels (Figure 4.13., 4.14).

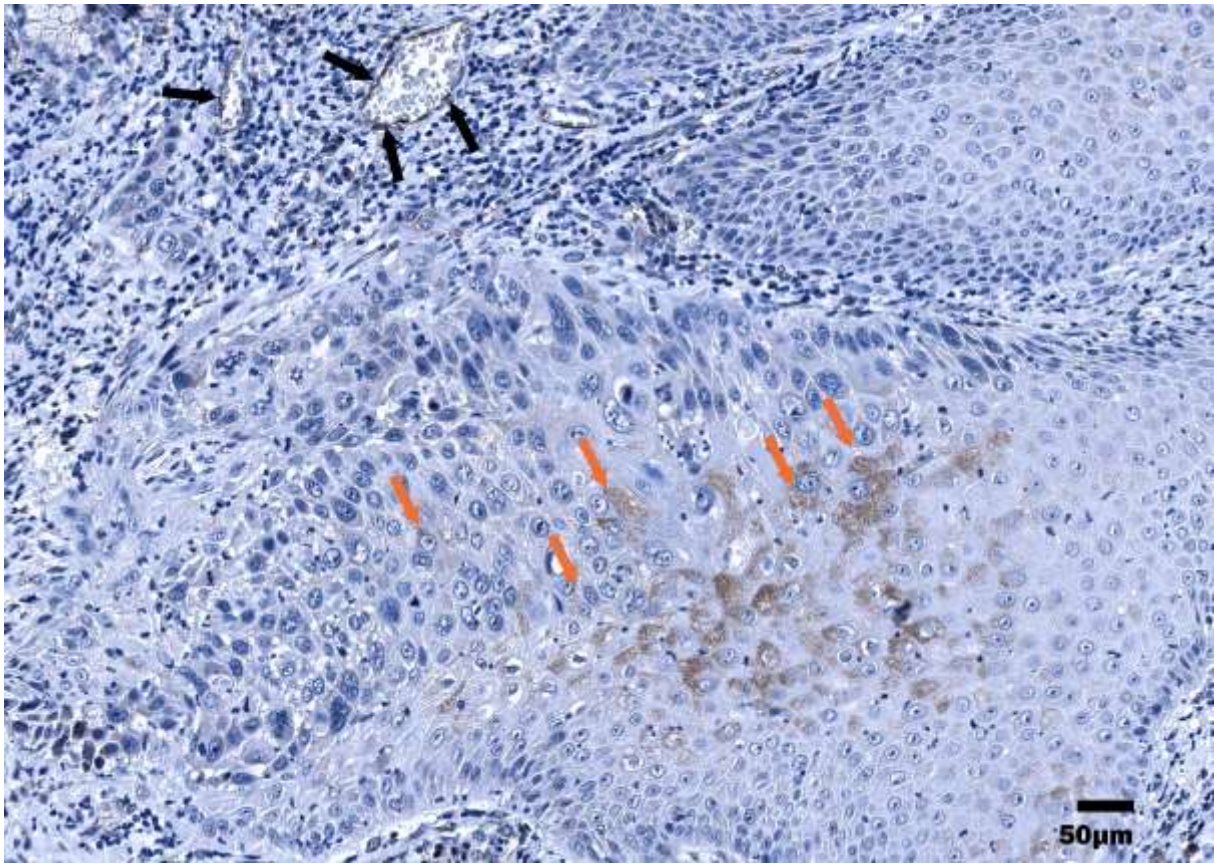

Figure 4.14 **IHC detection of HPV16 E6 oncoprotein, LSCC, differentiated suprabasal tumour cells demonstrating abundant HPV16 E6-positive cytoplasm and polymorphous nuclei (orange arrows), E6-positive endotheliocytes (black arrows) within a tumour stroma**

HPV16 E7 protein immunoexpression was confirmed in 20 out of 21 LSCC specimens (Figure 4.20C). 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 4.15, 4.16).

Strong immunoexpression of HPV16 E7 oncoprotein in the tumour nests was found in 8 out of 21 LSCC samples (Figure 4.17; Figure 4.20C, D). The presence of HPV16 E7 oncoprotein was also detected in the intimal aspect of small blood vessels.

In HPSCC samples, 18 out of 20 were positive for HPV16 E6 oncoprotein (Figure 4.20E, F). Most of the samples showed detectable levels of HPV16 E6 oncoprotein within the dysplastic epithelium, as indicated by cytoplasmic immunoreactivity. The expression of E6 oncoprotein within the tumour mass was generally low (Figure 4.18). Some endothelial cells in HPSCC samples also showed HPV16 E6 positivity.

A smaller number of HPSCC cases (13 out of 20) showed positivity for HPV16 E7 oncoprotein, primarily in the nucleus (Figure 4.20G, H). Positive reactions were observed in the tumour mass and differentiated suprabasal cells, as well as in endothelial cells (Figure 4.19).

Among the 42 samples, 7 (1 LSCC and 6 HPSCC) did not confirm the immunoexpression of HPV16 E6/E7 oncoproteins within the tumour mass. Only one HPSCC and one LSCC sample were negative for both, HPV16 E6 and E7, oncoproteins (tumour and dysplastic epithelium). Two samples showed matched HPV16 E6/E7 positivity, exclusively in the dysplastic epithelium. 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 4.20E). In general, a comparable pattern of HPV oncoprotein E6/E7 expression was observed within both the tumour mass and dysplastic epithelium in both LSCC and HPSCC, as illustrated in Figures 4.20A, C, and G.

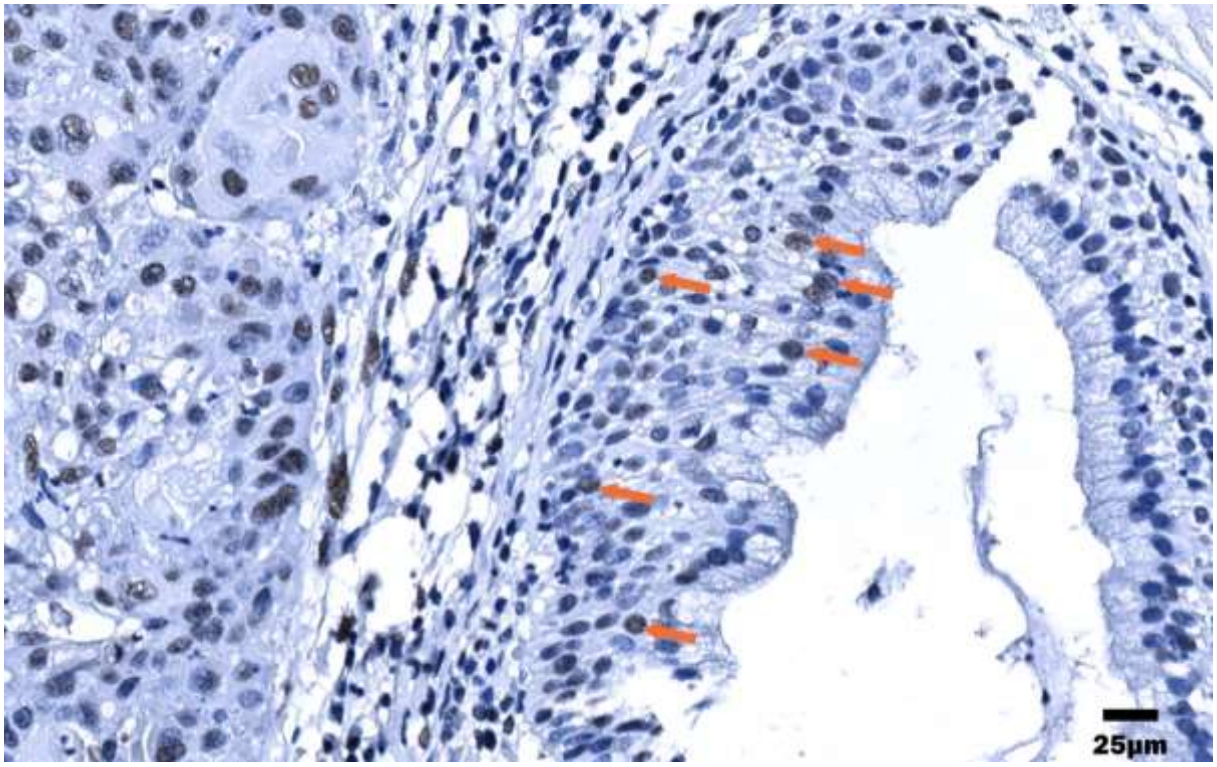

Figure 4.15 **IHC detection of HPV16 E7 oncoprotein; LSCC, tumour cells within a nest and some surface cells (orange arrows) demonstrating nuclear HPV16 E7 positivity**

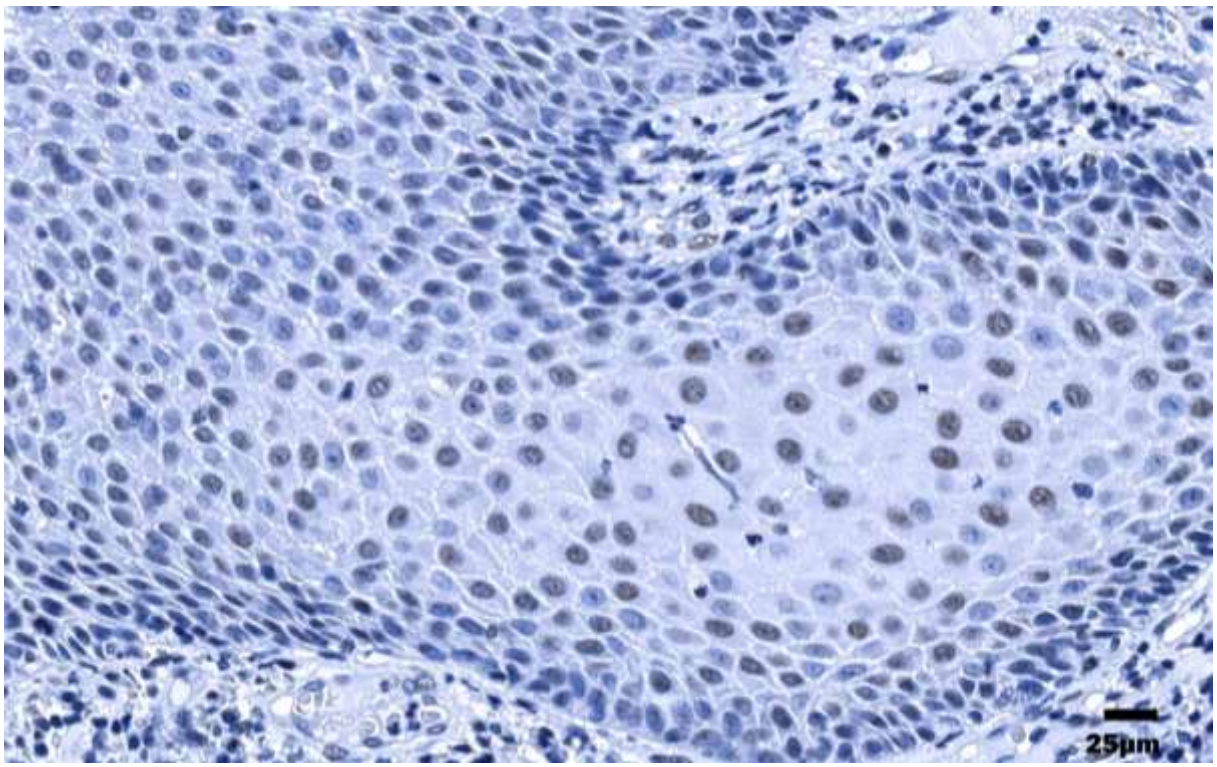

Figure 4.16 **IHC detection of HPV16 E7 oncoprotein; LSCC, numerous HPV16 E7-positive cells displaying nuclear immunostaining pattern**

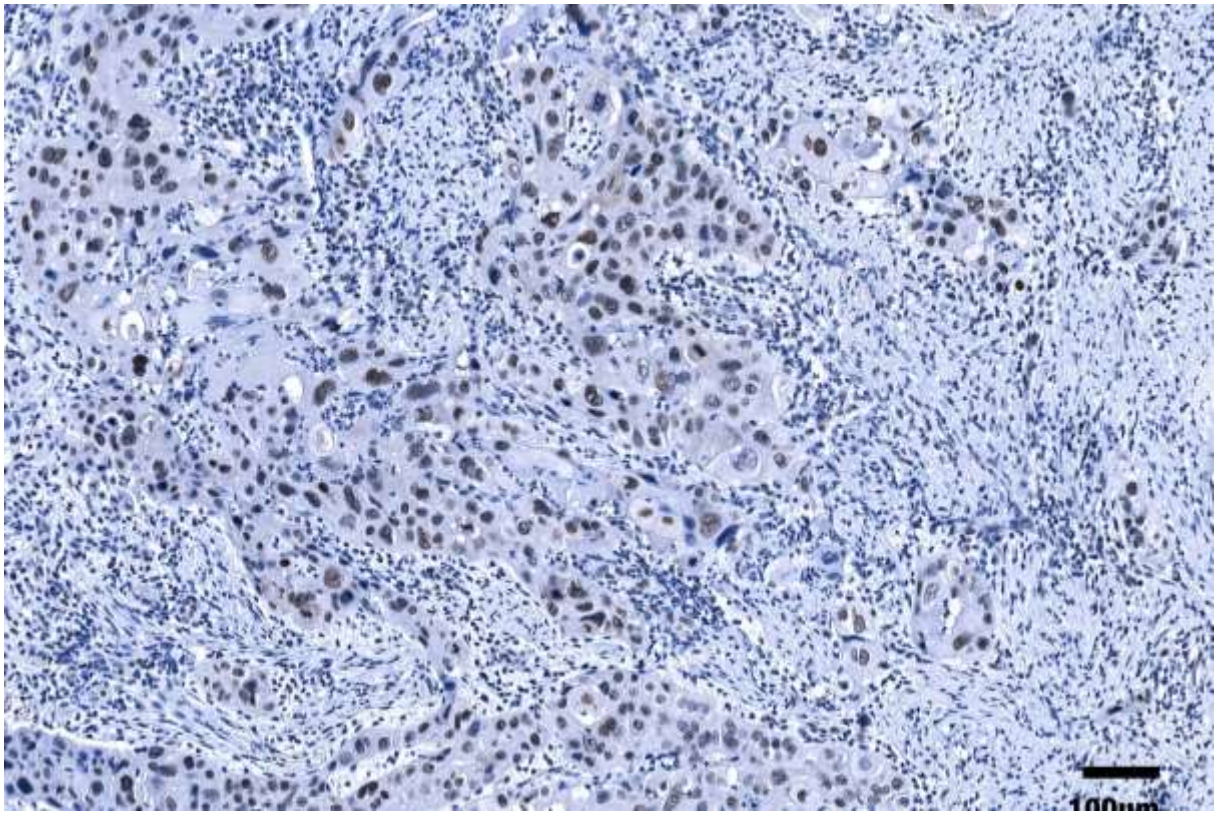

Figure 4.17 **IHC detection of HPV16 E7 oncoprotein; LSCC, highly polymorphous HPV16 E7-positive tumour cells demonstrating nearly total nuclear decoration**

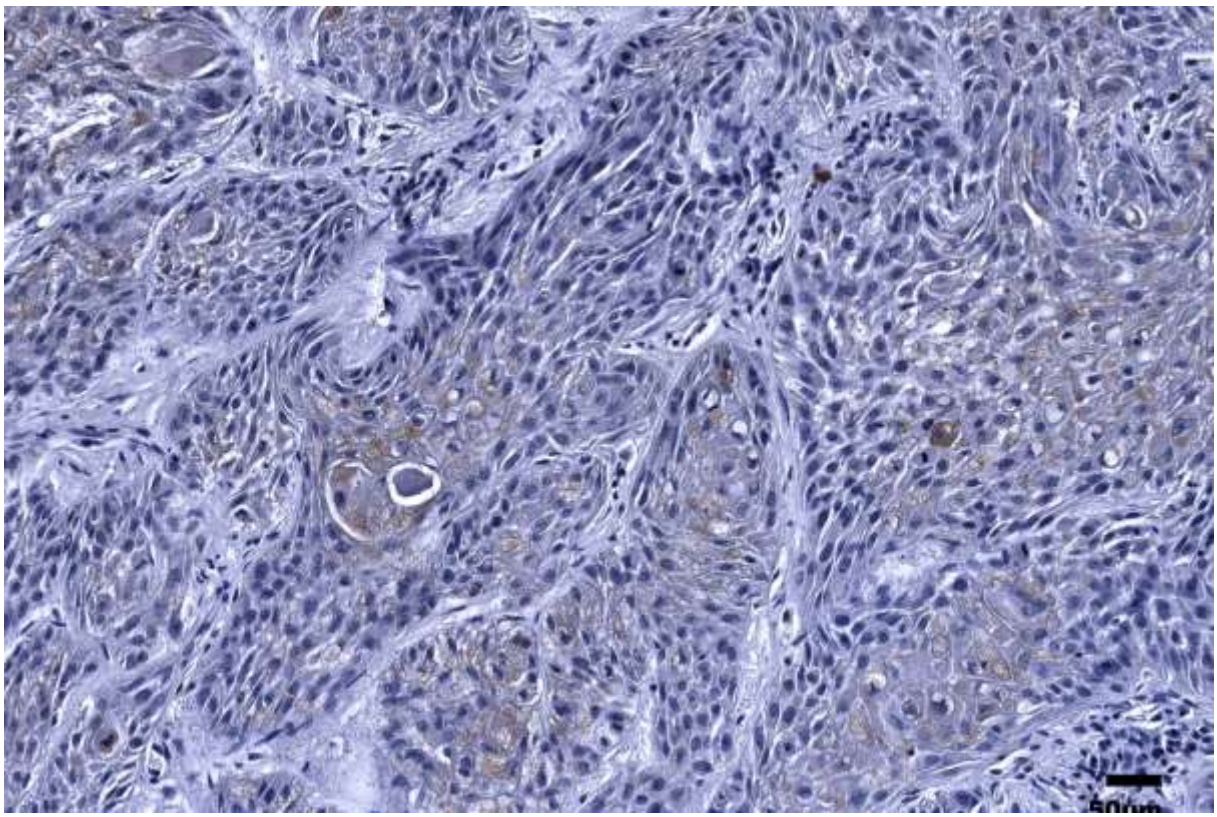

Figure 4.18 **IHC detection of HPV16 E6 oncoprotein; HPSCC, densely packed tumour cords demonstrating HPV16 E6 oncoprotein positivity, almost exclusively in more differentiated cells**

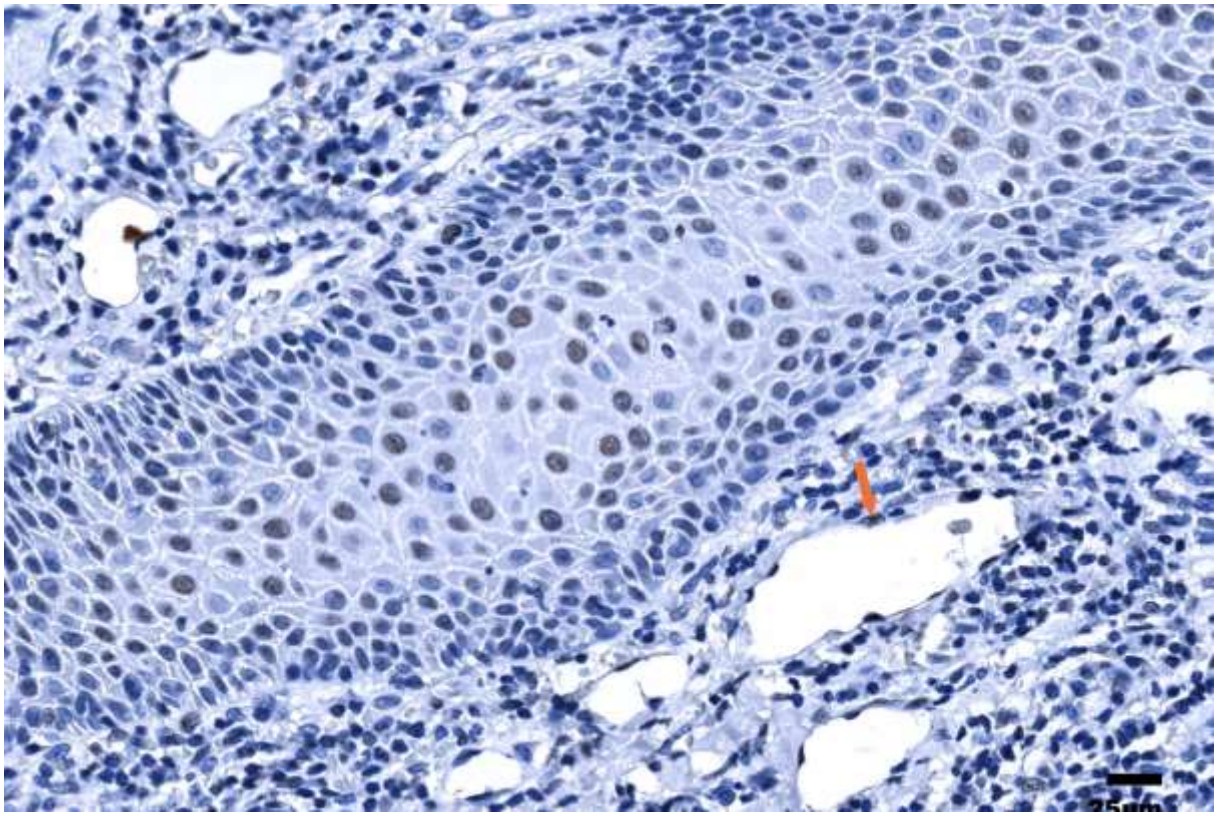

Figure 4.19 **IHC detection of HPV16 E7 oncoprotein; HPSCC, numerous HPV16 E7-positive cells displaying nuclear immunostaining pattern, endothelial (orange arrow) cells**

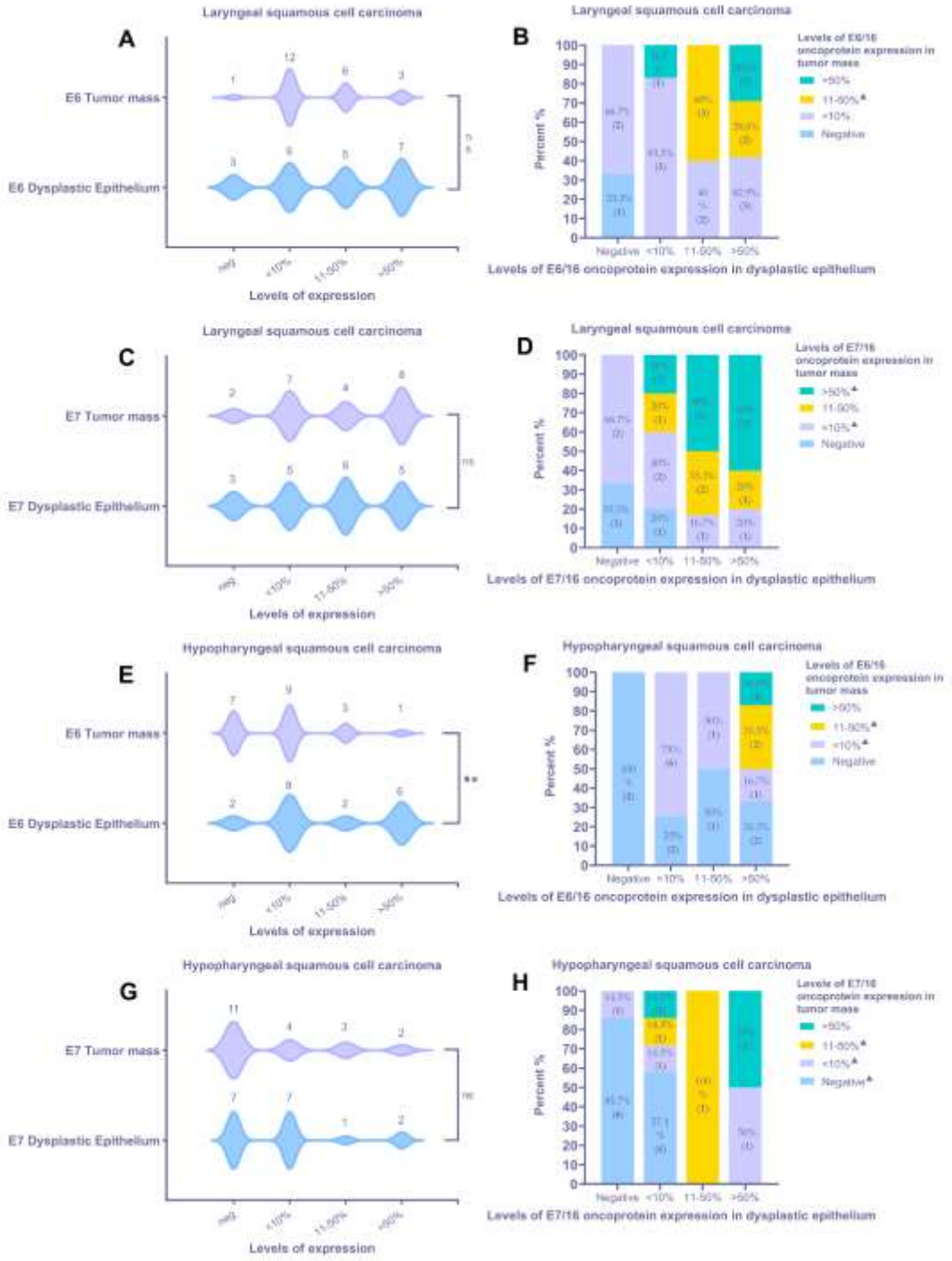

Figure 4.20 **Assessment of viral oncoproteins E6 and E7 in HPV16+ laryngeal and hypopharyngeal tumour tissue samples using IHC and statistics**

(**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.

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.

Immunofluorescence analysis confirmed the presence of HPV16 E7 in the cytoplasm and nuclei of the tumour cells (Figure 4.21).

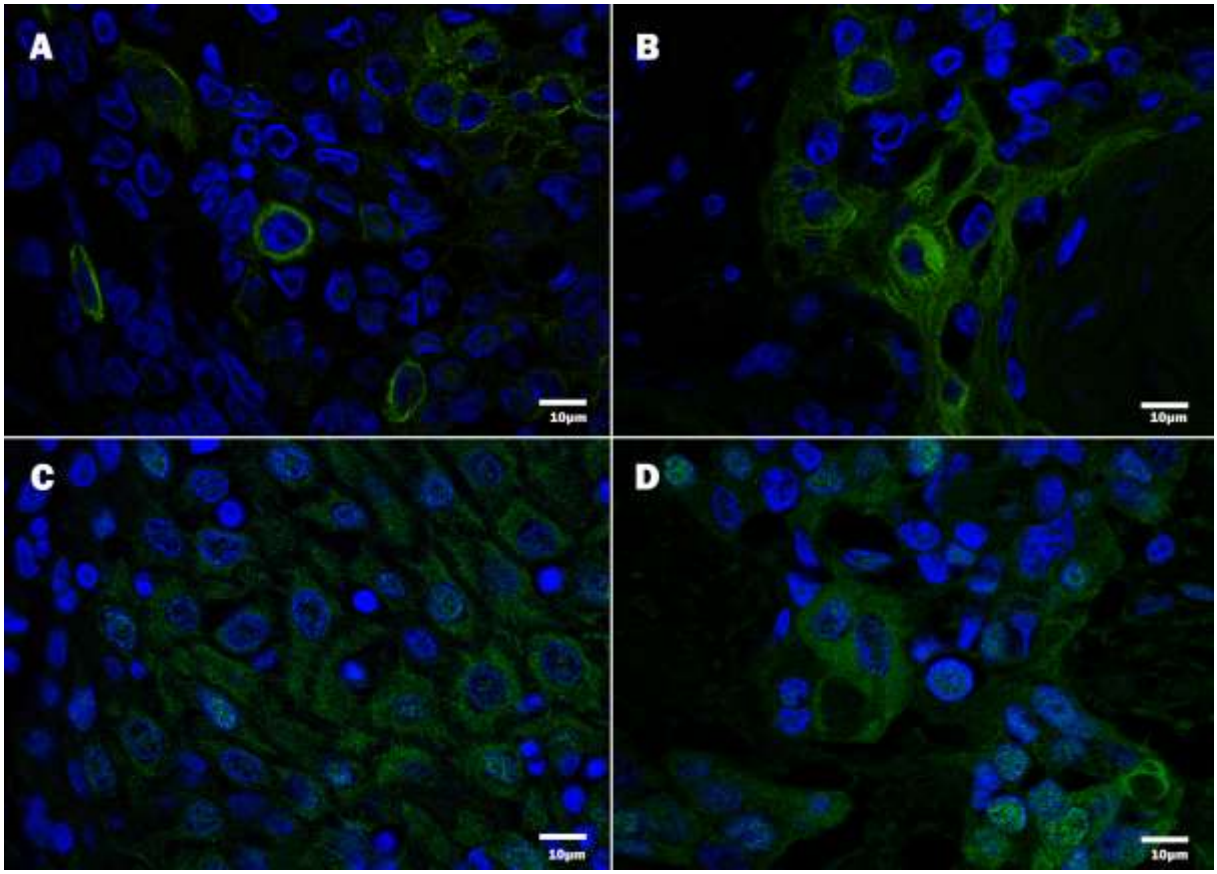

Figure 4.21 **Detection of HPV16 protein E7 by immunofluorescence, confocal microscopy**

DAPI—blue, HPV16 E7 immunopositive products—green: (**A, B**) HPV16 E7 positive tumour cells, displaying chiefly cytoplasmic positivity; (**C, D**) HPV16 E7 positive tumour cells, displaying cytoplasmic and nuclear positivity.

4.4 The third publication of 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. Out of 106 HNSCC samples, HPV DNA was detected in 92 samples (86.79 %). The presence of HPV DNA varied across different types of HNSCC: 29 out of 34 OPSCC samples (85.29 %), 32 out of 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 out of 106 HNSCC samples (65.09 %). HPV16 was prevalent in 26 out of 34 OPSCC samples (76.47 %), 22 out of 41 LSCC samples (53.66 %), and 20 out of 31 HPSCC samples (64.52 %). HPV coinfections with HPV16 were observed in 7 out of 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 samples

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 out of 26 OPSCC samples (57.7 %), 2 out of 22 LSCC samples (9 %), and none of the 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

Immunohistochemistry was performed to assess the overexpression of p16 in HNSCC samples (Figure 4.22). Among the 106 HNSCC samples, p16 overexpression was observed in 24 samples (22.64 %). Specifically, it was found in 16 out of 34 OPSCC samples (47.06 %), 6 out of 41 LSCC samples (14.63 %), and 2 out of 31 HPSCC samples (6.45 %). When considering HPV16 positivity, p16 overexpression was confirmed in 15 out of 26 HPV16-positive OPSCC samples (57.69 %), 5 out of 22 HPV16-positive LSCC samples (22.73 %), and 2 out of 20 HPV16-positive HPSCC samples (10 %).

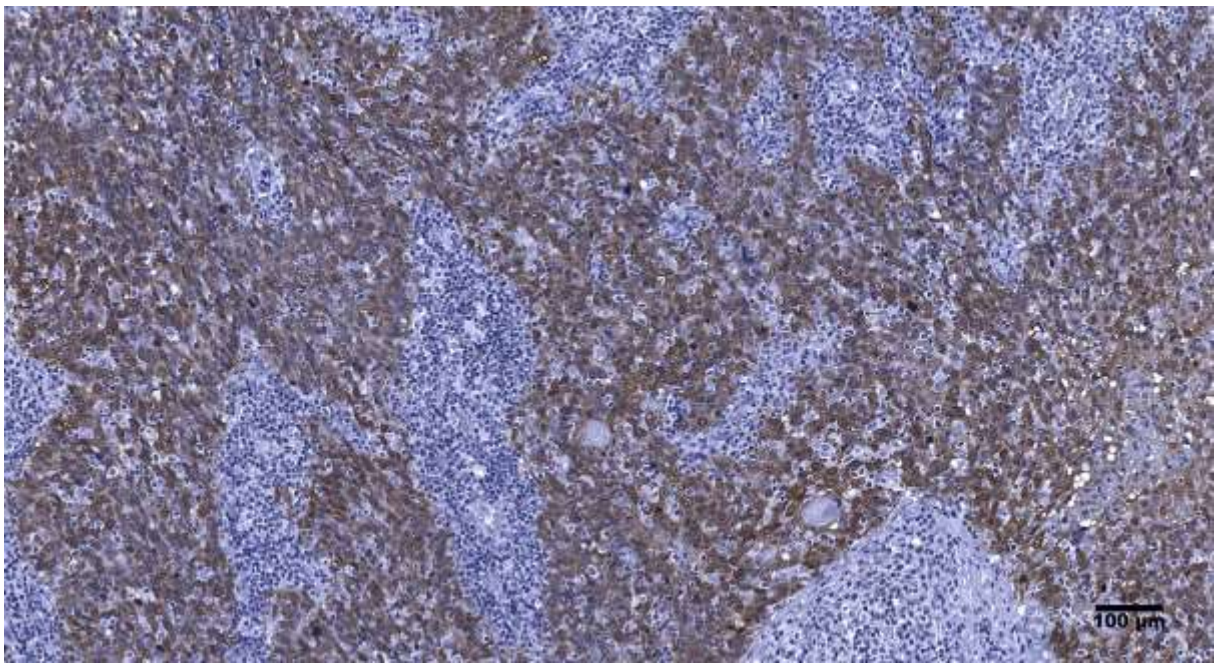

Figure 4.22 **IHC detection of p16 in OPSCC (palatine tonsil). Representative image from a case demonstrating >75 % p16-positive tumour cells displaying mostly nuclear and cytoplasmic expression**

4.4.4 Expression of p53 in HNSCC samples detected by IHC

The study revealed that p53 overexpression (p53+; Figure 4.23) was confirmed in 49 out of 106 HNSCC samples, accounting for 46.23 % of the cases. More specifically, among the different subtypes of HNSCC, p53 overexpression was observed in 17 out of 34 (50 %) of OPSCC samples, 21 out of 41 (51.22 %) LSCC samples, and 11 out of 31 (35.48 %) HPSCC samples.

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

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 out of 15 (73.33 %) OPSCC samples and 1 out of 2 (50 %) LSCC samples.

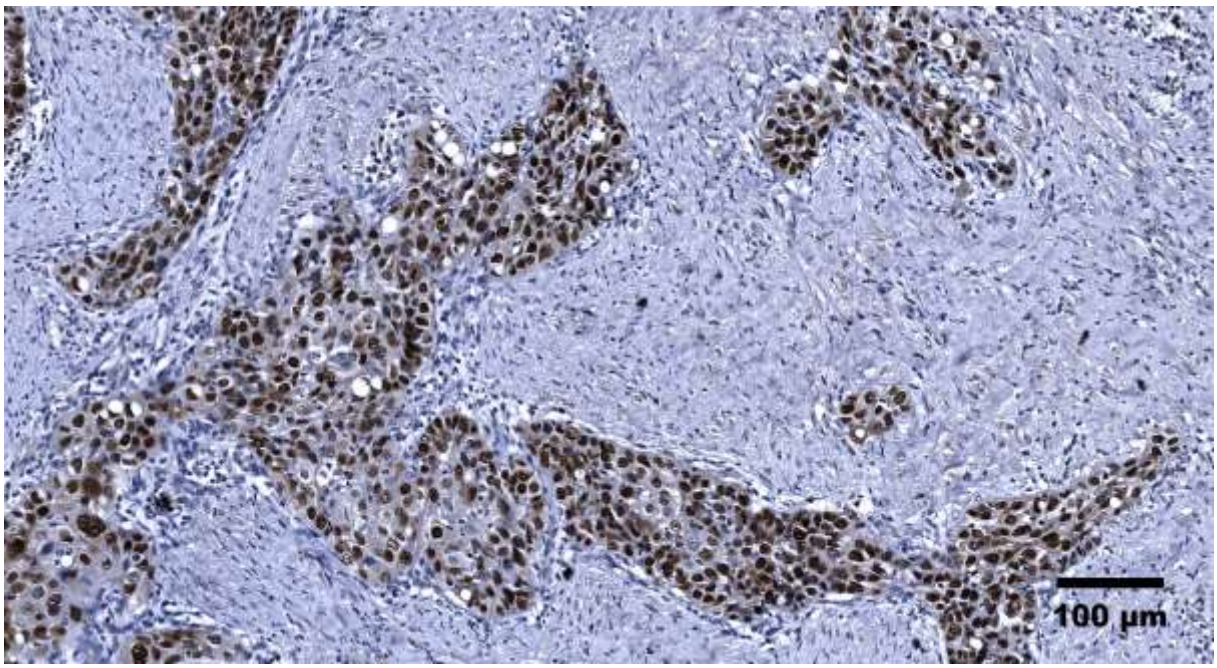

Figure 4.23 **IHC detection of p53 in LSCC. Representative image of p53 overexpression demonstrating uniform strong nuclear staining of tumour cells**

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

Overexpression of HPV16 E6 protein (Figure 4.24) was confirmed in 44 out of 106 (41.5 %) HNSCC samples. Specifically, it was observed in 21 out of 34 (61.8 %) OPSCC samples, 14 out of 41 (34.1 %) LSCC samples, and 9 out of 31 (29.0 %) HPSCC samples.

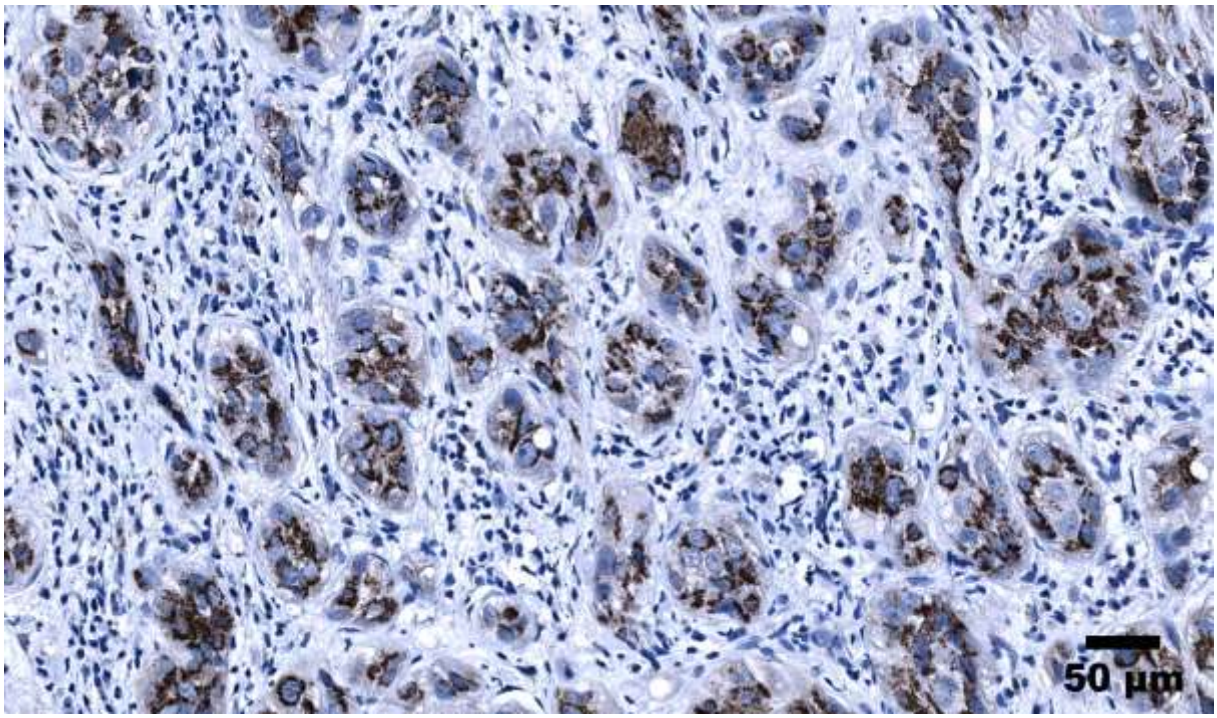

Figure 4.24 **IHC detection of HPV16 E6 in OPSCC (palatine tonsil). Representative image demonstrating cytoplasmic expression of HPV16 E6 protein confirmed in tumour cells organised in cords**

Similarly, overexpression of HPV16 E7 protein (Figure 4.25) was found in 39 out of 106 (36.8 %) HNSCC samples. More specifically, it was observed in 19 out of 34 (55.9 %) OPSCC samples, 14 out of 41 (24.1 %) LSCC samples, and 6 out of 31 (19.4 %) HPSCC samples.

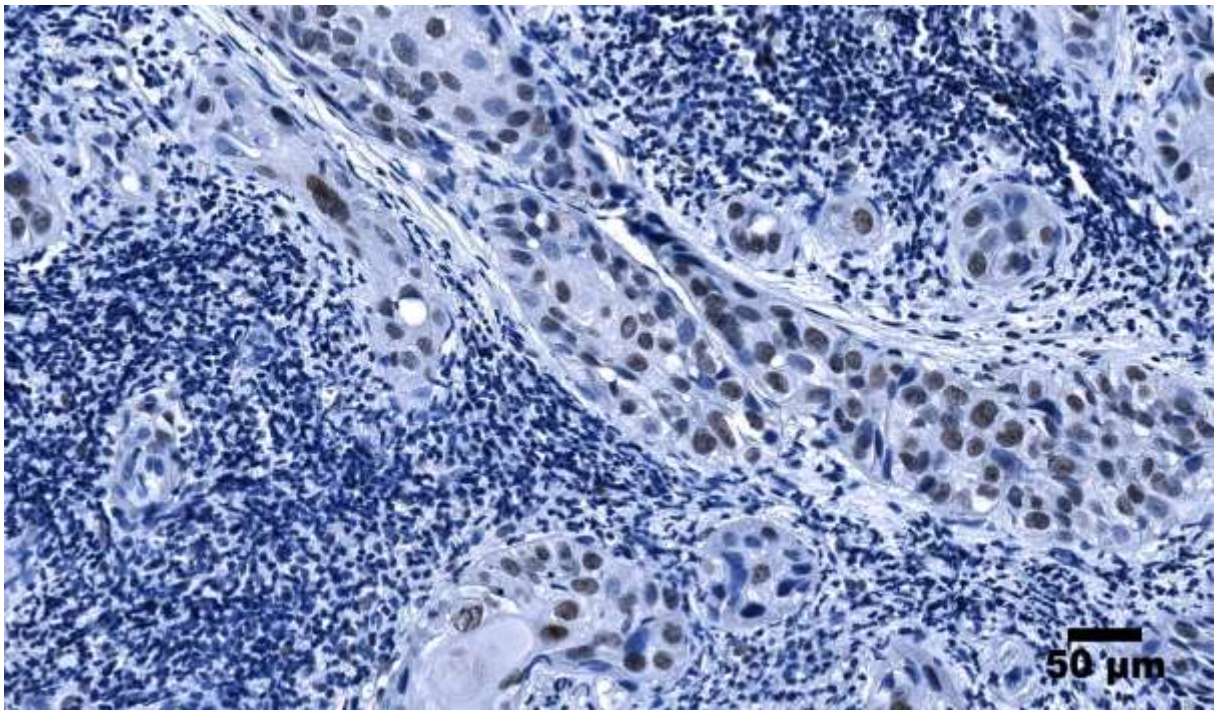

Figure 4.25 **IHC detection of HPV16 E7 in OPSCC (palatine tonsil). Representative image demonstrating nuclear expression of HPV16 E7 protein confirmed in the tumour cells organised as nests and cords**

4.4.6 Kaplan-Meier survival analysis

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

The five-year OS and DSS were assessed in HPV-positive and HPV-negative patients based on the primary tumour location. For patients with OPSCC, the OS rates were 26.82 % for HPV-positive patients and 0 % for HPV-negative patients, although the difference did not reach statistical significance ($p = 0.077$; Figure 4.26A). However, the DSS rates were 27.78 % for HPV-positive patients and 0 % for HPV-negative patients, showing statistical significance ($p < 0.05$; Figure 4.26B).

For patients with LSCC, the OS rates were 64.59 % for HPV-positive patients and 44.44 % for HPV-negative patients, demonstrating statistical significance ($p < 0.05$; Figure 4.26C). The DSS rates were 68.90 % for HPV-positive patients and 50% for HPV-negative patients, also showing statistical significance ($p < 0.05$; Figure 4.26D).

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

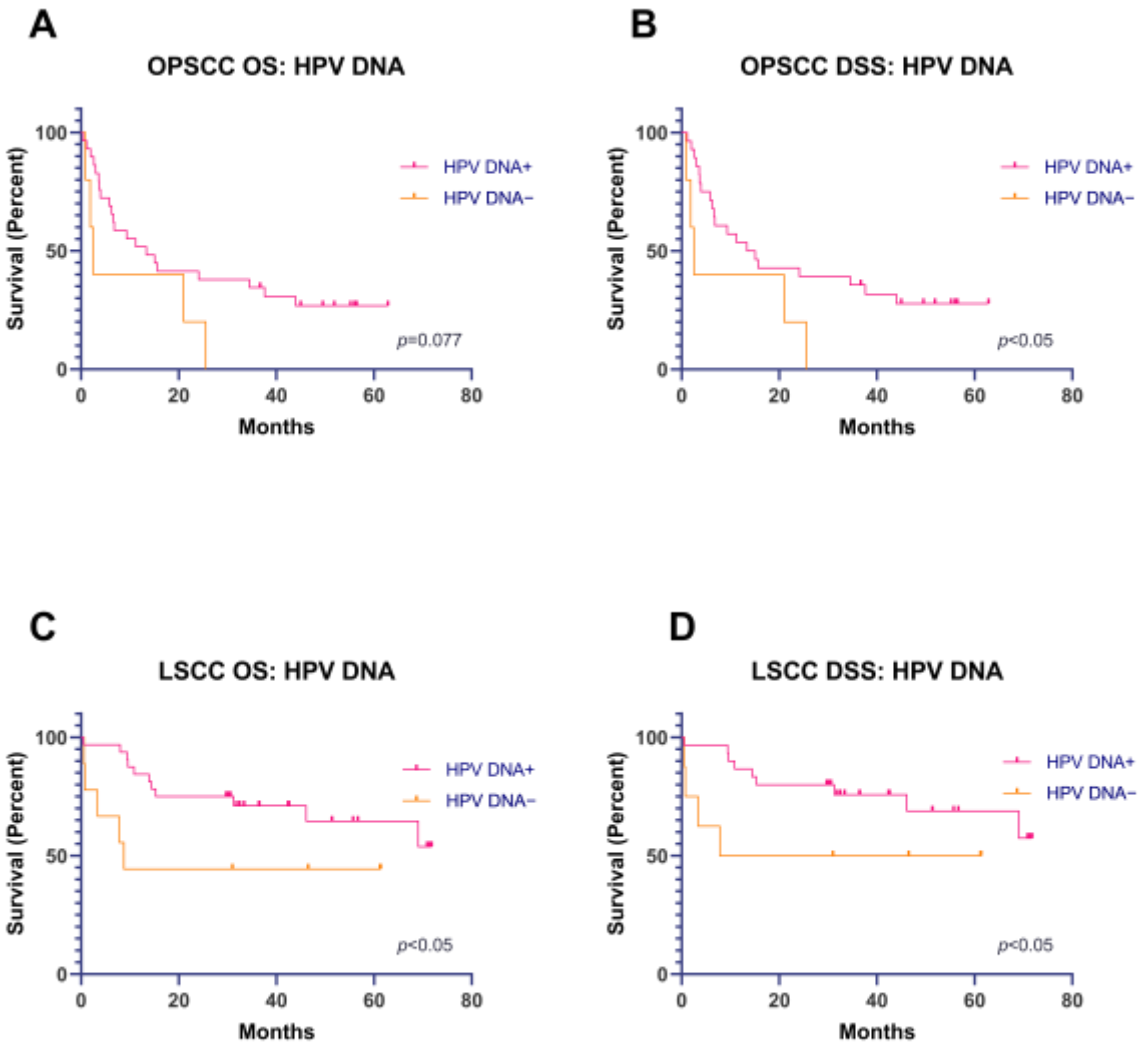

Figure 4.26 (**A, B) OS and DSS analyses (Kaplan–Meier), depending on the presence of HPV DNA (HR- and LR-) in OPSCC. (C, D) OS and DSS analyses (Kaplan–Meier), depending on the presence of HPV DNA (HR- and LR-) in LSCC**

4.4.8 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 4.27A, B) 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 4.27C, D) with significantly better survival for p53− group.

IHC overexpression of HPV16 E6 protein was associated with significantly better OS and DSS in patients with OPSCC (Figure 4.27E, F).

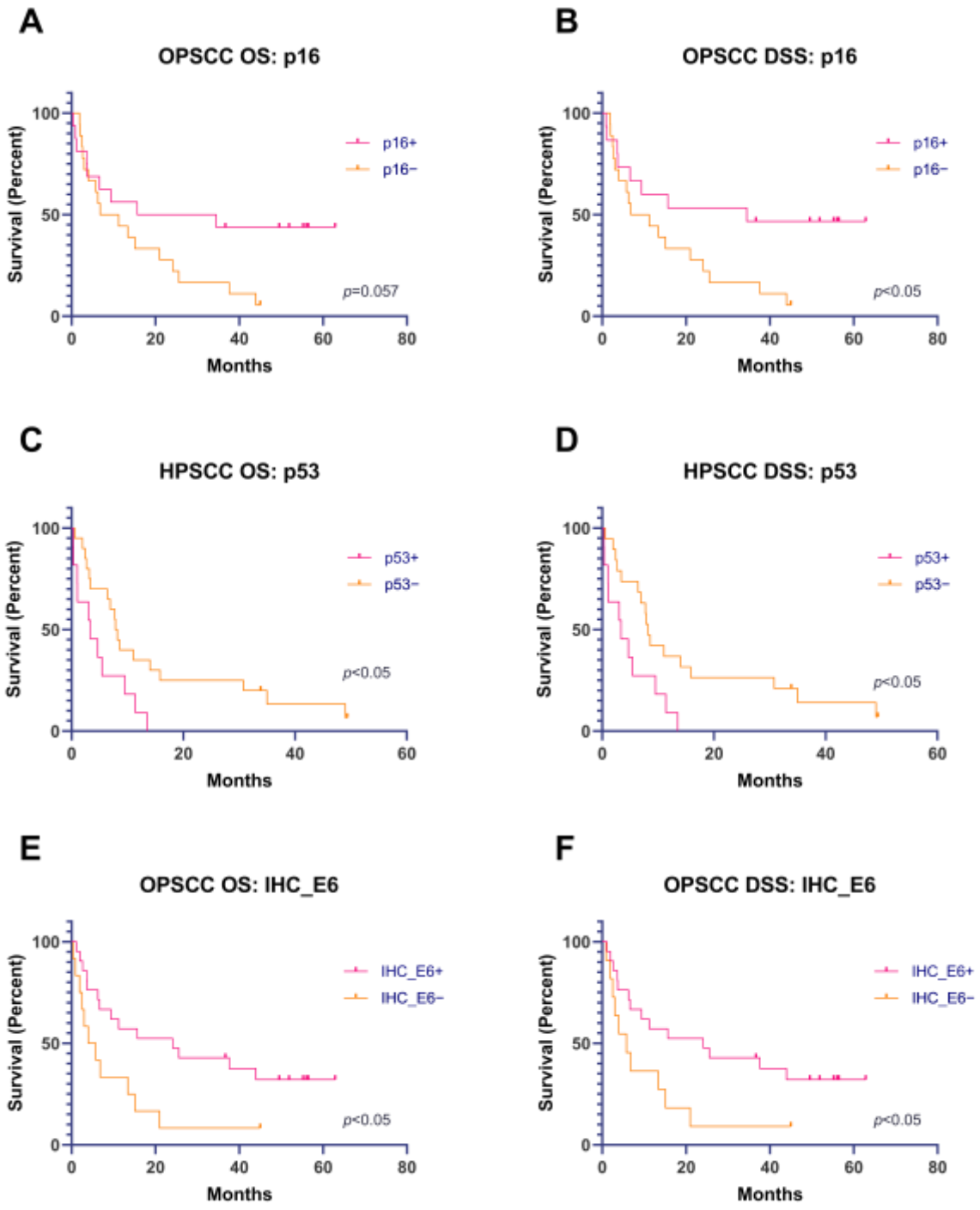

Figure 4.27 **(A, B) OS and DSS analyses (Kaplan–Meier), depending on the result of the IHC expression of p16 in OPSCC. (C, D) OS and DSS analyses (Kaplan–Meier), depending on the results of the IHC expression of p53 in HPSCC. (E, F) OS and DSS (Kaplan–Meier), de-pending on the results of the IHC expression of HPV16 E6 protein in OPSCC**

4.4.9 Multivariate Cox regression analysis

4.4.10 All HNSCC

A multivariate Cox regression analysis was performed for all patients with head and neck tumours (Table 4.5). The analysis included T, N, M, G, age, sex, applied treatment, IHC expression of p16, p53, E6 protein, and E7 protein, and the presence of HPV16 DNA and E6/E7 mRNA. The results showed that T1 tumours were associated with a lower risk of early death, and there was a trend toward higher early death risk with a higher T stage. A higher N stage was associated with a higher risk of early death, with N1 having a 4.98-fold greater risk compared to N0. Lower tumour differentiation grade (G) was associated with a higher risk of early death. Combined treatment (RT+ChT+/−OP) showed a lower risk of early death. IHC overexpression of HPV16 E6 protein was associated with a lower hazard ratio.

Table 4.5

Cox regression survival analysis for all HNSCC

Variables		**N = 106 $**	**β**	***p* ^**	**Survival**	
Name	**Groups ***				**Hazard Ratios (Exp(β))**	**95 % CI**
Sex	Female *	11			(1)	
	Male	92	1.024	0.0627	2.785	0.9886 to 8.688
Age			0.01344	0.4200	1.014	0.9810 to 1.048
p16	Negative *	81			(1)	
	Positive	22	0.5351	0.2412	1.708	0.6693 to 4.081
p53	Negative *	55			(1)	
	Positive	48	0.4982	0.1658	1.646	0.8141 to 3.358
IHC_E6	<10 % *	60			(1)	
	>10 %	43	−1.052	**0.0147**	0.3492	0.1464 to 0.8037
IHC_E7	<10 % *	65			(1)	
	>10 %	38	0.4807	0.2956	1.617	0.6590 to 4.024
Hazards	None *	15			(1)	
	Smoking	57	0.5992	0.2624	1.821	0.6636 to 5.526
	Smoking and alcohol abuse	31	−0.1794	0.7552	0.8358	0.2768 to 2.700
Location	Oropharynx *	32			(1)	
	Larynx	40	−0.7745	0.3747	0.4609	0.08271 to 2.545
	Hypopharynx	31	−0.5893	0.3045	0.5547	0.1772 to 1.693
T	1 *	10			(1)	
	2	18	−0.2886	0.6946	0.7493	0.1843 to 3.473
	3	44	0.5419	0.4727	1.719	0.4207 to 8.411
	4	31	0.9882	0.1683	2.686	0.7107 to 12.33

Table 4.5 continued

Variables		N = 106 $	β	*p* ˆ	Survival	
Name	**Groups ***				**Hazard Ratios (Exp(β))**	**95 % CI**
N	0 *	41			(1)	
	1	33	1.607	**0.0011**	4.988	1.944 to 13.55
	2	22	1.372	**0.0182**	3.943	1.277 to 12.62
	3	7	2.208	**0.0036**	9.098	2.042 to 40.88
M	0 *	98			(1)	
	1	5	1.104	0.1662	3.015	0.5851 to 13.67
G	1 *	15			(1)	
	2	76	−0.9494	**0.0413**	0.387	0.1556 to 0.9791
	3	12	−1.658	**0.0058**	0.1906	0.05657 to 0.606
HPV16 DNA	Negative *	37			(1)	
	Positive	66	0.4515	0.2491	1.571	0.7248 to 3.395
HPV16 E6/E7 mRNA	Negative *	87			(1)	
	Positive	16	−0.9763	0.1399	0.3767	0.09878 to 1.335
Treatment	RT *	37			(1)	
	OP	9	−1.042	0.3186	0.3528	0.03495 to 2.325
	RT+OP	35	−0.6763	0.2432	0.5085	0.1548 to 1.506
	RT+ChT (Cetuximab) +/−OP	9	−2.089	**0.0163**	0.1239	0.01986 to 0.6441
	Symptomatic	13	0.8416	0.0635	2.32	0.9130 to 5.507

* group of reference. $ Three were excluded due to missing values. ˆ statistically significant values ($p < 0.05$) are highlighted in bold.

4.4.11 OPSCC

The group consisted of 34 patients, of which 26 experienced events (death). Two patients were excluded from the analysis due to missing values. The Cox regression results for OPSCC are summarised in Table 4.6.

The Cox regression analysis revealed that several factors significantly affected the survival of OPSCC patients. These factors included the IHC expression of p16, p53, and HPV16 proteins E6 and E7, the T (tumour size), the applied treatment, and smoking.

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 tumours over-expressing p16 (p16+) had better survival outcomes compared to patients with p16-negative (p16−) tumours (Figure 4.28A). 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 (E7+) led to decreased survival, even in patients with p16+ tumours (Figure 4.28A).

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 4.28B). There was no difference in survival between patients with p53−/E6+ and p53+/E6− tumours.

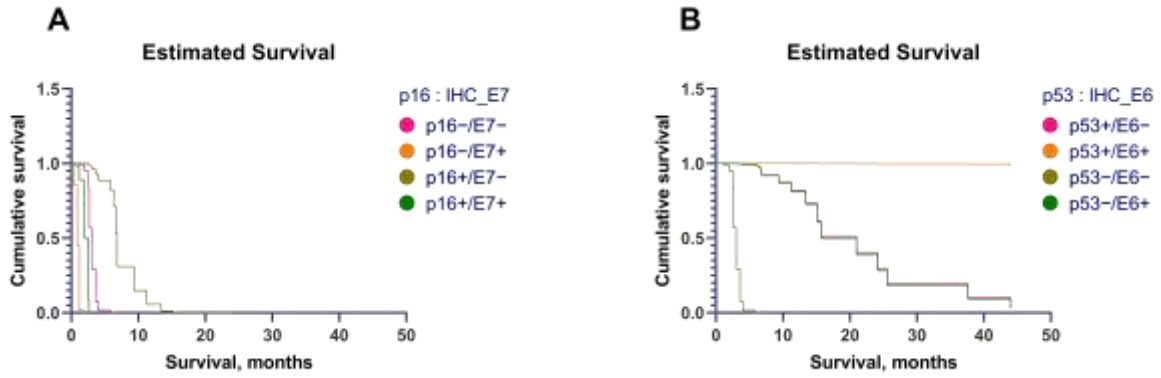

Figure 4.28 **(A) Estimated survival, depending on the IHC expression of p16 and E7 protein. (B) Estimated survival, depending on the IHC expression of p53 and E6 protein**

Additionally, the analysis showed that a larger tumour size had a negative impact on survival. Patients with larger tumours had a higher risk of early death. Regarding the lymph node involvement (N), the analysis suggested a lower risk of early death for tumours with lower N stage, although the difference was not statistically significant.

Furthermore, the type of treatment received by the patients was found to significantly influence survival outcomes. Patients who underwent radiotherapy had a significantly lower risk of early death compared to patients who received other treatment modalities.

Table 4.6

Cox regression survival analysis for OPSCC

Variables		N = 34 $	β	*p* ^	Survival	
Name	**Groups ***				**Hazard Ratios (Exp(β))**	**95 % CI**
Sex	Female *	7			(1)	
	Male	25	−3.121	0.0810	0.04411	0.001033 to 1.742
Age		32	−0.02128	0.8114	0.9789	0.8275 to 1.177
p16	Negative *	18			(1)	
	Positive	14	−3.548	**0.0532**	0.02879	0.0005461 to 0.9340
p53	Negative *	16			(1)	
	Positive	16	−6.206	**0.0028**	0.002018	1.930×10^{-5} to 0.08535
IHC_E6	<10 % *	12			(1)	
	>10 %	20	−6.171	**0.0265**	0.002089	1.830×10^{-6} to 0.1431
IHC_E7	<10 % *	14			(1)	
	>10 %	18	6.154	**0.0355**	470.6	8.716 to 604,132
Hazards	None *	8			(1)	
	Smoking	8	8.18	**0.0323**	3568	3.203 to 10,181,954
	Smoking and alcohol abuse	16	5.424	0.0801	226.9	0.4392 to 139,247
T	1	6	−4.794	**0.0137**	0.008275	0.0001011 to 0.2757
	2	6	−7.933	**0.0010**	0.0003588	1.456×10^{-6} to 0.02453
	3	4	−5.286	**0.0480**	0.00506	5.478×10^{-6} to 0.2114
	4 *	16				
N	0	1	−29.16	>0.9999	2.166×10^{-13}	-
	1	13	0.5427	0.7926	1.721	0.02756 to 200.9
	2	12	−3.366	0.1093	0.03453	0.0001571 to 1.714
	3 *	6			(1)	
M	0	32	-	-	-	-
G	1 *	5			(1)	
	2	21	1.356	0.4788	3.882	0.08016 to 198.0
	3	6	−0.8802	0.6145	0.4147	0.007811 to 11.00
HPV16 DNA	Negative *	8			(1)	
	Positive	24	1.07	0.4826	2.914	0.1090 to 47.14
HPV16 E6/E7 mRNA	Negative *	18			(1)	
	Positive	14	−1.53	0.3384	0.2166	0.003954 to 4.418
Treatment	RT *	15			(1)	
	OP	0	-	-	-	-
	RT+OP	2	8.757	**0.0100**	6352	7.160 to 9,678,504
	RT+ChT (Cetuximab) +/−OP	9	1.005	0.6443	2.731	0.06476 to 538.3
	Symptomatic	6	9.218	**0.0003**	10072	154.8 to 6,028,349

* group of reference. $ Two were excluded due to missing values. ^ Statistically significant values ($p < 0.05$) are highlighted in bold.

4.4.12 LSCC

The Cox regression analysis for LSCC did not show any variables significantly affecting survival (Table 4.7).

Table 4.7

Cox regression survival analysis for LSCC

Variables		N = 41 [$]	β	*p*	Survival	
Name	Groups *				Hazard Ratios (Exp(β))	95 % CI
Sex	Female *	2			(1)	
	Male	38	1.705	0.3963	5.503	0.09481 to 377.9
Age		40	0.0283	0.7890	1.029	0.8258 to 1.273
p16	Negative *	34			(1)	
	Positive	6	1.467	0.2088	4.336	0.4193 to 59.81
p53	Negative *	19			(1)	
	Positive	21	2.395	0.2148	10.97	0.2552 to 852.0
IHC_E6	<10 % *	26			(1)	
	>10 %	14	3.339	0.1686	28.2	0.5441 to 7569
IHC_E7	<10 % *	26			(1)	
	>10 %	14	1.518	0.3083	4.561	0.1710 to 86.44
Hazards	None *	4			(1)	
	Smoking	29	−5.135	0.0780	0.005887	9.676×10^{-6} to 0.9809
	Smoking and alcohol abuse	7	−6.277	0.0620	0.001879	1.366×10^{-6} to 0.8238
T	1	4	−8.155	0.0872	0.0002874	6.497×10^{-9} to 1.257
	2	5	−35.47	>0.9999	3.952×10^{-16}	-
	3	24	−3.532	0.2137	0.02925	0.0001130 to 9.026
	4 *	3			(1)	
N	0*	34			(1)	
	1	4	3.649	0.2089	38.43	0.1891 to 35704
	2	2	3.630	0.1058	37.73	0.4809 to 5214
	3	0	–	–	–	–
M	0	39	–	–	–	–
	1	1	–	–	–	–
G	1 *	4			(1)	
	2	34	−6.052	0.0701	0.002354	9.408×10^{-7} to 0.7631
	3	2	−33.81	>0.9999	2.08×10^{-15}	–
HPV16 DNA	Negative *	18			(1)	
	Positive	22	−4.551	0.1606	0.01055	1.135×10^{-5} to 4.306
HPV16 E6/E7 mRNA	Negative *	38			(1)	
	Positive	2	−29.46	>0.9999	1.612×10^{-13}	–
Treatment	RT *	1			(1)	
	OP	9	−0.3476	0.9027	0.7064	0.001802 to 275.3
	RT+OP	29	1.71	0.3728	5.53	0.1326 to 681.2
	RT+ChT (Cetuximab) +/−OP	0	–	–	–	–
	Symptomatic	1	4.611	0.1628	100.6	0.1665 to 90589

* group of reference. [$] One was excluded due to missing values.

4.4.13 HPSCC

The group consisted of 31 patients, of which 29 experienced events (death). The Cox regression analysis for HPSCC is summarised in Table 4.8.

The Cox regression model indicated that several factors statistically significantly affected the survival of HPSCC patients. These factors included the 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 4.29A, C). 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 4.29B, overlaying of the curves). 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 4.29D). The group of patients with p53−/E6− tumours had the worst survival outcomes.

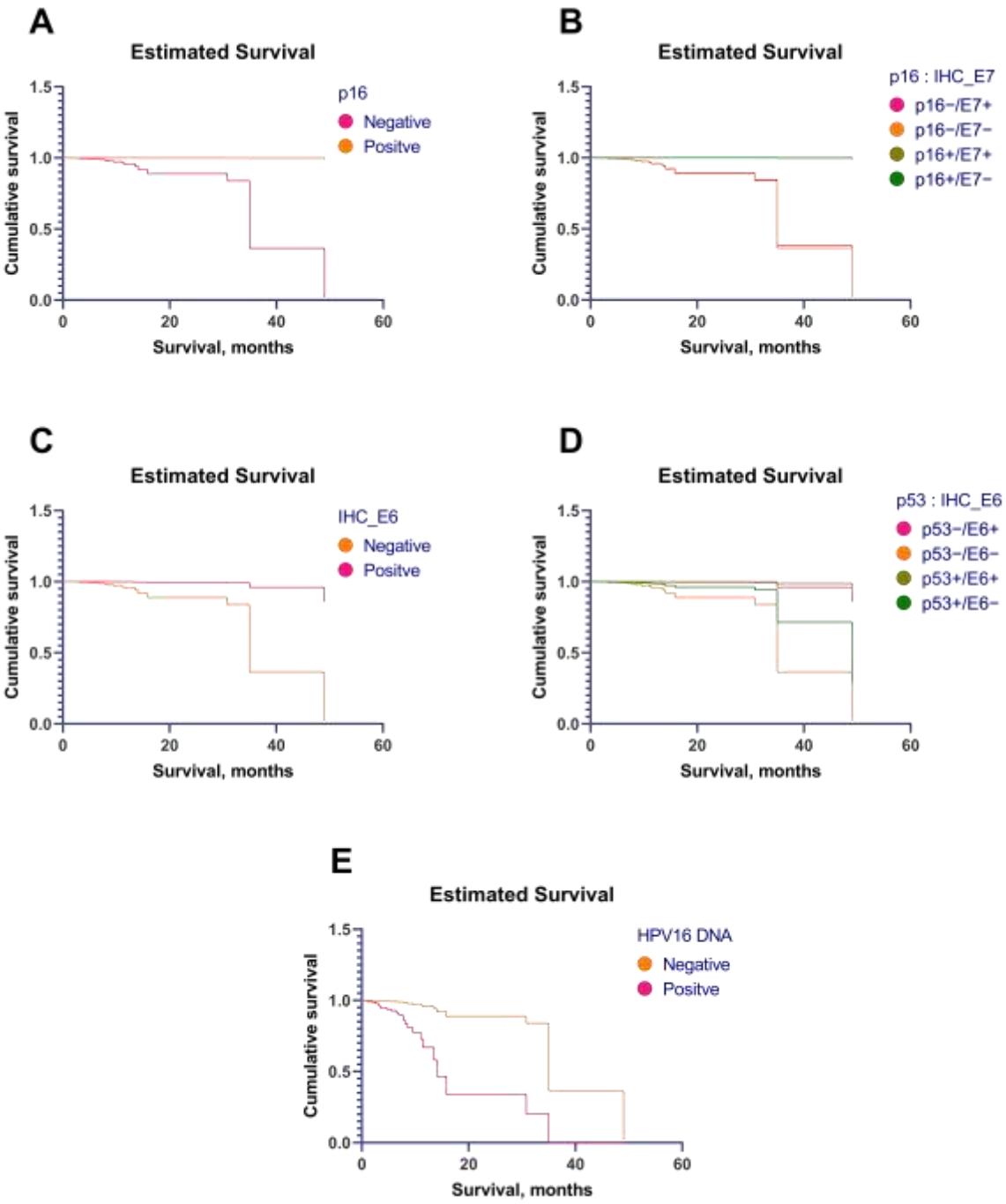

Figure 4.29 **(A) Estimated survival (Cox regression), depending on the IHC expression of p16. (B) Estimated survival (Cox regression) depending on the IHC expression of p16 and HPV16 E7 protein. (C) Estimated survival (Cox regression), depending on the IHC expression of HPV16 E6 protein. (D) Estimated survival (Cox regression), depending on the IHC expression of p53 and HPV16 E6 protein. (E) Estimated survival (Cox regression), depending on the presence of HPV16 DNA**

Furthermore, the presence of HPV16 DNA was associated with a significantly higher early death risk (Figure 4.29E). 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 (regional lymph node involvement) was associated with lower hazard ratios, indicating a reduced risk of early death. Lastly, the presence of distal metastases was found to be strongly associated with a 22-fold increase in the risk of death.

Moreover, it was noted that smoking patients had a 57-fold increase in the risk of early death compared to non-smokers/non-drinkers.

Table 4.8

Cox regression survival analysis for HPSCC

Variables					Survival	
Name	**Groups ***	**N = 31**	**β**	***p* ˆ**	**Hazard Ratios (Exp(β))**	**95 % CI**
Sex	Female *	2			(1)	
	Male	29	1.92	0.2873	6.823	0.2575 to 478.6
Age		31	0.0571	0.2702	1.059	0.9605 to 1.194
p16	Negative *	29			(1)	
	Positive	2	−6.638	**0.0049**	0.001309	5.631×10^{-6} to 0.08768
p53	Negative *	20			(1)	
	Positive	11	−1.099	0.2540	0.333	0.04332 to 2.109
IHC_E6	<10 % *	22			(1)	
	>10 %	9	−3.211	**0.0108**	0.04033	0.002158 to 0.3739
IHC_E7	<10 % *	25			(1)	
	>10 %	6	−0.04985	0.9711	0.9514	0.04166 to 10.39
Hazards	None *	3			(1)	
	Smoking	20	4.049	**0.0214**	57.36	2.263 to 3407
	Smoking and alcohol abuse	8	−0.8085	0.6127	0.4455	0.01394 to 9.424
T	1	0	-	-	-	-
	2	4	2.196	0.0950	8.986	0.7027 to 155.3
	3	16	−2.026	**0.0240**	0.1319	0.02240 to 0.7996
	4 *	11			(1)	
N	0 *	6			(1)	
	1	16	−2.825	**0.0421**	0.05932	0.003106 to 0.8054
	2	8	−2.719	**0.0235**	0.06597	0.005426 to 0.6552
	3	1	4.872	**0.0108**	130.6	2.628 to 7490
M	0 *	27			(1)	
	1	4	3.091	**0.0274**	21.99	1.535 to 460.4
G	1 *	6			(1)	
	2	21	−2.035	0.0912	0.1307	0.01189 to 1.553
	3	4	−2.087	0.1338	0.124	0.006553 to 1.883
HPV16 DNA	Negative *	11			(1)	
	Positive	20	2.205	**0.0194**	9.071	1.578 to 70.34
HPV16 E6/E7 mRNA	Negative *	31	-	-	-	-
	Positive	0	-	-	-	-
- Treatment	RT *	21			(1)	
	OP -	0	-	-	-	- -
	RT+OP	4	1.563	0.1378	4.771	0.5985 to 42.89
	RT+ChT (Cetuximab) +/−OP	0	-	-	-	-
	Symptomatic	6	0.17	0.8610	1.185	0.1367 to 7.244

* group of reference. ˆ Statistically significant values ($p < 0.05$) are highlighted in bold

5 Discussion

5.1 Lower T stage, lack of locoregional metastases, absence of bad habits, and surgical treatment result in improved survival rates and lower hazard ratios in OPSCC; HPV status should be assessed to conduct a more comprehensive prognosis assessment (retrospective study).

Survival analysis was conducted on patients with OPSCC treated at a single hospital in Latvia over a 10-year period. 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 (Winkelstein, 1990; Benhamou et al., 1992; 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). The study's survival estimations align with the importance of early diagnosis and support the need for timely intervention.

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 (Gatta et al., 2015; Gillison et al., 2019).

Tumours of the pharyngeal wall and palatine tonsils were associated with the worst OS and DSS outcomes, consistent with previous literature (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 showed better OS and DSS estimates compared to other modalities, with the best outcomes observed in the RT+OP group. While there were no significant differences in survival based on the specific type of surgery performed, significant differences were observed when any surgical intervention was compared to no surgery at all. Nevertheless, it is important to acknowledge that the study groups used in our research were characterised by unequal and relatively small numbers of patients. Reviewing the existing literature, it has become evident that surgical treatment has emerged as the essential and preferred treatment 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 (Rades et al., 2011; 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., 2017). Münscher et al. conducted a study that suggested the 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 (Cho et al., 2011; Lanzer et al., 2012; Donaduzzi et al., 2014; 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 SCC of the head and neck 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 radiotherapy 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 play a crucial role in selecting the appropriate treatment for patients with OPSCC. The tumour size, therapeutic modality “RT+OP”, hazardous habits (smoking, alcohol abuse), and the presence of locoregional lymph node metastases were identified as strong predictors of patient outcomes. Neck dissection appears to be necessary, and other studies have reported the effectiveness of ipsilateral elective neck dissection in clinically

negative necks (Kau et al., 2000; Fasunla et al., 2011; Psychogios et al., 2013). Regrettably, the present study was limited by the lack of data regarding the HPV status of the patients. This omission prevented the evaluation of the prognostic significance of HPV, as recommended by other researchers. HPV status has been recognised as an important factor in determining the prognosis of OPSCC, and its inclusion in the analysis could have provided valuable insights into the outcomes of the patients in this study (Ernster et al., 2007; Andrews et al., 2009; Gillison et al., 2019).

While this study did not find significant differences in survival based on tumour differentiation, previous studies have indicated that endophytic growth, perineural invasion, and extracapsular extension of tumours are associated with contralateral neck metastasis and lower 5-year OS (Capote-Moreno et al., 2010; Cho et al., 2011).

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 (O'Rorke et al., 2012; Chakravarthy et al., 2016; Sinha et al., 2018). Smoking and alcohol abuse are additional risk factors that should be considered in assessing disease outcomes.

The study has limitations, including its retrospective nature and 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 RT alone. Moreover, the study suggests the need for diverse chemotherapeutic interventions beyond cetuximab alone. Previous studies have advocated for supraomohyoid neck dissection as the primary treatment for clinically N0 tumours, which aligns with the findings of this study (Süslü et al., 2013). However, 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 (first publication of prospective part)

DNA extraction from FFPE tissue blocks and its use for testing has become more common in recent years. And 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 with good sensitivity and specificity (Cornall et al., 2017; del Pino et al., 2017; 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 and further tissue processing, etc. (Srinivasan et al., 2002; Ludyga et al., 2012). Biopsy material taken from hypopharyngeal cancers in many cases is in small amounts because biopsies are performed using local anaesthetic with indirect visualization. Nevertheless, our results show that even small amounts of DNA concentration can be successfully used for HPV DNA detection.

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-OPSCCs (second publication of prospective part)

Based on available data, it has been found that approximately 20 % of LSCC and 5 % of HPSCC cases in the USA are caused by HPV infection (Saraiya et al., 2015). The incidence of HPV-positive head and neck cancer in Europe is generally lower (Ndiaye et al., 2014), although it is higher in developed countries like the United Kingdom, Denmark, and Germany compared to less developed Eastern European countries (Chaturvedi et al., 2013; Reuschenbach et al., 2019; Wittekindt et al., 2019). These differences can be attributed to variations in lifestyles, preferences, sexual habits, and, most importantly, to the lack of appropriate HPV testing. Notably, smoking, a known significant factor in the development of head and neck cancer, is prevalent in Latvian society (Lifsics et al., 2020). Nevertheless, this study suggests that HPV plays a role in the carcinogenesis of non-oropharyngeal cancer, with HPV16 being the predominant type observed in LSCC and HPSCC, which aligns with findings from other studies (Kreimer et al., 2005; Ndiaye et al., 2014; Janecka-Widła et al., 2020).

This study emphasizes the high incidence of HPV-positive tumours and the involvement of high-risk HPV in the pathogenesis of HPSCC and LSCC in Latvia when compared to Europe and North America. The study demonstrates a higher prevalence of HPV16-positive tumours, specifically 53.7 % in LSCC and 64.5 % in HPSCC. However, further extensive investigation is required to determine whether HPV infection in tumour tissue is transcriptionally active (Jung et al., 2010). In this regard, the detection of HPV E6/E7 mRNA in LSCC and HPSCC tissue samples could provide additional clarity on this matter (Wittekindt et al., 2018). Another challenge is distinguishing primary tumours from those that have spread from different sites, such as the oropharynx, which is typically associated with HPV infection (Ndiaye et al., 2014). The late-stage disease often makes it difficult to identify the primary tumour site accurately. Therefore, optimizing diagnostic accuracy, especially in the advanced stages of malignancy, is of paramount importance. Nonetheless, there is evidence suggesting that late-stage hypopharyngeal cancer may exhibit a higher prevalence of high-risk HPV infection (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.

To the best of our knowledge, only a few previous studies have investigated the presence of HPV oncoproteins E6 and E7 in tumour and dysplastic epithelial cells using IHC (Phaëton et al., 2015; 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 (Kiyuna et al., 2019; Augustin et al., 2020; Chi et al., 2020). In this study, FFPE samples from HPV16-positive tumours (n = 42) identified by molecular biology methods were utilised. 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.

The objective of this study was to report on the characteristics of tumorigenesis in the larynx and hypopharynx, highlighting the status of HR-HPV DNA, p16, and E6/E7 oncoproteins assessed using molecular virology and IHC methods. Although many correlations did not reach statistical significance, weak to moderate positive correlations between molecular virology and IHC results may indicate active HPV infection in these samples. However, definitive conclusions about the activity of HPV infection (such as the detection of viral mRNA) require further investigation. PCR confirmed the presence of HPV DNA in the LSCC and HPSCC samples, but the applied molecular virology methods could not distinguish between active and latent infections. Nonetheless, the presence of HR-HPV E6/E7 proteins, known as significant contributors to tumour development, suggests the active involvement of HR-HPV in tumorigenesis.

Interestingly, in some HPV16-positive specimens, tumour cells stained negative for HPV16 E6/E7 oncoproteins, whereas dysplastic epithelium showed positivity. Additionally, some endothelial cells were positive for HPV16 E6/E7 proteins. These results highlight the limitations of PCR assays, which do not specify the source of genetic material. In general, the presence of high-risk HPV E6 and E7 oncoproteins suggests the possibility of cancerous transformation in these cells. While viral integration and dysregulation of E6 and E7 gene expression are common mechanisms in HPV-related cancers, including cervical cancer (Jeon & Lambert, 1995; Münger et al., 2004), viral integration occurs less frequently in HPV-associated head and neck SCCs. In these tumours, dysregulation of E6/E7 genes can occur in the episomal state, such as through disruption of HPV E2 binding sites by methylation (Reuschenbach et al., 2015; Vojtechova et al., 2016; McBride & Warburton, 2017). The absence of HPV16 E6/E7 oncoproteins in tumour cells, coupled with their presence in dysplastic epithelial and endothelial cells observed in this study, may indicate the absence of HPV integration. In advanced tumour stages, viral DNA may be cleared from the tumour itself, and other mechanisms of tumorigenesis may come into play.

This study identified a significant number of p16-negative/HPV-positive specimens in LSCC and HPSCC patients. These findings align with observations from other studies, suggesting that p16 may serve as a surrogate marker of HPV infection in OPSCC but may not be practical in laryngeal and hypopharyngeal cancers (Chung et al., 2014; Rosenthal et al., 2016; 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).

The strengths of this study lie in the use of a comprehensive range of HPV-specific tests, including HPV DNA PCR, detection of high-risk and low-risk HPV types, along with IHC staining of the HPV surrogate marker p16 and viral oncoproteins E6/E7, confirmed by both conventional and fluorescence-based immunodetection methods. However, several limitations should be considered when interpreting the data. The study involved a moderate number of samples, and the absence of HPV mRNA data is a limitation that could provide further insight into the activity of HPV infection in the analysed tumours. Additionally, some gender and tumour stage imbalances were observed, although they did not impact the overall results and can be explained by legal norms and inclusion criteria applied in the study.

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 (third publication of prospective part)

The primary objective of the present study was to examine 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. The initial analysis using the Kaplan-Meier survival method 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). One 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. Further investigation into the activity of HPV in HNSCC patients and its interaction with the immune system is warranted.

Numerous studies have established that patients with HPV-positive OPSCC exhibit higher 3- and 5-year survival rates compared to HPV-negative patients (You et al., 2019). However, this consensus primarily applies to HR-HPV types, particularly HPV16 and 18. Regarding LSCC, several studies have reported no significant improvement in survival for HPV-positive tumours (Ahmadi et al., 2018; Hughes et al., 2019; Lu et al., 2020). Nevertheless, recent investigations, including our own, have yielded similar results, indicating better survival outcomes for patients with HPV-positive LSCC (Kiyuna et al., 2019; 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; Saber et al., 2016; Cillo et al., 2020).

Head and neck cancers encompass various subsites, each with unique characteristics and prognoses. Sometimes studies analysing the effects of HPV on the survival of head and neck cancers can be confusing in that they unify the survival analysis without stratifying the primary

tumours by location, especially hypopharyngeal and laryngeal cancers, which are sometimes combined in non-oropharyngeal cancers (Deng et al., 2012; Dahm et al., 2018). In our view, this could lead to incorrect conclusions. The oropharynx, larynx, and hypopharynx are distinct locations with different prognoses based on lymphatic drainage patterns alone. In our study, when performing a Cox regression analysis encompassing all HNSCC cases, variables such as p16, p53, and others were not significant factors influencing patient survival. This highlights the importance of stratifying patients based on the primary tumour location to gain a more comprehensive understanding of potential risk factors.

This study reaffirmed the predictive role of p16 overexpression in OPSCC through univariate survival analysis, confirming that patients with p16-positive tumours have better survival rates (Chung et al., 2014; Wendt et al., 2021). This trend was further supported by the Cox regression analysis, which showed statistical significance and emphasised p16 as a distinct predictive marker for OPSCC. However, in the case of HPSCC and LSCC, the univariate survival analysis did not confirm this association. Nevertheless, the Cox regression analysis indicated better survival and a lower risk of death for patients with p16-positive HPSCC, suggesting the potential consideration of p16 as a predictive marker. Similar findings have been reported in several studies (Tribius et al., 2018; Shi et al., 2022). The association between p16 and HPV activity in non-oropharyngeal squamous cell carcinoma raises questions about its use as a surrogate marker for HPV infection and its suitability as a prognostic factor for survival. Several studies have shown that p16 often does not correspond to the HPV status in non-oropharyngeal cancers, but it does have prognostic value for survival (Stephen et al., 2013; Sánchez Barrueco et al., 2019; Gallus et al., 2022).

The lack of significance for many analysed variables in OPSCC during the univariate survival analysis in the present study may be attributed to the relatively small number of patients in this subgroup, which can impact the statistical power of the analysis. Additionally, the high number of smokers and alcohol abusers among the patients could also influence the significance of the results. This is accounted for in the Cox regression model.

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 E6 protein of HR-HPV. On the 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-positive 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-negative result when assessed using IHC. Published data suggest that HPV-driven tumours exhibit p53 downregulation (Ramesh et al., 2020; S. Wang et al., 2021; Benzerdjeb et al., 2021). Conversely, Hasegawa et al. reported that p53 overexpression correlates with a better response to chemotherapy and is associated with improved survival (Hasegawa et al., 2018). Similar results were demonstrated by Sun et al. (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 points (Zhou et al., 2016; Omura et al., 2017). 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. Given that E6 and E7 are recognised as the primary drivers of HPV-mediated carcinogenesis, we were interested in examining the impact of these proteins on survival using IHC. 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 at a certain stage of viral activity, 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 (S. Li et al., 2019). Unfortunately, this research did not investigate E6AP activity. A prospective study with multiple time points could provide a better understanding of the roles of HPV oncogenes in the progression of HNSCC tumours, as persistent HPV infection is a major factor in carcinogenesis (Byun et al., 2018). In this study, it is challenging to distinguish between persistent and non-persistent HPV infections since sampling was performed only once. However, in patients with HPSCC, E6 protein was detected through immunostaining, 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 IHC overexpression of HPV16 E7 protein in OPSCC is associated with a poorer prognosis according to 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, 2012, 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 intrinsic control of the kits utilised 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 immunohistochemistry represents a valuable prognostic indicator for, both, OPSCC and HPSCC.

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.
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Annexes

First Publication

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Experimental ONCOLOGY

SURVIVAL ANALYSIS OF OROPHARYNGEAL SQUAMOUS CELL CARCINOMA PATIENTS LINKED TO HISTOPATHOLOGY, DISEASE STAGE, TUMOR STAGE, RISK FACTORS, AND RECEIVED THERAPY

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***Background*: Survival of oropharyngeal squamous cell carcinoma (OSCC) patients depends on the risk and environmental factors, tumor biology, achievements in diagnostics and treatment approaches. *Aim*: To perform a survival analysis of the patients with OSCC treated over a 10-year period in a single hospital in Latvia linking these data to histopathological findings, risk factors and received therapy. *Materials and Methods*: The main outcome measures were overall and disease-specific survival (OS and DS) along with histopathology analysis. *Results*: Kaplan – Meier survival analysis showed better survival for females, younger patients lacking bad habits, operated and received radiotherapy, with lower T grade and disease stage. Cox regression showed diminished early death risk in patients with lower T grade, no regional metastases (N0) and bad habits, operated and received radiotherapy. A vast majority of tumors were localized in palatine tonsils and the base of the tongue. The localization did not correlate with mean survival time/survival. Lower OS ($p = 0.03$) and DS ($p = 0.026$) were estimated for patients with pharyngeal wall and tonsillar involvement compared to tumors localized in the soft palate. A histological variant of tumor seemed irrelevant estimating OS and DS, whereas therapeutic modalities significantly affected survival. *Conclusions*: OSCC patients with lower T grade, N0 status, lacking bad habits, and surgically treated had better survival.**
***Key Words*: oropharyngeal squamous cell carcinoma, survival rates, risk factors.**

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The oropharynx is one of the most common localizations for malignant neoplasms in the head and neck region. The GLOBOCAN data (2012) confirm over 140,000 new cases of pharyngeal cancer worldwide and age-standardized incidence of 1.9 per 100,000, whereas in Europe — about 34,000 new cases and age-standardized incidence of 2.9 [1].

Histopathologically, most malignancies found in the oropharynx (~ 90%) are squamous cell carcinoma (SCC) [2]. Although SCC of the oropharynx is diagnosed predominantly in people over the age of 45 years, some studies suggest an increased incidence of the disease in people less than 45 years of age, over the past 20–30 years [3]. Commonly, these tumors arise from certain regions — palatine tonsils, the base of the tongue, soft palate, and posterior pharyngeal wall and in greater than 60% of patients present with cervical lymph node involvement and 10–15% with distant metastases [2]. It has been found that the rates of lymph node metastasis vary considerably by localization with tumors of the tonsil and base of the tongue more likely presented with positive nodes than tumors of the soft palate and pharyngeal wall [4]. Approximately 60% of oropharyngeal SCCs (OSCC) have been found to be moderately differentiated, 20% well-differentiated, and 20% poorly differentiated [5]. Other tumors, namely minor salivary tumors (adenomas/adenocarcinomas), primary lymphoid tumors, undifferentiated tumors, various sarcomas, and mixed neoplasms also present in the oropharynx [6], and clinicopathological findings vary from country to country [7].

Major etiological and predisposing factors for this neoplasm include smoking and drinking habits, and several other factors such as human papillomavirus (HPV) and *Candida* infections, nutritional deficiencies and genetic predisposition [7–10]. Furthermore, it has been demonstrated that the carcinogenic effects of both alcohol and tobacco smoke on the oropharynx appear to function in dose-dependent manners [6], and increase 6–7-fold in individuals overusing tobacco or alcohol and as much as 15-fold with those who both smoke and drink alcohol [11].

Analysis of survival rates in the case of SCC reveals greatly varying data due to the variability of the observation period, patients' features, surgeons' expertise, percentage of starting tumors compared with advanced ones, quality of radiotherapy (RT), and the use of adjuvant treatments [12]. Pathologically, the significant predictors of 5-year disease-free survival proposed very recently by analyzing invasive tumor patterns of SCC were defined as the mode of invasion, worst pattern of invasion, and tumor budding as well as lymphovascular and perineural invasion [13]. The 5-year survival rate has been shown to range from 58% up to 94% [14]. A decrease in survival rate in a long-term follow-up happens mostly due to the development of new primary tumors, which have the same etiologic factors, and intercurrent deaths often caused by the same etiologic factors and by the age of the patients [15]. Other studies suggest an improvement in the 5-year

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Abbreviations used: ChT – chemotherapy; DS – disease-specific survival; H&E – hematoxylin and eosin; HPV – human papillomavirus; HR – hazard ratio; KSCC – keratinizing SCC; NKSCC – nonkeratinizing SCC; OS – overall survival; OSCC – oropharyngeal squamous cell carcinoma; RT – radiotherapy; SCC – squamous cell carcinoma; SUR – surgery.

overall survival (OS) and disease-specific survival (DS) rates during the past decade compared with the previous decade even despite older age, more advanced disease stage, and a higher rate of distant metastases, presumably due to the recent advances in tumor imaging and therapy [12, 14]. The world incidence of OSCC varies and estimated differences in the incidence and survival are generally related to the distinct risk and socioeconomic factors, environmental agents, public health awareness and accessibility of health services, as well as advances in diagnostics and therapy. Therefore, the aim of this study was to perform a survival analysis of the patients with OSCC treated over a 10-year period in a single hospital in Latvia correlating these data with histopathological findings, disease stage, tumor grade, nodal grade, patients' age and sex, habits (smoking, alcohol abuse), primary tumor location, and received therapy.

MATERIALS AND METHODS

We carried out a retrospective study of 247 patients diagnosed with OSCC, staged following the TNM classification of the International Union against Cancer (6th edition) for oropharyngeal carcinoma and treated in Riga Eastern Clinical University Hospital Stationary Oncology Centre of Latvia between January 1st, 2000 and December 31st, 2010. Patients are admitted to this hospital from all over the country, which has an estimated population of 1.91 million. The patients' data were collected from the Hospital Archive and The Centre for Disease Prevention and Control and included in the study when the diagnosis of OSCC was confirmed histologically. The study was approved by the Ethical Committee of Riga Stradins University.

The data collected were processed to calculate the overall and disease-specific 3 and 5-year survival rates for all patients. The Kaplan — Meier survival analysis was used for the estimation of statistical data. Statistical testing for differences in unadjusted survival rates was performed using the log-rank test. A Cox regression method was used to estimate hazard ratio (HR). Age, sex, T stage, N status, risk factors (smoking, alcohol abuse), therapy modality (RT, surgery (SUR), chemotherapy (ChT), symptomatic therapy and combinations of aforementioned, primary tumor location, histopathological variant of tumor were included as covariates in the survival model. ChT consisted of a single-agent regimen with cetuximab or platinum medication (cisplatin).

Statistical analysis of correlation of aforementioned covariates with survival, and mean OS time after diagnosis was performed. We used Pearson's chi-squared test or Fisher's exact test (depending on the size of the group) to find out if differences between analyzed groups are statistically significant, the value of $p < 0.05$ was considered significant. Cramer's V was used to measure an association between two nominal variables. For analysis of the correlation between nominal variables and mean survival time after diagnosis Kruskal — Wallis test or Mann — Whitney test (depending on the number of groups) were used.

Formalin-fixed paraffin-embedded OSCC samples obtained from all major subsites and sections cut off were retrieved from the archival files of the Department of Pathology Oncology Centre of Latvia, and pathology reports for all tumors were reviewed. Hematoxylin and eosin (H&E) stained sections were analyzed, and the tumors were classified according to their histologic features. Patterning of the invasion at the advancing tumor edge, the presence of perineural invasion, and immune system response as proposed by Brandwin *et al.* [16] and thereafter commented by the other scientists [17] were underestimated in the early years of this retrospective study. Therefore, the histopathological assessment was done not taking into account the revision of surgical margins and the evaluation of supplemental tissue. Microphotographs were obtained using Leitz DMRB bright-field optics equipped with a digital camera DC 300F.

RESULTS

The retrospective cohort consisted of 247 patients with pathologically confirmed OSCC, stage I–IV presented by the following subsites — palatine tonsils (n = 110, 44.52%), base of tongue (n = 76, 30.77%), soft palate (n = 20, 8.10%), and posterior pharyngeal wall (n = 41, 16.60%). Unfortunately, less than one-tenth of the cohort presented with stage I and II — 3 (1.22%) and 19 (7.72%) patients, accordingly, whereas a major portion — 224 (91.6%) revealed advanced disease stage. By gender, 8.10% (n = 20) of all reviewed patients were female and 91.90% (n = 227) — male. The mean patient age was 60 years (range 27–85), median — 60.20 years.

When the patients' data were collected and summed-up we found that most of the patients were regular smokers (75.95%, n = 180), habitual drinkers (35.19%, n = 82) or were exposed to both aforementioned major risk factors (31.47%, n = 73). The general characteristics of the patients are summarized in Table 1.

Table 1. Characteristics of the patients

Sex – n (%):	
Male	227 (91.90)
Female	20 (8.10)
Age – yr:	
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)
Tumor stage – n (%)**:	
T1	23 (9.39)
T2	59 (24.08)
T3	73 (29.80)
T4	90 (36.73)
Node 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)

Note: *Unknown for 1 patient; **unknown for 2 patients; ***unknown for 14 patients; ****unknown for 10 patients.

Female patients had significantly longer mean survival time than males, but we found no correlation between survival and gender ($V_1 = 0.09$, $p = 0.25$) as well as mean survival time and gender ($\eta = 0.17$). OS analysis showed better survival for females, but it wasn't significantly different when compared to males ($p = 0.06$). By contrast, DS survival in female patients appeared to be significantly better ($p = 0.0486$).

Additionally, survival was estimated subdividing the subjects into three age groups — younger than 55 years; 55 to 64 years old and older than 65 years. There were significantly more deceased patients in the subgroup with advanced age when compared to younger individuals ($p = 0.028$). However, no correlation was found between both age group and survival ($V_1 = 0.17$), and mean survival time ($\eta = 0.16$). Kaplan — Meier estimates showed a decrease in survival with increasing age, but the differences in OS and DS weren't statistically significant when all three age groups were considered ($p = 0.092$ and $p = 0.108$). In spite of that, pairwise comparisons showed statistically significant differences in survival between patients younger than 55 years and older than 64 years ($p = 0.048$). Table 2 deciphers a decrease in OS with more advanced age.

There was a moderate correlation between survival and disease stage ($V_1 = 0.32$, $p_s = 0.0014$). Kaplan — Meier survival analysis showed almost statistically significant (overall comparisons, $p = 0.058$) OS and DS differences according to the disease stage (see Table 2). In pairwise comparisons, a statistically significant difference in OS between stage I and stage II disease ($p = 0.139$), stage II and stage III disease ($p = 0.112$), stage III and stage IV disease ($p = 0.104$) was not found. Similar observations were made in pairwise comparisons between stages in DS.

Mean survival time and the positive outcomes (the patient survived) of the disease appeared to decrease with higher T grade, and there was a moderate correlation between outcome and T grade ($V_1 = 0.27$), whereas no correlation between mean survival time and T grade ($\eta = 0.2830$). Kaplan — Meier survival analysis showed a better OS and DS when lower tumor grade (T1–2) was compared to higher tumor (T3–4) grade (see Table 2).

There was no correlation between N status and mean survival time/survival (outcome). We found no statistical difference in OS and DS ($p = 0.11$ in both cases) according to N status (N0 *vs* N+; see Table 2).

Table 2. Kaplan – Meier analysis of potential prognostic factors for DS, OS

Variable	3-year Kaplan – Meier estimate, % (95% CI)		5-year Kaplan – Meier estimate, % (95% CI)	
	OS	DS	OS	DS
Age, years (n; %):				
<55 (62; 25.10)	25.8% (14.8–36.8)	24.1% (13.1–35.1)	22.6% (12.2–33.0)	20.7% (10.3–31.1)
55–64 (105; 42.51)	21.6% (13.6–29.6)	19.6% (11.8–27.4)	15.7% (8.6–22.8)	14,4% (7.3–21.5)
≥65 (80; 32.39)	14.1% (6.5–21.7)	12.3% (4.3–20.3)	7.7% (1.8–13.6)	7.7% (1.2–14.2)
	$p = 0.092$	$p = 0.108$	$p = 0.092$	$p = 0.108$
Sex:				
Male	19.8% (14.5–25.1)	19% (12.7–23.3)	14% (9.5–18.5)	12,8% (8.3–17.7)
Female	30% (10.0–50.0)	30% (10.0–50.0)	25% (6.0–44.0)	25% (6.0–44.0)
	$p = 0.06$	$p = 0.0486$	$p = 0.06$	$p = 0.0486$
Stage:				
I	100% (–)	100% (–)	100% (–)	100% (–)
II	36.8% (15.0–58.6)	37.5% (13.8–61.2)	31.6% (10.6–52.6)	31.3% (8.6–54.0)
III	21.7% (11.3–32.1)	23.6% (12.4–34.8)	13.3% (4.7–21.9)	14.5% (5.1–23.9)
IV	16.3% (10.6–22.0)	13.0% (7.5–18.5)	11.1% (6.2–16.0)	10.3% (5.4–15.2)
	$p = 0.0058$	$p = 0.0058$	$p = 0.0058$	$p = 0.0058$
T grade:				
T1	42.9% (21.7–64.1)	37.5% (13.8–61.2)	42.9% (21.7–64.1)	37.5% (13.8–61.2)
T2	34.5% (22.3–46.7)	35.8% (22.9–48.7)	22.4% (11.6–33.2)	22.6% (11.4–33.8)
T3	16.4% (8.0–24.8)	16.4% (7.6–25.2)	9.6% (2.9–16.3)	10.4% (3.1–17.7)
T4	11.4% (4.7–18.1)	8.5% (2.4–14.6)	6.8% (1.5–12.1)	6.1% (1.0–11.2)
	$p < 0.0001$	$p < 0.001$	$p < 0.0001$	$p < 0.001$
N status:				
N0	27.6% (17.6–37.6)	27.9% (17.3–38.5)	21.1% (11.9–30.3)	22.1% (12.3–31.9)
N+	19% (12.9–25.1)	16.8% (10.7–22.9)	12.3% (7.2–17.4)	10.7% (5.8–15.6)
	$p = 0.11$	$p = 0.11$	$p = 0.11$	$p = 0.11$
Primary tumor location:				
Palatine tonsil	18.5% (11.2–25.8)	16.8% (9.5–24.1)	12% (5.9–18.1)	9.9% (4.0–15.8)
Base of the tongue	24.3% (14.5–34.1)	22.7% (12.5–32.9)	17.6% (9.0–26.2)	18.2% (9.0–27.4)
Pharyngeal wall	15% (4.0–26.0)	13.5% (2.5–24.5)	7.5% (0–15.7)	8.1% (0–16.9)
Soft palate	40% (18.4–61.6)	43.8% (19.5–68.1)	35% (14.0–56.0)	37.5% (13.8–61.2)
Alcohol abuse and smoking:				
Neither	34% (20.5–47.5)	31.8% (18.1–45.5)	23.4%(11.2–35.6)	25% (12.3–37.7)
1 factor	22.7% (14.9–30.5)	20.4% (12.4–28.4)	16.4% (9.5–23.3)	14.3% (7.4–21.2)
Both	11.4% (4.0–18.8)	10.9% (3.3–18.5)	7.1% (1.0–13.2)	6.3% (0.4–12.2)
	$p = 0.002$	$p = 0.008$	$p = 0.002$	$p = 0.008$
Treatment (n):				
RT (175)	14% (8.7–19.3)	12.6% (7.5–17.7)	7.6% (3.7–11.5)	7.5% (3.4–11.6)
SUR (7)	42.9% (6.2–79.6)	40% (0–82.9)	42.9% (6.2–79.6)	40% (0–82.9)
RT+SUR (39)	52.6% (36.7–68.5)	54.8% (37.4–72.2)	42.1% (26.4–57.8)	41.9% (24.5–59.3)
RT+ChT (Cetuximab)+/–SUR (17)	23.5% (3.3–43.7)	25% (3.8–46.2)	17.6%(0–35.6)	18.8% (0–38.0)
RT+ChT (Cisplatin)+/–SUR (3)	33.3% (0–86.6)	33.3% (0–86.6)	33.3% (0–86.6)	33.3% (0–86.6)
Symptomatic (6)	0% $p < 0.001$	0% $p < 0.001$	0% $p < 0.001$	0% $p < 0.001$

A moderate correlation between smoking and survival ($V_1 = 0.21$, $P_x = 1.77 \cdot 10^{-3}$) was found, but there was no correlation between mean survival time and smoking ($\eta = 0.17$). Kaplan — Meier survival analysis showed a statistically higher OS and DS in subjects nonsmokers ($p < 0.05$). There was no correlation between alcohol abuse and survival/mean survival time.

Significantly higher OS was estimated for patients who didn't abuse alcohol ($p = 0.03$), whereas a decrease of the significance was found regarding DS ($p = 0.08$). However, there was a statistically significant decline in the OS and DS in the patients' group who smoked and abused alcohol simultaneously (yes *vs* no) (see Table 2, Fig. 1).

OSCC analyzed in the study developed from different subsites, but there was no impact of tumor location on mean survival time/survival. Worst OS ($p = 0.03$) and DS ($p = 0.026$) estimates were found for subjects presented with pharyngeal and tonsillar tumors, thus opposing estimates for patients presented with tumors of the soft palate (see Table 2).

Keratinizing SCC (KSCC) tissue samples showed large polygonal squamous cells with distinct cell borders and keratin formation revealing a spectrum of grades from well-differentiated to poorly differentiated tumors with various degrees of keratinization (Fig. 2, 3, 4). Keratin pearls were present. Squamous maturation was diffuse even in poorly differentiated tumors that lack keratinization. Keratinizing tumor samples with abundant eosinophilic cytoplasm were often composed in discrete nests and displayed nuclear pleomorphism (see Fig. 2 and Fig. 5). The infiltrative nests of tumor cells usually were found within stroma revealing prominent desmoplasia.

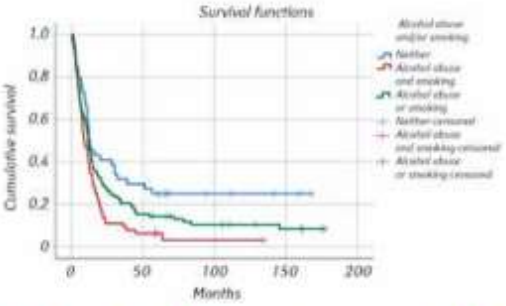

Fig. 1. Kaplan — Meier DS plot according to hazardous habits

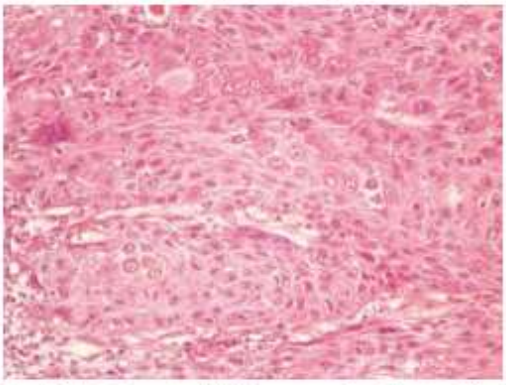

Fig. 2. Soft palate region. KSCC (verrucous type) showing folded and thickened neoplastic epithelium comprised of large polygonal cells with distinct cell borders and varying degree of eosinophilia. Nuclei are pleomorphic. H&E, original magnification, ×200

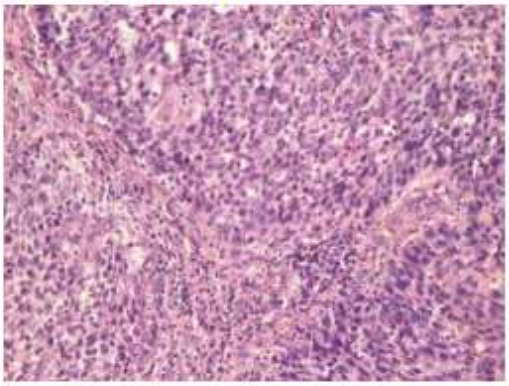

Fig. 3. Base of the tongue. NKSCC. Densely packed mitotically active epithelial cells forming the pushing and infiltrating masses of carcinoma. H&E, original magnification, ×200

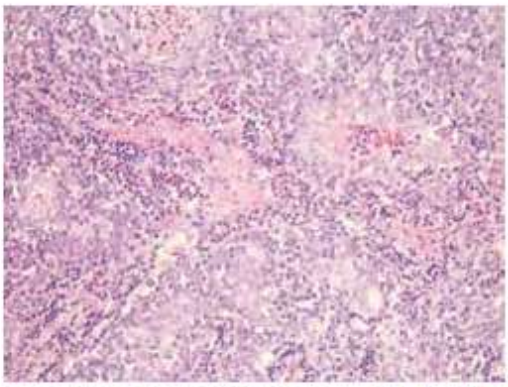

Fig. 4. Palatine tonsil. NKSCC. Nests of tumor cells with ill-defined borders and necrosis. H&E, original magnification, ×200

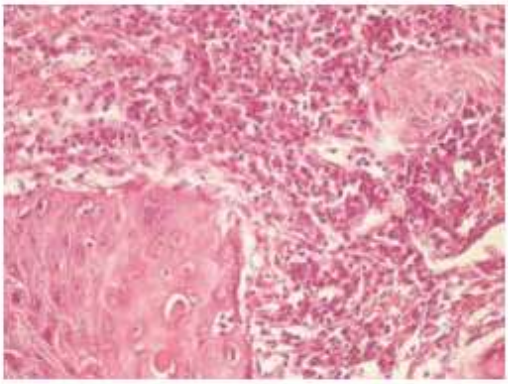

Fig. 5. Soft palate region. KSCC. Tumor cells demonstrate nuclear pleomorphism, mitotic and apoptotic features. Some tumor cells contact the nerve bundle. H&E, original magnification, ×250

Nonkeratinizing SCC (NKSCC) tumors often formed nests, sheets, and cords with well-defined borders. These tumors were characterized by relatively monomorphic, densely packed, ovoid, and spindle-shaped basaloid cells with indistinct cell borders. Mitotically active tumor cells revealed highly hyperchromatic nuclei and high nuclear-to-cytoplasmic ratio.

Although this study did not attempt to distinguish HPV driven tumors from those, which are HPV negative, we might speculate that KSCC are highly likely HPV negative whereas NKSCC highly suggestive of HPV association. Usually, these NKSCC formed sheets, nests, and cords with sharply defined borders; tumor cells displayed basaloid features and peripheral palisading (Fig. 6).

Most of the tumors were KSCC (n = 175, 70.85%), 19.43% were NKSCC (n = 48), 1.21% — undifferentiated carcinomas (n = 3), 1 (0.4%) — adenosquamous carcinoma, for the remainder of tumors keratinization pattern wasn't specified (n = 20, 8.10%). A histological variant of tumor seemed irrelevant estimating OS and DS ($p > 0.05$). Furthermore, a correlation between histological variant and mean survival time/survival was not found.

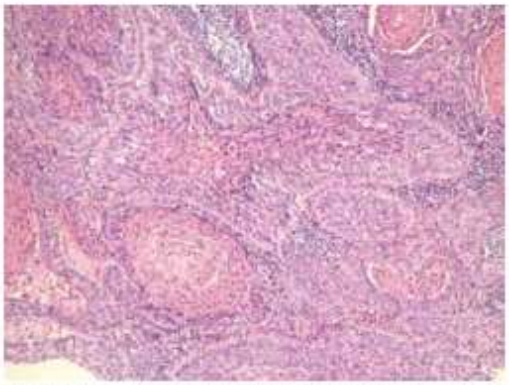

Fig. 6. Base of the tongue. NKSCC. Nests and cords of tumor cells with basaloid features, peripheral palisading, intraluminal necrosis, keratocysts. H&E, original magnification, ×100

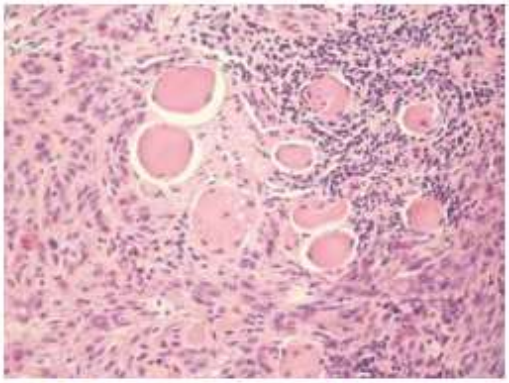

Fig. 7. Posterior pharyngeal wall. Poorly differentiated SCC. Tumor nests and nodules reveal muscular invasion; lymphoplasmacytic infiltration. H&E, original magnification, ×250

When recognizing that histological grade based on the amount of keratinization is not a consistent predictor of clinical behavior we fixed the presence of perineural spread, lymphovascular and muscular invasion to better understand aggressive behavior of the tumor. We found that perineural invasion and lymphovascular invasion are frequently observed in SCCs causing a decrease of survival (see Fig. 6). Additionally, the islands and cords of malignant cells infiltrated the underlying skeletal muscle tissue when the deeper invasion of the tumor masses took a place (Fig. 7).

We found a strong correlation between survival and therapy ($V_1 = 0.32$), but no correlation between therapy and mean survival time ($\eta = 0.33$). There were significant OS and DS differences ($p < 0.001$) between therapeutic modalities (Table 2), with better survival in SUR and RT+SUR groups. Pairwise comparisons revealed significant OS differences only in RT *vs* RT+SUR, RT+SUR *vs* RT+ChT (cetuximab)+/–SUR groups ($p < 0.05$), and borderline significance in RT *vs* SUR group, showing higher survival in those patients who underwent SUR and lowest survival in RT group. Similar observations were made performing pairwise comparisons between therapy modalities and DS.

Most of the patients didn't receive SUR as therapeutic modality (n = 196), 10 patients had primary tumor excision, 28 underwent neck dissection, and 13 had both, primary tumor excision and neck dissection. When suggesting the outcome of the disease and the impact of SUR as well as the type of operation done, we found that the number of deceased patients was much higher when no operation was done (Table 3). Furthermore, mean OS time after establishing the diagnosis of the disease was significantly longer in surgically treated patients; however, the correlation between mean OS time and the aforementioned treatment modality was not found. Kaplan — Meier analysis showed significant differences in survival (OS and DS) depending on whether the patient was operated on or not, with a much higher survival rate in patients who underwent SUR ($p < 0.0001$). However, OS and DS pairwise comparison of SUR type didn't show any significant differences ($p = 0.29$ for OS and $p = 0.11$ for DS).

Cox regression method was applied in two stages: (1) all the factors were analyzed without distinguishing subgroups of each factor (univariate analysis; Table 4); and then (2) subgroups of each factor were assessed (multivariate analysis, see Table 5). T grade ($p < 0.00001$), N status ($p = 0.017$) and sex ($p = 0.049$) appeared to have a statistically significant or probable impact on the mortality after detection of the disease in the common comparison model (see Table 4) (value of B is positive). Individually for grade T, the risk of death increases by 39% (Exp (B) = 1.39) if T grade increases with other values remaining constant. By contrast, the risk of death increases by 51% (Exp (B) = 1.51) in case of N status, if there is a change from N0 to N + when other values remain fixed. Finally, the risk increases

Table 3. Breakdown of patients by type of operation and outcome of the disease

Type of operation	N of patients (incidence, %)*	Outcome of the disease (therapy)	N of patients (incidence, %)	Statistical analysis between groups			
Primary Tu excision	10	Positive (survived)	3 (30.00)	All groups		Only operations	
	(4.05; 19.61)	Negative (deceased)	7 (70.00)	P_x	V_x	P_x	V_x
Neck dissection	28	Positive (survived)	5 (18.52)	7.11×10^{-4}	0.33	0.19	0.26
	(11.34; 54.90)	Negative (deceased)	22 (81.48)				
Both	13	Positive (survived)	6 (46.15)				
	(5.26; 25.49)	Negative (deceased)	7 (53.85)				
None	196	Positive (survived)	12 (6.25)				
	(79.35; -)	Negative (deceased)	180 (93.75)				

Note: *The incidence among all patients and the incidence only between operations.

Table 4. Cox proportional hazard, univariate analysis

Variable	B	P	Exp(B)	CI 95% Exp(B)
Sex	0.53	$4.88 \cdot 10^{-3}$	1.70	1 ... 2.88
Age groups	0.14	0.14	1.15	0.95 ... 1.4
Alcohol abuse and/ or smoking	0.02	0.83	1.02	0.85 ... 1.22
T grade	0.33	$2.40 \cdot 10^{-5}$	1.39	1.2 ... 1.63
N status (N0 *vs* N+)	0.41	$1.35 \cdot 10^{-3}$	1.51	1.09 ... 2.09
Therapy	−0.10	0.14	0.90	0.79 ... 1.03
Primary tumor location	−0.08	0.29	0.92	0.79 ... 1.07
Histological variant	0.07	0.36	1.07	0.92 ... 1.25

by 70% (Exp (B) = 1.70) within the gender axis (female > male). Other features in a particular regression model didn't have a statistically significant impact on the risk of earlier death. Cox regression plot for cumulative survival shows that 50% of patients die before 12 months after the diagnosis of cancer (Fig. 8).

It was found that T2 grade, N status, presence of one of the hazardous habits (smoking or alcohol abuse) and treatment modality — RT+SUR have a statistically significant impact on the risk of death when accounting nine factors and analyzing the HR between subgroups of factors (see Table 5). Patients with T2 grade tumor have 57% and 77% reduction in the risk of early death when compared to patients with T3 and T4 grade tumors. Finally, we found that there is a 34% reduction in the risk of early death when N0 status is compared to N+.

Significantly ($p = 0.0467$) lower early death risk was determined for patients exposed to SUR in combination with RT ($p = 0.002$) when compared to other treatment modalities, including RT alone or in combination with cetuximab (Fig. 9). When compared to symptomatic treatment, RT+SUR therapy has 300% or 3 times lower early death risk, but compared to RT+ChT (cetuximab) +/−SUR therapy — 154% or 1.54 times lower early death risk. When a combination of two — RT+SUR treatment modalities are compared to RT or SUR alone, there is 2.02 and 1.27 times greater death hazard estimated for RT and SUR, respectively.

Cox regression multivariate analysis showed that alcohol abuse and/or smoking significantly increase the risk of early death. Results of the Cox proportion hazard model are summarized in Tables 4 and 5.

DISCUSSION

We performed a survival analysis of the patients with OSCC treated over a 10-year period in a single

Table 5. Cox proportional hazard model, multivariate analysis

Variables	P	Exp (B) or HR*	CI 95% Exp (B)	HR comparing to other groups**
Sex (female > male)	0.11	0.63	0.36 ... 1.11	
Age group	0.15			
<55 years old	0.10	0.70	0.46 ... 1.06	
55–64 years old	0.08	0.74	0.52 ... 1.04	
>64 years old		(1.00)		
Alcohol abuse and/or smoking	0.06			
None	0.43	0.84	0.55 ... 1.29	
1 of aforementioned	**0.051**	1.42	1 ... 2.01	
Both		(1.00)		
T grade	$3.51 \cdot 10^{-3}$			
1	0.13	0.60	0.31 ... 1.17	1.06
2	$\mathbf{6.72 \cdot 10^{-3}}$	**0.57**	**0.37 ... 0.85**	
3	0.51	0.89	0.62 ... 1.26	1.57
4		(1.00)		1.77
N status (N0 > N+)	$\mathbf{1.58 \cdot 10^{-3}}$	**0.66**	**0.47 ... 0.93**	
Therapy	0.09			
RT	0.42	0.67	0.26 ... 1.75	2.02
OP	0.20	0.42	0.11 ... 1.56	1.27
RT+SUR	$\mathbf{4.67 \cdot 10^{-2}}$	**0.33**	**0.11 ... 0.98**	
RT+ChT (cetuximab) +/−SUR	0.70	0.80	0.26 ... 2.44	2.41
RT+ChT (platinum) +/−SUR	0.44	0.51	0.09 ... 2.82	1.54
Symptomatic		(1.00)		3.00
Primary tumor location	0.55			
Palatine tonsil	0.19	1.48	0.82 ... 2.64	
Base of the tongue	0.37	1.32	0.72 ... 2.4	
Pharyngeal wall	0.20	1.52	0.79 ... 2.92	
Soft palate		(1.00)		
Histological variant	0.73			
KSCC	0.78	0.93	0.54 ... 1.59	
NKSCC	0.90	0.96	0.52 ... 1.78	
Carcinoma, undifferentiated (Epit)	0.35	1.84	0.51 ... 6.67	1.91
SCC, BCN (unspecified)		(1.00)		1.04

Note: *HR – hazard ratio-calculation using the last group as a reference; **calculated for significant groups (bold) against others, taking a significant group as a reference.

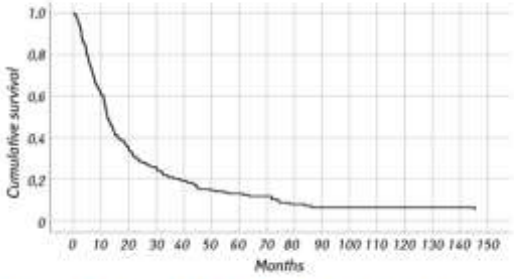

Fig. 8. Cox regression plot for cumulative survival (overall) accounting for all covariates (sex, age group, T grade, N status, alcohol abuse and/or smoking, therapy, primary tumor location, histological variant)

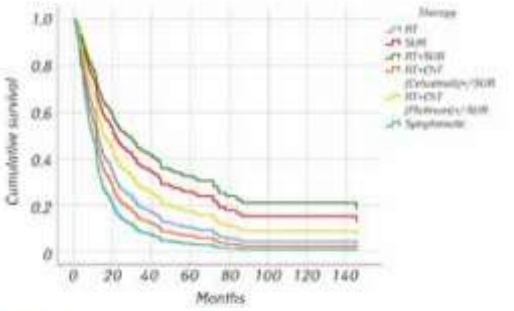

Fig. 9. Cox regression plot for cumulative survival. Covariates — sex, age group, T grade, N status, alcohol abuse and/or smoking, therapy, primary tumor location, histological variant. Plot for therapy

hospital in Latvia making attempts to link the data with disease stage, tumor stage, patients' age and sex, habits (smoking, alcohol abuse), histopathological variant of the tumor, primary tumor localization, and received therapy.

We found that two-thirds of the patients (76%) were smokers, whereas one third — (35%) had drinking problems. Regarding the relevance of habits, our study confirmed the independent role of these risk factors in survival (OS and DS), where smoking seems to play a more important role in survival, especially DS. Moreover, the combination of these two factors significantly decreases survival (DS and OS). Similar evidence has been reported previously [18–21]. Furthermore, according to our Cox hazard model (multivariate analysis) an early death risk is higher when at least one of the risk factors is present.

Our study showed that a vast majority of patients were diagnosed in advanced disease stages (III and IV) resulting in poorer outcome prognosis. Kaplan — Meier estimates of OS and DS for disease stage showed worse survival for late disease stages. Our investigation revealed that of 247 subjects used in the present study, there were only 3 and 19 patients with stage I and stage II disease, accordingly. The importance of early cancer diagnosis and fast referral to the specialist has been previously highlighted [22]. Our estimations of survival appear to support this.

OSCC is an aggressive tumor commonly diagnosed in advance stages and characterized by a high rate of lymphatic metastasis [23]. This was also true for our study where 68.4% of patients presented with clinically positive neck disease (locoregional spread of cancer to neck lymph nodes). Furthermore, N+ patients had higher early death risk (Cox regression multivariate analysis), although there were no significant differences in OS and DS.

We found that lower T grade tends to correlate with better disease outcome. This statement was confirmed by Kaplan — Meier estimates of OS and DS, which showed a significant ($p < 0.001$) decrease in survival by T grade revealing the longest survival for lower T grades. Nevertheless, it is worth noting that our estimates of survival are lower than those demonstrated in the western hemisphere [24].

Our study showed the worst OS and DS for tumors of pharyngeal wall and palatine tonsils, and these data partially agree with the literature [6]. In our study, the majority of the patients had palatine tonsil and base of tongue SCC associated with poorer survival.

In the given study, better OS and DS estimates were demonstrated for the surgically treated patients. Indeed, it may be argued that there is a selection bias in the treatment modalities. The present study brought evidence that SUR might have a clear role in better disease outcome, and the best outcome was demonstrated for the RT+SUR group. We were not able to show any significant differences in survival based on the type of SUR applied (primary tumor excision, neck dissection or both), however, these appeared when SUR *vs* no SUR at all was compared. However, we must admit that the numbers of patients constituting the study groups we used were unequal and not very high. Reviewing the literature, we found that surgical treatment has emerged as the necessary treatment modality for most patients [25].

Furthermore, other studies have shown a survival benefit in operated patients, even when stratified by HPV status [26–28]. However, controversy in results when HPV status was taken into account appears to be elucidated [29]. In his study, Münscher *et al.* showed that the HPV status seemed to have no impact on survival [29]. We hope that our observations have highlighted the necessity of further studies when OSCC outcome is compared in patients with uni- or bilateral neck dissection.

However, there are some studies that state no difference in long-term survival between uni- and bilateral neck dissection in patients with contralateral clinically negative neck [30–33].

Gillison *et al.* in their study have proved the superiority of cisplatin plus RT as opposed to cetuximab plus RT in HPV-positive OSCC [34]. Unfortunately, we should confirm that cetuximab is the only chemotherapeutic agent for the head and neck used in Latvia when treating SCC. There is compelling evidence for reconsidering the chemoradiotherapy regimen. In this study, performing survival analysis of patients with OSCC we found that younger patients had lower early death risk than older ones. Furthermore, by reviewing

the literature one should note that RT produces the long-lasting depression of the immune system and makes some OSCC patients more susceptible to tumor recurrence and worse survival [35].

Prognostic factors have been recognized to be important in selecting the appropriate treatment for the patient. In the current study, we made attempts to predict the course of OSCC investigating the possible prognostic factors. We found that the patient's eventual outcome is strongly predicted by the T stage, therapeutic modality received (RT+SUR), hazardous habits (smoking, alcohol abuse), and the presence of lymph node metastases. Collectively, these results are suggestive of neck dissection necessity, and other studies have reported on the effectiveness of ipsilateral elective neck dissection in clinically negative necks [36–38]. Unfortunately, in our study, data on the HPV status were lacking cutting off the evaluation of the prognostic value of this factor recommended by other scientists [39–44].

In the given study, statistically significant differences in survival rates estimated for patients with OSCC revealing various types of tumor differentiation were not found. Unfortunately, completeness of records deciphering the differentiation of tumor cells, the type of growth (exophytic or endophytic), and the presence of perineural invasion were not absolute. However, some previous studies have demonstrated that endophytic growth, perineural invasion, and extracapsular extension of tumor allow suggesting on contralateral neck metastasis and lower 5-year OS [33, 45].

Problems related to early diagnostics of tumors are well recognized worldwide based on statistical data analysis, we suggest that the majority of patients are diagnosed with stage IV OSCC which means a worse outcome of the disease. Effective measures must be taken to ensure OSCC diagnosis at the early stages. Supportive evidence on the necessity of neck dissection as one of the therapeutic modalities (best results in RT+SUR group) was found by us.

The incidence of OSCC has grown in the last two decades, which, at least partly, may be explained by a contributive role of HPV. HPV positive OSCC has a better prognosis than HPV negative; therefore, HPV status should be determined for prognostic reasons and selection of an appropriate treatment plan. Indeed, bad habits as smoking and alcohol abuse are risk factors that should be included in assessing the disease outcome.

The limitation of the study is that it is a retrospective analysis with a relatively small population. It is also difficult to assess the importance of treatment modalities because some patients treated with RT alone presented with an advanced stage of cancer at the time of diagnosis and poor general health, furthermore, the chemotherapeutical interventions should be presented by more treatment schemes than cetuximab alone. Other studies reporting on similarity in regional recurrence rates observed in patients with SCC of the tongue when selective and radical neck dissections were performed have suggested on supraomohyoid neck dissection as a primary treatment for patients with clinical N0 tumor [46]. This statement agrees with the study results and suggestions, however, our study didn't attempt an assessment of various neck dissection types as well as comparison of SUR and other treatment modalities.

CONCLUSION

Collectively, the study showed that patients with lower T grade, N0 status, lacking bad habits and when SUR was applied as one of the treatment modalities had better 3 and 5-year OS and DS, and lower HR. Future studies leading to more efficient research should be undertaken combining tests for HPV validation with traditional histopathology methods independently performed in several institutions.

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DETECTION AND GENOTYPING OF HUMAN PAPILLOMAVIRUS IN HYPOPHARYNGEAL CARCINOMA SAMPLES

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The incidence of hypopharyngeal cancer globally is about 0.8 per 100 000. Globally, approximately 38 000 cases of head and neck cancer are considered yearly to be high-risk human papillomavirus (HR-HPV) related. Biopsy material fixation in formalin and embedding in paraffin (FFPE) creates many challenges. The extraction of nucleic acid material requires a more complicated approach, and often the extracted DNA is fragmented. The aim of the study was to compare several HR-HPV detection methods in nucleic acid material extracted from FFPE samples. The extracted DNA was analysed with different molecular biology methods to assess DNA quality and to determine the presence of HPV DNA with various HPV detection systems. The results were compared and statistically analysed. There was good agreement between two real-time PCR methods — Anyplex II HPV28 and Sacace HPV High-Risk Screen Real-TM Quant. We failed to reach a conclusion on agreement between real-time PCR methods and HPV16 type-specific primer PCR. There was moderate positive correlation between Anyplex II HPV28 semiquantitative results and Sacace quantitative results. We suggest that real-time PCR assays detecting smaller DNA amplicons are good and reliable methods for detecting HPV genetic material in FFPE samples.

Key words: *hypopharyngeal squamous cell carcinoma, FFPE, HPV.*

INTRODUCTION

Worldwide there are approximately 600 000 new cases of head and neck cancer registered yearly (Dayyani *et al.*, 2010). That makes this region of the body to be the 6th most common for cancer to appear in. The incidence of hypopharyngeal cancer is relatively low globally — 0.8 per 100 000 (1.4 in men and 0.3 in women) (Shield *et al.*,, 2017). Most head and neck cancers are squamous cell carcinomas (SCC).

In the last 20 years, the association between head and neck, especially, oropharyngeal squamous cell carcinoma and the presence of human papillomavirus (HPV) infection, has been established (Gillison *et al.*, 2000; Gillison and Shah, 2001; Veyer *et al.*, 2019). Globally, every year approximately 38 000 cases of head and neck cancers are considered to be HPV-related (Plummer *et al.*, 2016; de Martel *et al.*, 2017). HPV16 and 18 have been generally recognised as the most frequent causative HPV types in head and neck cancer, particularly oropharyngeal SCC (Anonymous, 2007; Gillison *et al.*, 2014; 2015; de Martel *et al.*, 2017). There is still a pending question on the significance of the role of HPV infection in the development of hypopharyngeal and laryngeal SCC (Dahm *et al.*, 2018). It was reported that about 5–7% of laryngeal cancers and as low as 0% of hypopharyngeal cancers were associated with high-risk (HR) HPV infection (Combes and Franceschi 2014; Plummer *et al.*, 2016).

Of all head and neck SCC, hypopharyngeal manifests most aggressively. Conventionally, diagnosis is made based on histopathological examination of the tumour. Often, the amount of tissue is limited for extended examination, including for DNA and RNA extraction and testing. Even greater hurdles appear when biopsy material is formalin-

fixed and embedded in paraffin (FFPE). Furthermore, DNA appears fragmented when applying a complicated approach for extracting nucleic acid material.

Fortunately, the development of FFPE DNA extraction kits has allowed the use of molecular biology methods for analysis of materials that have been stored for a long period and have small size.

Therefore, this study aimed to compare several methods of HR-HPV assessment in nucleic acid material extracted from hypopharyngeal SCC FFPE samples and to test different detection systems.

MATERIALS AND METHODS

Clinical material. We carried out a retrospective study of 31 patients diagnosed with hypopharyngeal SCC, staged following the TNM classification of the International Union against Cancer (7^{th} edition), and treated in Rīga East Clinical University Hospital, Stationary Oncology Centre of Latvia. The patients' data were collected from the Hospital Archive.

Collection of biopsy samples and tissue processing. Hypopharynx biopsy samples from patients hospitalised in Rīga East Clinical University Hospital, Oncology Centre of Latvia, were acquired between 2015 and 2019. All included patients belonged to a cohort declared and approved by Rīga Stradiņš University Ethics Committee (Decision No. 3/24.09.2015). The diagnosis of hypopharyngeal SCC was histopathologically confirmed. Biopsies were taken prior to treatment. Three to six 10 µm thick sections cut from FFPE samples were used for DNA extraction. Each sample was sectioned separately with a clean blade to exclude cross-contamination of specimens.

DNA extraction. Thirty-one hypopharyngeal SCC FFPE samples were used for DNA extraction with a *blackPREP FFPE DNA Kit* (Analytik Jena, Germany) in accordance with the manufacturer's protocol.

The quality and quantity of DNA were estimated spectrometrically (Nanodrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Beta- (β-) globin PCR with appropriate primers was used to determine the quality of isolated DNA (Vandamme *et al.*, 1995). Only β-globin positive samples were used for further analysis.

HPV DNA detection using MY09/11 and GP5+/6+ consensus primers. Polymerase chain reaction (PCR) with consensus primers MY9/MY11 and GP5+/6+ was used to detect the range of HR-HPV and low-risk HPV (LR-HPV) types (Shikova *et al.*, 2009; Şahiner *et al.*, 2014). The results were visualised by electrophoresis in 1.7% agarose gel. Amplification products of 450 base pairs (bp) and 150 bp length for MY09/11 and GP5+/6+ correspondingly were considered HPV positive. Positive and negative controls were included in each reaction.

Table 1. Primers used in the study

Primers	Sequence (5'-3')	Amplicon (bp)
Consensus primers		
MY09	CGTCC(AC)A(AG)(AG)GGA(T)ACTGATC	450
MY11	GC(AC)CAGGG(AT)CATAA(CT)AATGG	
GP5+	TTTGTTACTGTGGTAGATACTAC	150
GP6+	GAAAAATAAACTGTAAATCATATTC	
Type-specific primers		
16.L1-1	TGCTAGTGCTTATGCAGCAA	152
16.L1-2	ATTTACTGCAACATTGGTAC	
18.1	AAGGATGCTGCACCGGCTGA	216
18.2	CACGCACACGCTTGGCAGGT	

HPV genotyping using isolated HPV16 and HPV18 primers. The primers used to detect HPV16 and HPV18 specific genomic sequences are summarised in Table 1 (Shikova *et al.*, 2009). Amplification using HPV16 specific primers produces 152 bp long amplicons and using HPV18 specific primers — 216 bp amplicons. The results were visualised by electrophoresis in 1.7% agarose gel. Reactions were performed with the use of positive and negative controls. Positive and negative controls were included in each reaction.

HPV detection and genotyping by Anyplex II HPV28. Anyplex II HPV28 multiplex real-time PCR (RT-PCR) was performed as recommended by the manufacturer (Seegene, South Korea). A specimen of 5 µl DNA was added in each of two sets (wells) with 20-µl PCR reaction mix. Set A consisted of primer mix for 14 HR-HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and set B consisted of primer mix for five possible HR-HPV types (HPV26, 53, 69, 73, and 82) and nine LR- HPV types (HPV6, 11, 40, 42, 43, 44, 54, 61, and 70). Both primer sets were designed for the HPV L1 gene and produced 100 and 200 bp long amplicons, respectively.

Melting curves were obtained at 30, 40, and 50 cycles allowing semiquantitative specimen analysis and differentiating between high (+++), medium (++), or low (+) viral loads, and there were internal positive and negative controls. The kit had DNA quality control by detecting the β-globin gene. The results were analysed using the Seegene Viewer software (Seegene).

HPV detection by Sacace HPV High-Risk Screen Real-TM Quant. 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 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). The kit contains quantitative standards with the known concentration of HPV DNA, used for calculation of the viral load.

Statistical data analysis. Seegene results were assessed semiquantitatively and coded as follows: 1 – negative; 2 – for +; 3 – for ++; and 4 – for +++. Viral load (copies/10^5 cells) from the Sacace assay was expressed in $\log_{10}$ and submitted to statistical analysis. For negative samples, $\log_{10}$ random values of 0 (viral load of approximately 1 copies/10^5 cells) as a mean and SD of 0.1 were assigned (generated with GraphPad Prism random number generator).

Cohen's κ test was used to assess agreement between the HPV detection methods with 1 – indicating perfect agreement; 1 to 0.81 – very good agreement; 0.80 to 0.61 – good agreement; and 0.60 to 0.21 – moderate to a poor agreement.

The Mann–Whitney test was used to assess if the differences of nonparametric data were significant. Relations between viral load (Sacace assay) and semiquantitative results (Anyplex assay) were investigated using nonparametric Spearman's correlation analysis (Mukaka, 2012; Akoglu, 2018).

A nonlinear regression model was used to graphically assess the relationship between the viral load and the semiquantitative results of Anyplex II HPV28 assay.

RESULTS

Thirty-one patients with hypopharyngeal SCC were included in this study. Most patients were male (93.5%, n = 29), with only two female patients (6.5%). The mean patient age was 66.3 years (range 44-83.3), median — 65.2 years. Unfortunately, all of the patients presented with advanced disease stage — 11 (35.5%) with stage III, and 20 (64.5%) — with stage IV.

DNA extraction from FFPE samples was an easy and not time-consuming procedure when using the *blackPREP FFPE DNA Kit*. 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 gene-positive, which made them viable for further analysis.

Of the 31 hypopharyngeal SCC FFPE samples, only 1 was positive for HPV using MY09/11 consensus primers. In contrast, GP5+/6+ consensus primers were much more efficient with positivity of 100% (n = 31) for HPV DNA.

Further HPV genotyping using HPV16 and HPV18 specific primers showed positivity for HPV16 only. Of 31 samples, 15 (48.4%) were positive for HPV16.

Anyplex II HPV28 assay showed HPV positivity for 14 (45.2%) samples. In one case, there was co-infection by 2 HPV types (type 16 and 56), with the remaining 13 cases positive for HPV16.

The Sacace HPV High-Risk Screen Real-TM Quant test showed HPV positivity for 12 (38.7%) samples in HEX channel, which corresponded to the HPV A9 group (16, 31, 33, 35, 52, 58).

Comparison of HPV detection and genotyping results between HPV16 specific primers, Anyplex II HPV28 assay, and Sacace HPV High-Risk Screen Real-TM Quant. The same DNA extracts from the 31 selected FFPE samples tested by GP5+/6+ 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.

Although, HPV16 specific primers' PCR and Anyplex assay showed similar positivity in overall count sense (15/14 positive out of 31), only 9 cases were identical. The remaining positive cases were discordant, meaning cases positive in PCR with HPV16 specific primers were negative in Anyplex II HPV28 assay, and *vice versa*. We could not assess the agreement of both tests because of the high p-value of Cohen's kappa (Cohen's κ coefficient = 0.288, p = 0.156). 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 that the agreement between these two methods could not be assessed with significance.

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

HPV viral load. As previously mentioned, HPV High-Risk Screen Real-TM Quant (Sacace Biotechnologies, Italy) is an *in vitro* real-time amplification test for quantitative detection of HPV. Although it does not specifically show the type of HPV, it has three channels for different types of HPV. The analysed samples showed positive signals in the HEX channel only, which corresponded to HPV16.

There was a moderate positive correlation between viral load (assessed by Sacace assay) and semiquantitative Seegene assay results estimated semiquantitatively (Spearman's correlation coefficient = 0.60, CI 0.30–0.79, p = 0.0004), Figure 1.

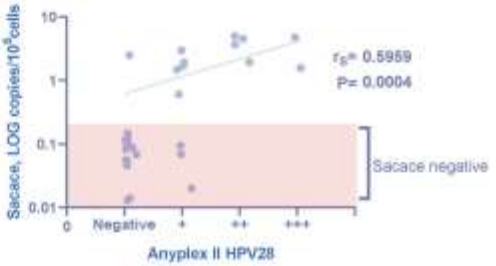

Fig. 1. Correlation between two RT-PCR assays.

DISCUSSION

All methods were used on the same DNA extracts, which makes the results highly accurate and applicable for assessment of agreement between different HPV detection methods.

As shown in different studies, the Anyplex II HPV28 assay is an appropriate and dependable HPV detection method with good sensitivity and specificity (Cornall *et al.*, 2017; Del Pino *et al.*, 2017; Baasland *et al.*, 2019; Veyer *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.*, 2019). In our study we were unable to surely conclude agreement/disagreement between Anyplex II assay and HPV16-specific primer PCR results. Simultaneously, we had multiple HPV16 positive samples by HPV16 specific primers' PCR, diagnosed as negative in Anyplex II HPV28 assay and *vice versa*. This suggests the need for multiple detection methods for FFPE DNA extracts.

Previous studies have demonstrated that genetic material extracted from FFPE is highly variable in terms of DNA quality and quantity (Lillsunde Larsson *et al.*, 2015). Various factors can affect the results of assessment – reagents used in a fixation procedure, the amount of tissue submitted to fixation and further tissue processing, etc. (Srinivasan *et al.*, 2002; Ludyga *et al.*, 2012). This applies even more for hypopharyngeal biopsy material, as biopsies are performed using local anesthetic with indirect visualisation. Nevertheless, our results show that even small amounts of DNA concentration can be successfully used for HPV DNA detection.

MY09/11 consensus primers produce 450 bp long amplicons. In our study, we had only one (of a total of 31) HPV-positive specimens with the use of these primers. In contrast, GP5+/6+ primers produce 150 bp long amplicons, which resulted in 100% positivity in our study. Therefore, we suggest that primers for shorter HPV DNA 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 can 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 estimating the viral load.

In conclusion, it seems that RT-PCR assays detecting smaller DNA amplicons are good and reliable for detecting HPV genetic material in FFPE samples. However, confirmation of HPV detected using additional HPV methods may be applicable when searching for overall sensitivity.

The author declares that there is no conflict of interest.

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CILVĒKA PAPILOMAS VĪRUSA (CPV) NOTEIKŠANA UN TIPĒŠANA *HYPOPHARYNX* PLAKANŠŪNU KARCINOMAS PARAUGOS

Hypopharynx vēža ikgadēja pasaules incidence ir aptuveni 0,8 uz 100 000. Tiek uzskatīts, ka aptuveni 38 000 galvas un kakla vēža gadījumu ir CPV infekcijas izraisīti. Ir daudz grūtību, kas saistītas ar biopsijas materiāla, kas fiksēts formalīnā un ieguldīts parafīnā, molekulāri bioloģisko analīzi. DNS izdalīšana no šādiem paraugiem bieži vien ir laikietilpīga, grūtāka, un izdalītā DNS nereti ir fragmentēta. Šī pētījuma mērķis bija salīdzināt vairākas augsta riska CPV noteikšanas metodes no parafīna blokiem izdalītajā ģenētiskajā materiālā. Tika noteikta izdalītās DNS kvalitāte, kā arī CPV DNS klātbūtne, izmantojot vairākas CPV noteikšanas "sistēmas". Rezultāti tika salīdzināti un statistiski analizēti. Starp divām reālā laika polimerāzes ķēdes reakcijas (PĶR) metodēm (*Anyplex II HPV28* un *Sacace HPV High-Risk Screen Real-TM Quant*) bija vērojama laba sakritība. Mēs nevarējām izdarīt statistiski ticamus secinājumus par rezultātu sakritību starp reālā laika PĶR metodēm un HPV16 specifisko praimeru PĶR. Daudziem paraugiem bija ne tikai identiski, bet arī pretēji rezultāti šo metožu lietojumā. Starp *Anyplex II HPV28* puskvantitatīvajiem rezultātiem un *Sacace* kvantitatīvajiem rezultātiem bija mērena pozitīva korelācija. Secinājums: reālā laika PCR testi, ar kuru palīdzību var noteikt mazākus DNS amplikonus, ir labas un uzticamas metodes CPV ģenētiskā materiāla noteikšanai parafīna bloku paraugos.

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Article

Identification of High-Risk Human Papillomavirus DNA, p16, and E6/E7 Oncoproteins in Laryngeal and Hypopharyngeal Squamous Cell Carcinomas

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Abstract: Human papillomavirus (HPV) was proven to play a significant role in cancer development in the oropharynx. However, its role in the development of laryngeal (LSCC) and hypopharyngeal squamous cell carcinoma (HPSCC) remains to be clarified. High-risk HPV (HR-HPV) viral proteins E6 and E7 are considered to be pertinent to HPV-related carcinogenesis. Hence, our aim was to estimate LSCC and HPSCC for HR-HPV DNA, p16, and E6/E7 oncoprotein status by using molecular virology and immunohistochemistry methods. The prevalence of HPV16 infection was 22/41 (53.7%) and 20/31 (64.5%) for LSCC and HPSCC, accordingly. The majority of HPV16+ tumor samples were stage III or IV. In most samples, the presence of either HPV16 E6 or HPV16 E7 viral protein in dysplastic or tumor cells was confirmed using immunohistochemistry. Our results suggest a high prevalence of HPV16 as a primary HR-HPV type in LSCC and HPSCC. The lack of HPV E6/E7 oncoproteins in some tumor samples may suggest either the absence of viral integration or the presence of other mechanisms of tumorigenesis. The utilization of p16 IHC as a surrogate marker of HR-HPV infection is impractical in LSCC and HPSCC.

Keywords: HPV; larynx; hypopharynx; squamous cell carcinoma; PCR; immunohistochemistry; p16 regulatory protein; E6/E7 viral oncoproteins

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1. Introduction

Laryngeal and hypopharyngeal cancers belong to a large group of squamous cell carcinomas of the head and the neck. The annual incidence of these types of tumors in the world population exceeds 250,000 new registered cases [1]. Among these two anatomically closely related cancers, hypopharyngeal tumors are known to be associated with worse outcomes [2].

In the last three decades, the contribution of HPV to the development of oropharyngeal squamous cell carcinoma (OPSCC) was proven [3,4]. Furthermore, HPV-associated OPSCC is reported as biologically and clinically distinct from tobacco and alcohol-related OPSCC [5]. A better prognosis of HPV-positive (HPV+) OPSCC, compared to HPV-negative (HPV-) OPSCC, with 80% or higher three year overall survival for locally advanced disease was confirmed [3,6–9]. Currently, the biological role of HPV in the pathogenesis of laryngeal and hypopharyngeal squamous cell carcinomas (LSCC and HPSCC) appears controversial and has not been sufficiently studied [10,11].

Accumulating evidence suggests the epithelium of skin and mucosa is infected through superficial defects, and, upon establishment of viral genomes in the nucleus

of infected cells as episomes, the early viral genes E1, E2, E6, and E7 are expressed. Furthermore, HPV episomes are maintained in poorly differentiating but actively dividing basal epithelial cells by replicating along with cellular DNA [4,12,13]. Along with the natural upward migration and the further differentiation of epithelial cells, the productive phase of the viral life cycle is triggered, allowing for the continued expression of E6 and E7 in differentiating cells [14]. Therefore, from an oncogenic standpoint, high-risk HPV (HR-HPV) E6 and E7 proteins are of the utmost importance [15].

While the viral oncoprotein E6 induces the degradation of p53, leading to the inhibition of elimination by apoptosis in affected epithelial cells, oncoprotein E7 inactivates tumor suppressor proteins of the pRb family, promoting the transcription of p16 [5,13,15–17]. Upon proteasomal degradation of pRb, p16 becomes overexpressed and, therefore, applicable for immunohistochemical (IHC) detection of HPV-driven tumors [18,19]. The necessity of the inclusion of p16 status when diagnosing OPSCC was confirmed by updating a TNM Classification of Malignant Tumors, and p16 IHC proved to be a reliable and, therefore, stand-alone test for the detection of HPV in OPSCC [5,20]. Furthermore, the eighth edition of the AJCC Cancer Staging Manual separated HPV positive OPSCC from HPV negative OPSCC, highlighting the biological role and the prognostic significance of p16 [21,22]. However, the question of whether HPV infection actively contributes to cancer development needs a substantial examination [23]. The presence of the p16-positive (p16+) OPSCCs in HPV-cases was demonstrated by previous studies, thus suggesting the existence of other mechanisms of p16 overexpression [24–26]. Simultaneously, contrary conditions demonstrating the presence of p16-negative (p16-) but HPV RNA-positive tumors were reported [27]. This is the reason for the suggested multimodality testing for OPSCC—both p16 and HPV DNA/ RNA detection [28].

Application of p16 for the assessment of transcriptionally active HR-HPV infection in non-OPSCCs and discussion around it highlight the complexity arising when exploring LSCC and HPSCC [29–31]. Currently, it is recommended that HPV testing on head and neck cancers should be limited to assays for HR-HPV types, and it should be routinely performed on (but also limited to) OPSCC and metastatic SCCs in neck lymph nodes from unknown primary sites [24,30,31]. The prevalence of HPV+ HPSCC and LSCC varies depending on region and study center reports, suggesting 5–20% of laryngeal cancers and as little as 0% of hypopharyngeal cancers are associated with HR-HPV infection [32–35]. Previous studies validated HPV-specific testing modalities such as HPV DNA-ISH, DNA polymerase chain reaction (PCR), mRNA RT PCR, and mRNA ISH for viral oncoproteins E6 and E7 as well as p16 IHC, including those performed on formalin-fixed, paraffin-embedded (FFPE) specimens in OPSCC; however, more clarity is needed to better explore these tests applicable to LSCC and HPSCC cases [36].

This study aimed to estimate LSCC and HPSCC for HR-HPV DNA, p16, and E6/E7 oncoprotein status by using molecular virology and immunohistochemistry methods.

2. Materials and Methods

2.1. Patients' Characteristics

Seventy-two patients, 68 (94.4%) males (median age 64.9 (range 44.2–83.3)) and 4 (5.6%) females (median age 70.8 (range 53.5–77.5)) with histologically confirmed LSCC and HPSCC, treated at the Latvian Oncology Centre between January 2015 and August 2019, were enrolled in the study.

The clinical data of patients included information on TNM stage, smoking and drinking habits, and clinical features of the disease at the time of presentation. Forty-one of 72 patients had LSCC; for 31 patients, the primary tumor site was the hypopharynx. Most patients (88.9%) were smokers; 15 (20.8%) were heavy drinkers [37]. The patients' data are summarized in Table 1.

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.

Table 1. Patients' characteristics.

	Cases (n = 72)	
	LSCC (n = 41)	HPSCC (n = 31)
Sex:		
Male	39	29
Female	2	2
Hazardous habits		
Smoking	37	27
Excessive drinking	8	7
Age (median)	64.3	65.9
T grade:		
T1	4	0
T2	8	4
T3	24	16
T4	5	11
N grade		
N0	35	6
N1	4	16
N2	2	8
N3	0	1
M grade		
M0	40	27
M1	1	4
Stage:		
I	4	0
II	7	0
III	22	10
IV	8	21

2.2. DNA Extraction

Total DNA was extracted from either fresh frozen biopsies and surgical materials (34 LSCC and 3 HPSCC) or FFPE tumor tissue blocks (28 HPSCC and 7 LSCC).

DNA extraction from the fresh frozen tumor tissue samples was carried out with the phenol/chloroform extraction method.

DNA extraction from FFPE tumor samples was carried out with the blackPREP FFPE DNA Kit (Analytik Jena, Germany) following the manufacturer's protocol. Three to six 10 µm thick sections cut from FFPE samples were used for DNA extraction. Each sample was sectioned separately with a sterile blade to exclude cross-contamination of specimens.

The concentration and the quality of the extracted DNA were measured spectrophotometrically (Nanodrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Beta- (β-) globin PCR with appropriate primers was used to determine the quality of isolated DNA [38]. All samples were β-globin positive.

2.3. HPV DNA Detection Using MY09/11 and GP5+/6+ Consensus Primers

Initially, separate regular polymerase chain reactions (PCR) with consensus primers MY9/MY11 and GP5+/6+ were used for the detection of a broad range of HPV types [39,40]. The results were visualized using 1.7% ethidium bromide electrophoreses gel. The amplification products of 450 base pairs (bp) and 150 bp length for MY09/11 and GP5+/6+, correspondingly, were considered HPV-positive (Table 2). Positive and negative controls were included in each reaction.

Table 2. Oligonucleotide primers used for HPV DNA detection.

Primers	Sequence (5'-3')	Amplicon (bp)
β-globin primers		
GS 268	ACACAACTGTGTTCACTAGC	200
GS 269	TGGTCTCCTTAAACCTGTCTTG	
Consensus primers		
MY09	CGTCC(AC)A(AG)(AG)GGA(T)ACTGATC	450
MY11	GC(AC)CAGGG(AT)CATAA(CT)AATGG	
GP5+	TTTGTTACTGTGGTAGATACTAC	150
GP6+	GAAAAATAAACTGTAAATCATATTC	
Type-specific primers		
16.L1-1	TGCTAGTGCTTATGCAGCAA	152
16.L1-2	ATTTACTGCAACATTGGTAC	
18.1	AAGGATGCTGCACCGGCTGA	216
18.2	CACGCACACGCTTGGCAGGT	

2.4. HPV Genotyping

Two types of primers were used: the type-specific primers for HPV 16 and 18 (L1) and the Anyplex II HPV28 multiplex real-time-PCR (RT-PCR).

Genomic sequences of HPV16 and HPV18 type-specific primers' are summarized in Table 2 [40]. Amplimers of 152 bp in length were produced by HPV16 primers, whereas 216 bp long amplimers were produced by HPV18 primers. The results were visualized by electrophoresis in 1.7% agarose gel. Each reaction included positive and negative controls.

Anyplex II HPV28 multiplex RT-PCR was performed as recommended by the manufacturers (Seegene, South Korea). In total, 5 µL of specimen DNA were added in each of the two sets (wells) with 20 µL PCR reaction mix. Set A consisted of a primer mix for 14 HR-HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and set B consisted of a primer mix for five possible HR-HPV types (HPV26, 53, 69, 73, and 82) and nine LR- HPV types (HPV6, 11, 40, 42, 43, 44, 54, 61, and 70). Both primer sets were designed for the HPV L1 gene and produced 100 and 200 bp amplicons, accordingly. Melting curves allowing the semiquantitative assessment and the differentiation between high (+++), moderate (++), or low (+) viral load were obtained at 30, 40, and 50 cycles and had positive and negative internal controls.

The kit had DNA quality control detecting the β-globin gene; all analyzed samples were β-globin positive. The results were analyzed using the Seegene Viewer software (Seegene, South Korea).

2.5. Immunohistochemistry

LSCC specimens were obtained during laryngectomy and cordectomy. A small piece of surgically obtained LSCC was further processed for molecular testing, whereas the remaining material as a FFPE tissue was submitted to IHC.

In contrast, HPSCC specimens were mostly obtained during a biopsy procedure and further processed as FFPE tissue samples at Latvian Oncology Centre. Only tumor tissue samples confirmed by histopathological examination as HPSCC were used in the study.

HPV 16 E6/E7 proteins and p16 were assessed immunohistochemically. Histological sections of 4–5 µm were cut from FFPE tissues and mounted on slides. The consecutive sections were used as negative controls of the immunohistochemical reactions and for hematoxylin and eosin (H&E) staining to confirm the diagnosis. Immunohistochemistry (IHC) was performed manually using sections collected on SuperFrost Plus slides (Gerhard Menzel GmbH, Germany). Immunostaining was carried out following the previously used IHC protocol [41,42].

The sections were incubated at 4 °C overnight with the following primary antibodies: monoclonal mouse anti-CDKN2A/p16INK4a antibody (Abcam, Cambridge, UK,

1:300 dilution, ab201980); monoclonal mouse anti-HPV16 E6 + HPV18 E6 antibody (Abcam, Cambridge, UK, prediluted, ab51931), which recognize the HPV early antigen E6 of HPV 16 and 18 [43–45]; mouse monoclonal anti-HPV16 E7 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:50 dilution, sc-6981).

The amplification of the primary antibody and the visualization of reaction products were performed by applying the HiDef Detection HRP Polymer system and diaminobenzidine tetrahydrochloride substrate kit (Cell Marque, Rocklin, CA, USA). The sections were counterstained with Mayer's hematoxylin, washed, mounted, and covered with coverslips. The immunohistochemical controls included the omission of the primary antibody. The assessment of immunostaining was performed by two independent observers blinded to clinicopathological data.

The sections were photographed by a Leitz DMRB bright-field microscope using a DFC 450C digital camera or scanned with a Glissando Slide Scanner (Objective Imaging Ltd., Cambridge, UK) with a 10×, 20×, and 40× objective.

Cells that were labeled with anti-CDKN2A/p16INK4a, anti- HPV16 E6 + HPV18 E6, and anti-HPV16 E7 antibody and that displayed brown reaction products were considered immunopositive.

The assessment of immunostaining of p16 was carried out by determining positive vs. negative structures with a cut-off at 50% tumor cells independently of the reaction proposed by Hong et al., (2013) [46]. The immunostaining assessment for E6 and E7 viral proteins was performed semiquantitatively in 20 randomly selected visual fields of each sample (magnification 400×) representing the tumor and the surface epithelium of the regions of interest. The levels of E6 and E7 were graded as negative—0%, weak—≤10%, moderate—11–50%, and strong—>50%, respectively.

2.6. Immunofluorescence

To better visualize the cellular distribution and the localization of the HR-HPV16 E7 oncoprotein, the tumor tissue specimens were processed for fluorescence-based immunodetection. The sections immunoreacted with mouse monoclonal anti-HPV16 E7 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:50 dilution, sc-6981) overnight at 4 °C were washed in PBS and incubated with goat anti-mouse IgG-FITC: sc-2010 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA 1:300) as the secondary antibody. Then, sections were counterstained with 4′,6-diamidino-2phenylindole (DAPI) (Thermo Fisher Scientific, Invitrogen, Renfrew, UK, 1:3000) and mounted in Prolong Gold with DAPI (Thermo Fisher Scientific, Waltham, MA, USA). Imaging was performed using an Eclipse Ti-E confocal microscope (Nikon, Tokyo, Japan).

The workflow of the present study is summarized in Appendix A.

2.7. Statistical Data Analysis

All the statistical analyses were performed using the GraphPad Prism 9 (demo, GraphPad Software, La Jolla, CA, USA). Anderson–Darling, D'Agostino and Pearson, and Shapiro–Wilk normality tests were applied to assess numerical data distribution. The comparison of means between different groups of numerical variables was performed using one-way ANOVA. For data with a non-Gaussian distribution, Kruskal–Wallis or Friedman's test (for paired groups) followed by the two-stage step-up method of Benjamini, Krieger, and Yekutieli as a false discovery rate controlling test were used. To compare numerical values between two groups, the Mann–Whitney test or the Wilcoxon test (for pared groups) was applied. The relations between the analyzed groups were investigated using nonparametric Spearman's correlation analysis [47]. The IHC results were expressed as violin plots and stacked bar graphs, and a p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. HPV DNA Detection Using MY09/11 and GP5+/6+ Consensus Primers

Eleven (15.3%) out of 72 tumor samples were positive for HPV genomic sequences using PCR with MY9/11 consensus primers. In turn, 55 (76.4%) samples demonstrated positivity using PCR with GP5+/6+ primers. By summing up, when tested with consensus PCRs, 61 (84.7%) tumor tissue samples were found positive for HPV DNA—31 HPSCC and 30 LSCC samples.

3.2. HPV Genotyping Using HPV16 and HPV18 L1 Primers

All the tumor tissue samples (n = 72) were subjected to HPV genotyping using HPV16 and HPV18 L1 primers. A total of 2/72 tumor tissue samples (both LSCC), positive when detected by HPV16 L1 primers, were negative in consensus PCRs. No specific HPV-18 genomic sequence was found in any of the samples.

Overall, 26/72 (36.1%) samples were positive for HPV16—10 LSCC and 16 HPSCC samples. In total, 63 HPV+ samples were considered applicable for further analysis; among them, 61 were selected using consensus PCRs, whereas 2 additional samples were selected by HPV16 L1 PCR.

3.3. Detection of HPV Using Anyplex II HPV28 RT-PCR

All 63 HPV-positive samples confirmed using either consensus primers or HPV16-specific primers were further explored by Anyplex II HPV28 multiplex RT-PCR. All samples were β-globin positive (internal control). When assessed by Anyplex II HPV28 multiplex RT-PCR, 28/63 samples were HPV-negative. HPV16 monoinfection was confirmed in 32/63 samples, whereas HPV16 and HPV31 coinfection was confirmed in 2/63 samples, and HPV16 and HPV56 coinfection in 1/63 samples. When HPV+ samples were stratified by the location, 19 LSCC and 13 HPSCC presented as HPV16+, 2 LSCC presented as demonstrating HPV16 and HPV31 coinfection, and 1 HPSCC presented as demonstrating HPV16 and HPV56 coinfection.

Interestingly, seven (one LSCC and six HPSCC) HPV16+ tumor tissue samples, confirmed by applying HPV16 L1 PCR, were negative according to Anyplex II HPV28 RT-PCR, thus contributing to a total number of 42/72 (58.3%) HPV16+ samples. The prevalence of HPV16 infection, including multiple infections in a sample, was 22 of 41 (53.7%) for LSCC and 20 of 31 (64.5%) for HPSCC (Supplementary Table S1). Most of the HPV16+ samples were stage III or IV tumors (Figure 1a).

Twenty-one HPV16+ samples presented with low, nine with moderate, and two with high viral load, respectively, when detected using the Anyplex II assay. The presence of multiple HPV infections was confirmed in three samples (one HPSCC and two LSCC). A low viral load for both HPV types was established in the HPSCC sample presented with HPV16 and 56 coinfections. Similarly, a low viral load for both HPV types was confirmed in the LSCC sample presented with HPV16 and 31 coinfections. By contrast, a low viral load was confirmed for HPV16, whereas a high viral load was confirmed for HPV31, assessed in the remaining LSCC sample with HPV 16 and 31 coinfections, thus suggesting the dominance of HPV31. The HPV16 RT-PCR data are summarized in Figure 1b.

In most LSCC samples (12/22), the expression of the E6 oncoprotein in the tumor mass was low (Figure 3a). By contrast, a dysplastic epithelium demonstrated an almost equal distribution of expression levels; six, five, and seven cases presented with levels <10%, 11–50%, and >50%, respectively. In 3/22 cases, dysplastic epithelial cells were E6-negative; however, among three tumors, two neoplasms presented with low immunopositivity (Figure 3b). In most specimens, positive staining in the invasive front of tumor mass was noticed, commonly presented as the decoration of the suprabasal cells (Figure 2c). Furthermore, the expression of the HPV E6 viral protein in the endothelial cells of small blood vessels was demonstrated (Figure 2b,c).

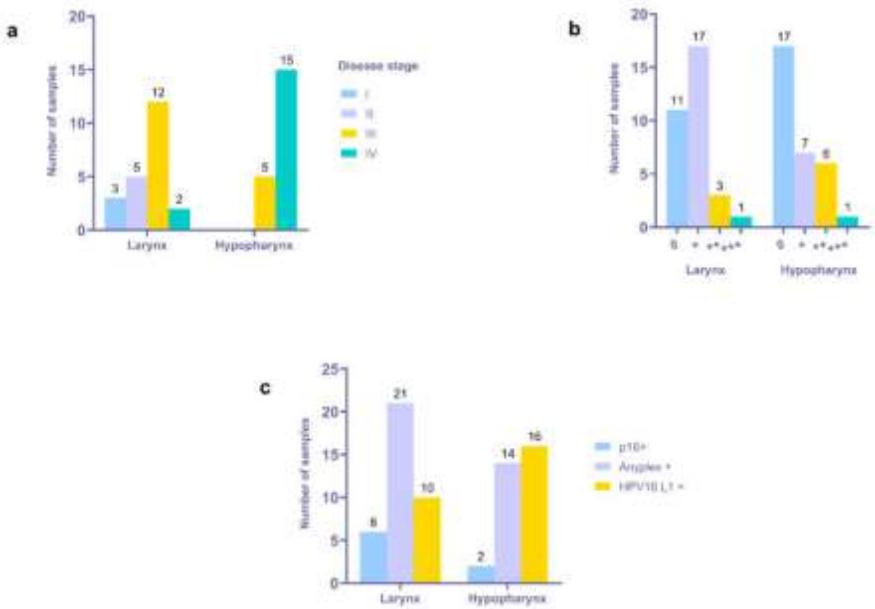

Figure 1. Distribution of HPV16+ tumor samples according to location, disease stage, and PCR data. (**a**) Distribution of HPV16+ tumor samples according to location and disease stage. (**b**) Distribution of HPV16+ samples according to location and Anyplex assay results; 0—negative, +—low viral load, ++—moderate viral load, +++—high viral load. (**c**) Cross-reference of p16 and HPV status.

3.4. Immunohistochemical Detection of p16^{INK4a}

Immunohistochemically, the expression of p16INK4a was confirmed in 11.1% of the tumor tissue samples. Out of 41 LSCC and 31 HPSCC, in six and two samples, respectively, were found p16+. Cross-referencing p16 and HPV status, the tumors were stratified as follows: 7/72 (9.7%)—p16+/HPV+, 1/72—p16+/HPV-, 8/72 (11.1%)—p16-/HPV-, and 56/72 (77.8%)—p16-/HPV+. Most of the p16+/HPV+ tumors were LSCC (n = 6), whereas two were HPSCC (Figure 1c). The only p16+/HPV- tumor was LSCC. Six out of seven p16+/HPV+ tumors had HPV16 mono-infection, whereas one had HPV16 and 31 coinfections. A total of 35 out of 56 p16-/HPV+ tumors, 27 LSCC and 29 HPSCC, were positive for HPV16 when explored by Anyplex II RT-PCR and HPV16 L1 primers' PCR, whereas two had the aforementioned HPV coinfections.

3.5. Immunohistochemical Detection of HPV16 E6 and E7 Oncoproteins in LSCC and HPSCC

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

The expression of E6 in HPV16+ LSCC specimens was detected in 21 of 22 cases. The immunoreactive structures were revealed both within the tumor mass and the surface epithelium of the region of interest, demonstrating dysplastic features. One specimen contained only the tumor nest. In 3/22 cases, immunoexpression of HPV16/18 E6 oncoprotein in the tumor mass was strong (>50%); furthermore, among them, 2/22 cases presented with strong positivity in a dysplastic epithelium (Figures 2a and 3a,b).

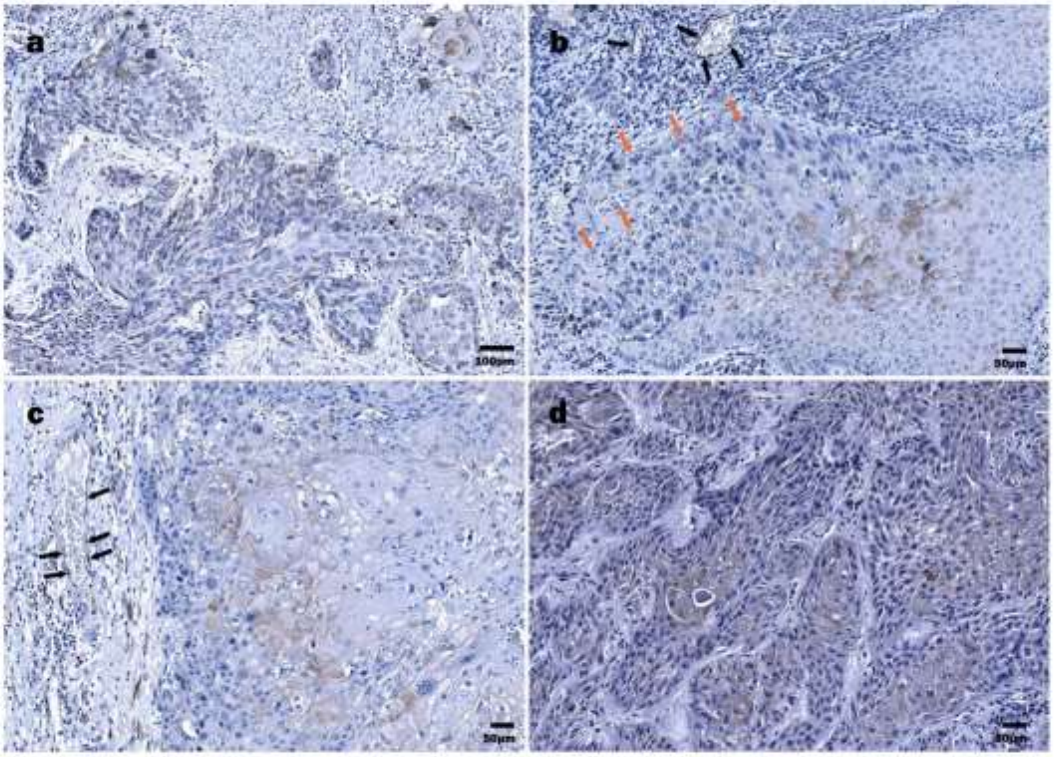

Figure 2. Immunohistochemical detection of HPV16/18 E6 oncoprotein: (**a**) LSCC, tumor cords and nests composed of diffusely distributed E6 protein-positive cells interspersed by the E6 oncoprotein-negative cells; (**b**) LSCC, differentiated suprabasal tumor cells demonstrating abundant HPV16 E6-positive cytoplasm and polymorphous nuclei (orange arrows), E6-positive endotheliocytes (black arrows) within a tumor stroma; (**c**) LSCC, HPV16/18 E6 positivity in suprabasal, more differentiated tumor cells; E6-positive endothelial cells (black arrows); (**d**) HPSCC, densely packed tumor cords demonstrating HPV16 E6 oncoprotein positivity, almost exclusively in more differentiated cells.

HPV16 E7 protein immunoexpression was confirmed in 20/21 LSCC specimens (Figure 3c). In two cases, the specimen did not contain an epithelial region, being predominantly tumor. In one case, there was not enough material for suitable immunohistochemical detection of the HPV16 E7 protein. Cells labeled by the HPV16 anti-E7 antibody commonly displayed a nuclear staining pattern and rarely showed nuclear and cytoplasmatic staining pattern. The presence of HPV16 E7 oncoprotein expression was confirmed in an affected epithelium, both stratified squamous and pseudostratified ciliated (Figure 4a). Dominant immunostaining was confirmed in basal and suprabasal cells (Figure 4b). Strong immunoexpression of the HPV16 E7 oncoprotein in the tumor nests was found in 8/21 LSCC samples (Figure 3c,d and Figure 4c). Finally, HPV16 E7 oncoprotein immunopositivity was demonstrated along an intimal aspect of small blood vessels.

Eighteen out of 20 HPSCC samples were HPV16 E6 oncoprotein positive. However, immunohistochemically HPV16 E6 positivity in a tumor mass was confirmed in 13/20 cases (Figure 3e,f). In most samples, cytoplasmatic immunoexpression of the HPV16 E6 oncoprotein within the dysplastic epithelium was evidenced. Commonly, the expression of the HPV16 E6 oncoprotein within a tumor mass was low. Immunostaining within differentiated tumor cells was also demonstrated (Figure 2d). Similar to the LSCC samples, some endothelial cells were found to be HPV16 E6-positive.

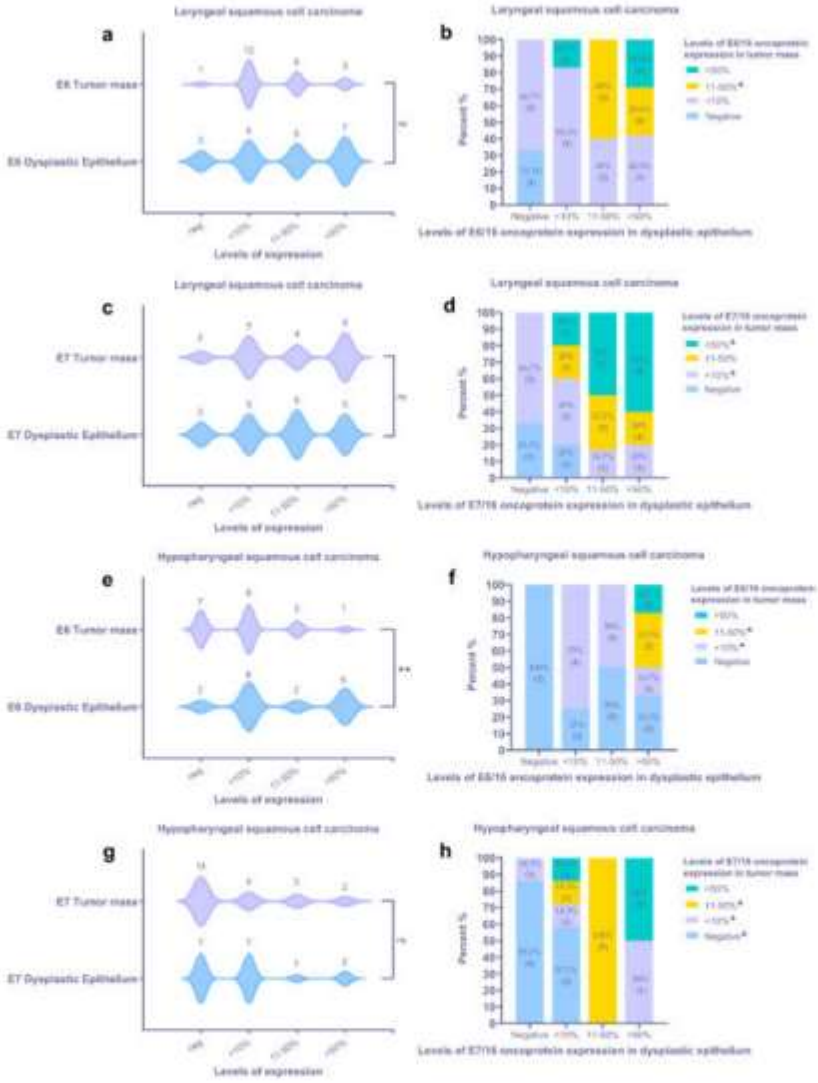

Figure 3. Assessment of viral oncoproteins E6 and E7 in HPV16+ laryngeal (**a**–**d**) and hypopharyngeal (**e**–**h**) tumor tissue samples using IHC and statistics: (**a**,**c**) characterization of HPV oncoprotein E6 (**a**) and E7 (**c**) immunoexpression within a tumor mass and dysplastic epithelium of LSCC samples; (**b**,**d**) the IHC expression levels for HPV oncoprotein E6 (**b**) and E7 (**d**) in a tumor 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 tumor mass and dysplastic epithelium of HPSCC samples; (**f**,**h**) the IHC expression levels for HPV oncoprotein E6 (**f**) and E7 (**h**) in a tumor 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 an epithelial region suitable for assessment and, therefore, were excluded from crosstab analysis.

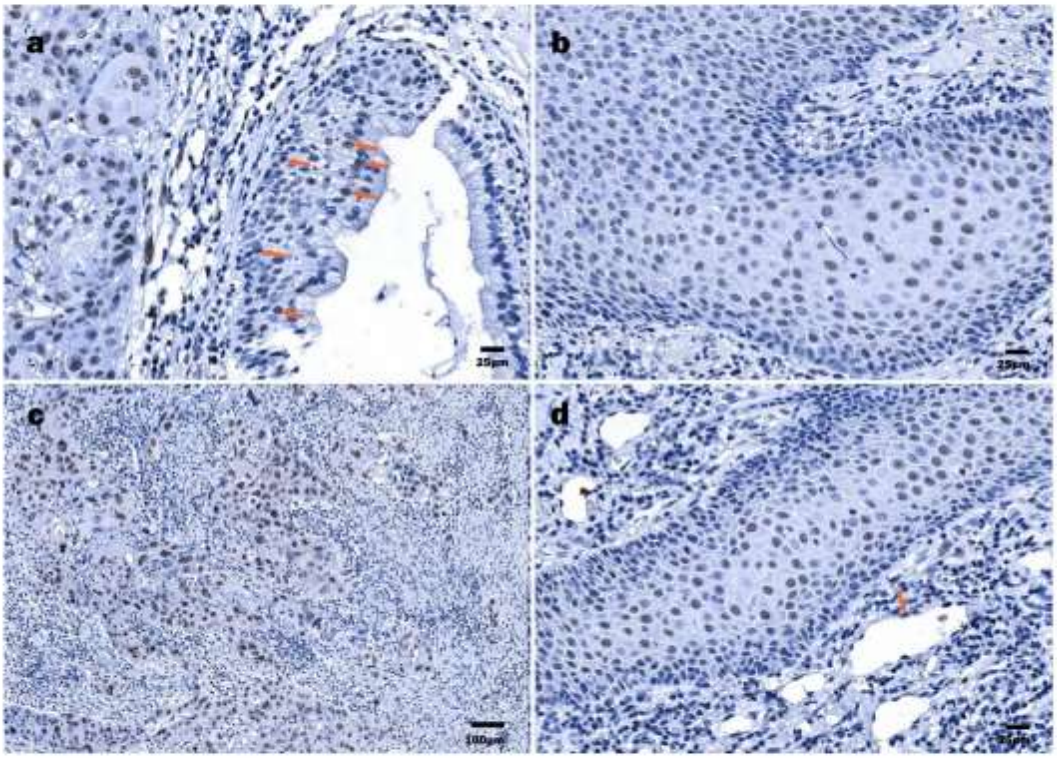

Figure 4. Immunohistochemical detection of HPV16 E7 oncoprotein: (**a**) LSCC, tumor cells within a nest and some surface cells (orange arrows) demonstrating nuclear HPV16 E7 positivity; (**b**) LSCC, numerous HPV16 E7-positive cells displaying nuclear immunostaining pattern; (**c**) LSCC, highly polymorphous HPV16 E7-positive tumor cells demonstrating nearly total nuclear decoration; (**d**) HPSCC, numerous HPV16 E7-positive cells displaying nuclear immunostaining pattern, endothelial (orange arrow) cells.

An even smaller number of HPV16 E7 oncoprotein positive HPSCC cases were found when compared to HPV16 E6-positive cases, represented by 13/20 cases (Figure 3g,h). Furthermore, a positive reaction within a tumor mass was confirmed in 9/20 cases. Among them, a strong tumor immunoreaction was demonstrated in 2/20 HPSCC samples. The expression was nuclear only. In differentiated suprabasal cells and endothelial cells, a positive HPV16 E7 oncoprotein reaction was found (Figure 4d).

Collectively, the immunoexpression of HPV16 E6/E7 oncoproteins within a tumor mass was not confirmed in 7/42 samples (one LSCC and six HPSCC). Five of these samples were HPV16+ with the Anyplex RT-PCR and two with HPV16 L1 PCR, all of them p16-. Immunohistochemically, only one HPSCC and one LSCC sample were both HPV16 E6 and E7 oncoprotein negative; simultaneously, the HPSCC sample was HPV16+ by HPV16 L1 PCR and the LSCC sample by Anyplex RT-PCR. Matched HPV16 E6/E7 positivity was demonstrated in 2/42 samples within the dysplastic epithelium. No significant differences in tumoral or dysplastic epithelial HPV16 E6/E7 oncoprotein expression were found, except a significant difference demonstrated for E6 oncoprotein positivity in the HPSCC samples (Figure 3e). Overall, a similar tendency of HPV oncoprotein E6/E7 expression was demonstrated within a tumor mass and the dysplastic epithelium in both LSCC and HPSCC (Figure 3a,c,g).

The results of semiquantitative RT-PCR and IHC E6/E7 oncoprotein immunoexpression were further submitted to nonparametric correlation analysis. A moderate positive correlation ($r_S = 0.445$, $p = 0.056$) between semiquantitative RT-PCR and HPV16 E7 IHC data was demonstrated in the LSCC tissue samples, particularly in the dysplastic epithelium. Additionally, there were weak-to-moderate positive correlations found in the HPSCC tissue samples; however, they failed to reach statistical significance (Supplementary Figure S1).

Additionally, we performed nonparametric correlation analysis of p16 and E6/E7 IHC, HPV16 L1 PCR, and Anyplex RT-PCR results. No statistically significant correlations were found in LSCC and HPSCC samples for the aforementioned data (Supplementary Figure S2).

To ensure better visualization of the E7 oncoprotein, in addition to bright-field optics, fluorescence-based immunodetection was applied. By immunofluorescence, the presence of HPV16 E7 was confirmed in the cytoplasm and the nuclei of the tumor cells (Figure 5). Notably, nuclear E7 immunopositivity was confirmed and found to be in agreement with the bright-field optics observations; however, the intensity of staining greatly varied. The cytoplasmic E7 oncoprotein targeted was expressed in occasional or multiple cells constituting a tumor mass.

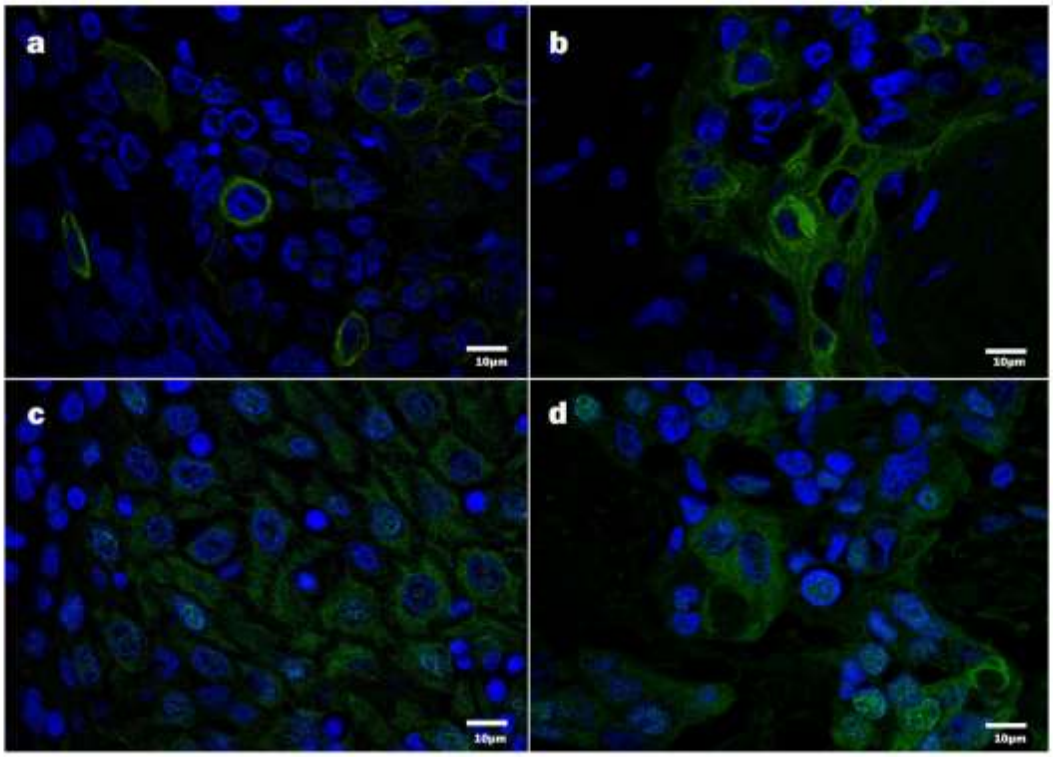

Figure 5. Detection of HPV16 protein E7 by immunofluorescence, confocal microscopy, DAPI—blue, HPV16 E7 immunopositive products—green: (**a**,**b**) HPV16 E7 positive tumor cells, displaying chiefly cytoplasmic positivity; (**c**,**d**) HPV16 E7 positive tumor cells, displaying cytoplasmic and nuclear positivity.

4. Discussion

According to the available data, around 20% of LSCC and 5% of HPSCC are attributable to HPV infection in the USA [48]. In Europe, the incidence of HPV+ head and neck cancer is lower [33]. However, it is higher in developed countries, such as the United Kingdom, Denmark, and Germany, than in less developed ones (mostly Eastern European countries) [49–51]. This difference may be explained by different lifestyles, preferences, and sexual habits. Notably, smoking, recognized as a significant factor influencing the development of head and neck cancer, is a common hazard in Latvian society [52]. However, the present study suggests a role of HPV in the carcinogenesis of non-oropharyngeal cancer and points at HPV16 being a predominant HPV type confirmed in LSCC and HPSCC. These results are consistent with the data published by other authors [33,53,54].

This study highlights the high incidence of HPV+ tumors and HR-HPV's role in the pathogenesis of HPSCC and LSCC in Latvia when compared to Europe and North America. Higher prevalence of HPV16 + tumors—53.7% and 64.5% in LSCC and HPSCC, respectively, were demonstrated in the given study. However, the question of whether HPV infection in the tumor tissue is transcriptionally active needs further extensive investigation and remains open [55]. In this context, the detection of HPV E6/E7 mRNA in LSCC and HPSCC tissue samples may add clarity to this problem [56]. The other problematic issue is the necessity of better distinction between primary tumors and those that are an extension from different sites, for example, the oropharynx, which is generally considered to be most associated with HPV infection [33]. Often, in the late stages of the disease, it is hard to distinguish the primary tumor site. Therefore, optimization of diagnostic accuracy at the early and especially at the late stages of the malignant process is of pivotal significance. In this study, we confirmed the presence of HPV16 in a large portion of LSCC and HPSCC characterized as stage III and IV tumors, thus suggesting a possible linkage between the late stage of a tumor and a higher prevalence of HR-HPV infection. This evidence is in agreement with the results published by other authors reporting on the late stages of hypopharyngeal cancer presenting with a high prevalence of HR-HPV infection [57]. Further studies unraveling the intimate relationships between the stage of neoplasm and HPV status could be of interest.

To the best of our knowledge, few studies previously explored the presence of HPV oncoproteins E6 and E7 in tumor and dysplastic epithelial cells by IHC [58–60]. Previously, some HPV DNA and RNA in situ hybridization results obtained by other authors using FFPE samples and conventional light microscopy were reported [5,30,61]. In this study, FFPE samples selected from the HPV16+ tumors (n = 42) detected by molecular biology methods were used. Most HPV16+ samples demonstrated either oncoprotein E6/16 or E7/16 positivity. However, the absence of oncoprotein E6/E7 immunostaining, evidenced in some samples, is likely to suggest other, non-HPV-related mechanisms of tumor development.

The given study aimed to report on the peculiarities of tumorigenesis in the larynx and the hypopharynx and the likely differences between these two sites, highlighting HR-HPV DNA, p16, and E6/E7 oncoprotein status assessed using molecular virology and IHC methods. Even though most correlations failed to reach statistical significance, weak to moderate positive correlations between the molecular virology and the IHC results may indicate active HPV infection in these samples. However, the data about the activity of HPV infection (detection of viral mRNA) could clarify this hypothesis. In this study, PCRs confirmed the presence of HPV DNA in the LSCC and HPSCC samples; still, the molecular virology methods applied failed to distinguish between active and latent infection. On the other hand, the presence of HPV E6/E7 proteins, known as significant contributors to tumor development, suggests active participation of HR-HPV infection in tumorigenesis.

Interestingly, in some HPV16+ specimens, tumor cells stained negative for HPV16 E6/E7 oncoproteins, whereas dysplastic epithelium stained positive. Finally, some endothelial cells were found to be positive for HPV16 E6/E7 proteins. These results reflect the limitations of PCR assays, which do not specify the source of the genetic material. In

general, the presence of HR-HPV E6 andE7 oncoproteins suggests a possibility of cancerous transformation of these cells. Viral integration and dysregulation of E6 and E7 gene expression is a common tumorigenesis mechanism confirmed in HPV-related cancers in general and in cervical cancer in particular [16,62]. However, in HPV-associated head and neck SCCs, viral integration occurs less regularly. In these tumors, dysregulation of the E6/E7 genes can be induced in the episomal state, for example, by the disruption of the HPV E2 binding sites by methylation [63–65]. The lack of HPV16 E6/E7 oncoproteins in the tumor cells and, contrarily, the appearance of these in dysplastic epithelial and endothelial cells demonstrated in our study may reflect the absence of HPV integration. In the advanced tumor stages, viral DNA could be cleared from the tumor itself. Other mechanisms of tumorigenesis could also exist.

Some authors suggested that HR-HPV infection may contribute to laryngeal carcinogenesis via integration of the viral DNA in the host cell genome and a further increase in p16 expression [66]. In this study, however, high numbers of p16-/HPV+ specimens in LSCC and HPSCC patients were demonstrated. Furthermore, there were no significant correlations found between the p16, E6/E7 IHC, and PCR data. These results are in agreement with other authors, suggesting p16INK4a can be used as a surrogate marker of HPV infection in OPSCC but appears impractical in laryngeal and hypopharyngeal cancers [31,67,68].

The use of broad spectrum of HPV-specific tests such as HPV DNA PCR, detection of HR-HPV and LR-HPV types, along with IHC staining of HPV surrogate marker p16 and viral oncoproteins E6/E7, confirmed by conventional and fluorescence-based immunodetection methods, may be considered as the strength of this study. A few limitations should be considered when interpreting our data. A moderate number of samples were used in this study. The second limitation is related to the absence of HPV mRNA data. These data would be of interest, bringing clarity to the question regarding the activity of HPV infection in analyzed tumors. Finally, some imbalance in gender and tumor stage characteristics, but not affecting the overall results, should be explained by the legal norms and the inclusion criteria used in the given study.

5. Conclusions

In conclusion, this study based on HPV testing assays and a robust platform of IHC methods used to further explore p16 status and the presence of viral oncoproteins E6/E7 confirmed a high prevalence of HPV16 genotype in laryngeal and hypopharyngeal cancers. The absence of the HPV E6/E7 oncoproteins in some tumor samples suggests a mechanism different from the viral integration tumorigenesis mechanism. Unlike in OPSCC, the application of p16 IHC as a surrogate marker of active HR-HPV infection in LSCC and HPSCC appears impractical.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v13061008/s1, Table S1: The results of different PCRs, Figure S1: Correlation between the Anyplex II HPV 28 assay semiquantitative (viral load) data and viral E6/E7 oncoprotein expression determined immunohistochemically in LSCC and HPSCC tissue samples, Figure S2: Correlation between the p16 IHC results, viral E6/E7 oncoprotein expression determined immunohistochemically, and PCR results in LSCC and HPSCC tissue samples.

Author Contributions: Conceptualization, A.L.; methodology, A.L., V.G. and M.C.; formal analysis, A.L.; investigation, A.L., M.C.; resources, A.L., R.D. and M.C.; data curation, A.L.; writing—original draft preparation, A.L.; writing—review and editing, A.L., V.G., M.M., S.S. and M.C.; visualization, A.L. and S.S.; supervision, V.G., M.M., M.C. and S.S.; project administration, A.L., V.G., M.M.; All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: 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.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

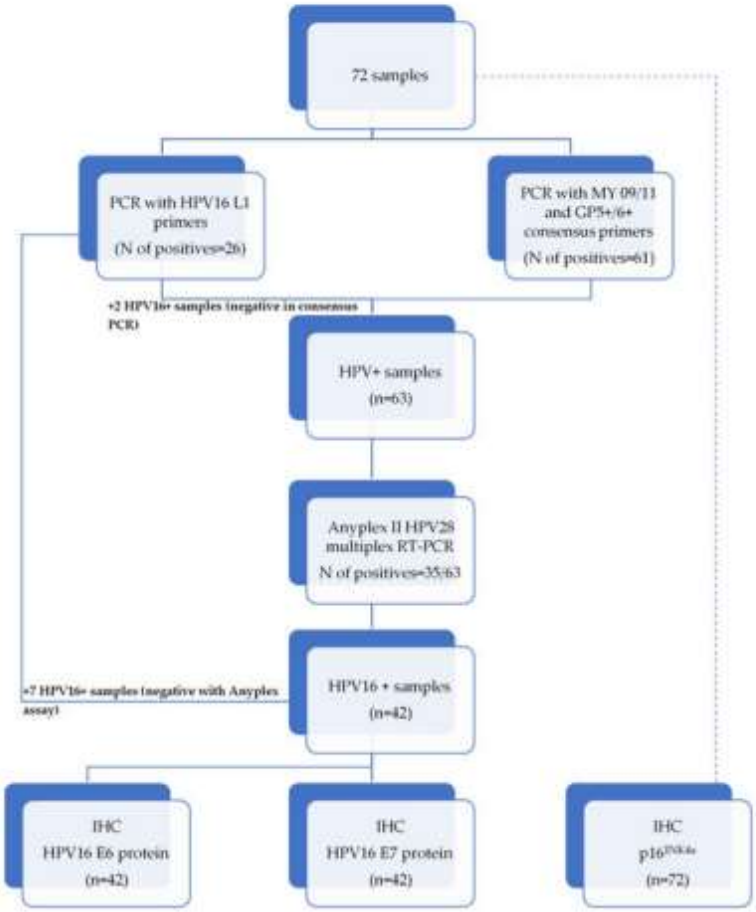

Figure A1. The workflow scheme of the study.

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Article

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

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Simple Summary: The role of human papillomavirus (HPV) in the survival of patients with head and neck squamous cell carcinoma (HNSCC) is an important topic. The recognition of additional markers could play a significant role in survival prognosis. Our study aimed to assess the roles of different molecular and immunohistochemical factors in the survival of patients with HNSCC. We analyzed 106 HNSCC samples and confirmed the roles of HPV DNA and p16, p53, and HPV16 E6 and E7 proteins in different subgroups of HNSCC. In addition to p16, the immunohistochemical overexpression of HPV16 E6 protein should be used for patient survival prognosis.

Abstract: The role of HPV in the survival prognosis of patients with head and neck squamous cell carcinoma, especially patients with laryngeal squamous cell carcinoma (LSCC) and hypopharyngeal squamous cell carcinoma (HPSCC), is still somewhat ambiguous. The present study aimed to explore the significance of tumor suppressor proteins and HPV16 E6 and E7 oncoproteins in the assessment of survival in patients with oropharyngeal squamous cell carcinoma (OPSCC), LSCC, and HPSCC associated with high-risk (HR-) and low-risk (LR-) HPV infections. By utilizing molecular and immunohistochemical investigations of HNSCC samples and patient data, univariate and multivariate survival analyses were conducted. The presence of HPV DNA (LR- and HR-HPV) was associated with a better 5-year OS and DSS for OPSCC and LSCC. The IHC overexpression of HPV16 E6 protein and p16 protein was associated with better survival in the univariate (for OPSCC) and multivariate (OPSCC and HPSCC) survival analyses. The overexpression of p53 was associated with better survival in OPSCC. HPV infection plays a significant role in the tumorigenesis of HNSCC, and the immunohistochemical assessment of HPV16 E6 protein expression should be interpreted as a useful prognostic marker for OPSCC and HPSCC.

Keywords: oropharynx; larynx; hypopharynx; squamous cell carcinoma; HPV; PCR; immunohistochemistry; p16; p53; E6/E7 viral oncoproteins; survival analysis

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1. Introduction

As one of the most common cancers globally, HNSCC accounts for more than 660,000 new cases and 325,000 deaths annually [1]. According to the GLOBOCAN data, 98,412 new cases of OPSCC, 98,412 new cases of LSCC, and 84,254 new HPSCC were registered in 2020 [2].

LR-HPV types encompass the majority of known HPV (more than 200) types and are not usually associated with cancer development [3]. By contrast, HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 are viewed as HR-HPV types. HR-HPV infection

has long been recognized as an etiologic factor of anogenital cancers and has also relatively recently been recognized as an etiologic factor of some head and neck cancers. While HR-HPV infection and HPV-16 are most commonly strongly linked to OPSCC development (with HPV prevalence ranging from 45 to 90%) [4], in other head and neck cancers such as LSCC and HPSCC, the role of HR-HPV is still debated, 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 seem to be distinct from their HPV-negative counterparts in various aspects from the molecular mechanisms of transformation and tumor progression to epidemiology and, importantly, patient survival. The HPV status in squamous cell carcinomas overall has been demonstrated to be a prognostic factor for survival, and HPV-associated OPSCCs specifically are associated with a reduced risk of death and a reduced risk of recurrence [5], yet for other head and neck squamous cell carcinomas (HNSCC) (e.g., LNSCC and HPSCC), such an association has not been confidently established [6–14]. Additionally, HPV-positive head and neck cancers have several molecular signatures: degradation of wild-type p53 and a lack of mutations in the p53-encoding gene, decreased expression of pRb, and subsequent increased expression of p16. These molecular differences could help distinguish HPV-associated cancers, thus aiding in treatment adjustment, and could serve as prognostic markers [5].

The oncogenic potential of HPV is mainly dependent on two of its early proteins: E6 and E7. These viral proteins interact with important cell cycle regulators (tumor suppressors) of the infected epithelial cells, causing their uncontrolled proliferation. Since HPV oncoprotein expression is considered necessary for carcinogenesis as well as causality, their expression could serve as a prognostic marker. Some researchers suggest that HPV-related head and neck cancers have a better prognosis due to a more aggressive and specific immune response to tumor-expressing HPV antigens, including E6 and E7. Some studies have demonstrated that T cells from patients with OPSCC proliferate and synthesize inflammatory cytokines upon HPV16 E6 and E7 oncoprotein recognition, and T cells from patients with HPV-related head and neck cancer show increased responses to E7 epitopes [6,15–17].

One of the oncoproteins, E6, promotes proteasomal p53 degradation via E6-associated ubiquitin ligase, thus deregulating cell cycle checkpoints, avoiding apoptosis, and inactivating one of the p53 targets, p21, which prevents cells from entering the S phase via cell cycle arrest in the G1 phase [18]. In non-HPV-associated cases of head and neck cancers, the p53-encoding gene is often mutated, resulting in a loss of p53 function or even gain of functions that promote invasion, metastasis, and cancer cell proliferation [19]. Studies have shown that patients whose HNSCCs are positive for HPV and lack p53 expression (due to p53 degradation via E6) have a better prognosis and better overall survival [20].

E7, on the other hand, strongly binds pRb and induces its proteasomal degradation, thus releasing a transcription factor called E2F, which again drives the cells to enter the S phase of the cell cycle [21,22]. Another consequence of the E7-mediated pRb degradation is the overexpression of p16, a potent tumor suppressor. The detection of p16 overexpression has been adopted as a molecular hallmark of HPV-associated OPSCCs, with studies demonstrating its positive effect on patient survival. Studies have demonstrated its positive effects on patient survival in OPSCC. For other HNSCCs of non-oropharyngeal subsites, such an association has not been established [23,24], with studies reporting a lack of p16 expression, even in the presence of HPV mRNA, or similar p16 expression levels, regardless of HPV positivity [25,26].

This study aimed to explore the significance of various molecular and cellular markers of HR-HPV and LR-HPV in the assessment of survival in patients with OPSCC, LSCC, and HPSCC.

2. Materials and Methods

2.1. Patients' Characteristics

A total of 106 patients (95 (89.6%) males and 11 (10.4%) females) with histologically confirmed OPSCC, LSCC, and HPSCC treated at the Latvian Oncology Centre between January 2015 and August 2019 were enrolled in the study.

The sex, age, TNM stage, differentiation grade (G) of the tumor, smoking and drinking habits at the time of presentation, 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 (Table 1).

Table 1. 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 grade:			
• T1	6	4	0
• T2	6	8	4
• T3	6	24	16
• T4	16	5	11
N grade:			
• 0	1	4	0
• 1	15	7	0
• 2	12	22	10
• 3	6	8	21
M grade:			
• 0	34	40	27
• 1	0	1	4
G grade *:			
• 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 missing value in the OPSCC group. ˆ One patient had a missing value in the LSCC group. RT—radiotherapy, OP—surgery treatment, ChT—chemotherapy.

2.2. DNA Extraction

Fresh frozen cancer tissues (24 OPSCC, 34 LSCC, and 2 HPSCC) or formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks (10 OPSCC, 28 HPSCC, and 8 LSCC) were used to extract DNA material for further investigation.

The DNA extraction from fresh frozen tissue material was performed with the standard phenol/chloroform extraction method.

FFPE cancer samples were processed using a blackPREP FFPE DNA Kit (Analytik Jena, Germany) following the manufacturer's protocol. To avoid cross-contamination, separate sterile blades were used for each specimen.

To assess the concentration and quality of the extracted DNA, a spectrophotometric analysis was performed (Nanodrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Beta-(β-) globin was used as a quality control for the isolated DNA [27]. Only β-globin-positive samples were included in the further investigation of the gathered specimens.

2.3. RNA Extraction

Fresh frozen cancer tissue materials (24 OPSCC, 34 LSCC, and 2 HPSCC) or FFPE cancer tissue blocks (10 OPSCC, 28 HPSCC, and 8 LSCC) were processed for total RNA extraction.

Standard RNA extraction with TRIzol LS Reagent from Thermo Fisher Scientific was accomplished 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. Each sample was sectioned separately with a new sterile blade.

A spectrophotometric analysis was used to assess the concentration and quality of the extracted RNA.

2.4. HPV DNA Detection Using MY09/11 and GP5+/6+ Consensus Primers

A polymerase chain reaction (PCR) with the consensus primers MY9/MY11 and GP5+/6+ was used for the initial detection of the broad range of HPV types (HR-HPV and LR-HPV types) [28,29]. Electrophoresis in a 1.7% ethidium bromide gel was used to assess the PCR results. Amplification products of appropriate lengths for the primers that were used were considered HPV-positive. Each reaction included positive and negative controls.

2.5. HPV Genotyping

Consensus PCR-positive samples were further subjected to HPV genotyping. Primers for HPV 16 and 18 (L1) and the Anyplex II HPV28 multiplex real-time-PCR (RT-PCR) were used for HPV genotyping.

The results were visualized via electrophoresis in 1.7% agarose gel with an assessment of appropriate amplification products [29]. Positive and negative controls were used in each reaction.

Anyplex II HPV28 multiplex RT-PCR was used following the manufacturer's recommendations (Seegene, Seoul, Republic of Korea).

2.6. HPV16 E6/E7 mRNA Detection

The detection of E6/E7 mRNA was performed using real-time PCR with the PreTect HPV-Proofer kit. The PreTect HPV-Proofer assay qualitatively detected the presence of HPV E6/E7 oncogene mRNA from HPV types 16, 18, 31, 33, and 45. It had an intrinsic sample control to assess specimen quality. Specimens with positive intrinsic controls were considered valid. Only HR-HPV-positive samples were used for E6/E7 mRNA detection.

2.7. Immunohistochemistry

OPSCC, LSCC, and HPSCC specimens were further processed as FFPE samples. The expression of HPV16 E6/E7 proteins, p53, and p16 proteins were assessed immunohistochemically.

For this purpose, 4–5 µm-thick FFPE tumor sections were mounted on SuperFrost Plus slides (Gerhard Menzel GmbH, Braunschweig, Germany) (Gerhard Menzel GmbH, Braunschweig, Germany). We used a previously tested and approved IHC protocol [30,31].

Briefly, after the standard preparation process, the sections were incubated overnight with the primary antibodies at 4 °C. We used a monoclonal mouse anti-CDKN2A/p16INK4a antibody (Abcam, Cambridge, UK, 1:300 dilution, ab201980); a monoclonal mouse anti-p53 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, 1:50 dilution, sc-47698); a monoclonal mouse anti-HPV16 E6 + HPV18 E6 antibody (Abcam, Cambridge, UK, prediluted, ab51931) [32–34]; and a monoclonal mouse anti-HPV16 E7 antibody (Santa Cruz Biotechnology, Inc., 1:50 dilution, sc-6981). We used a HiDef Detection HRP Polymer system and a diaminobenzidine tetrahydrochloride substrate kit (Cell Marque, Rocklin, CA, USA) to visualize the products of IHC reactions. Counterstaining of cell nuclei within a tumor section with Mayer's hematoxylin was used. In the negative controls of reactions, primary antibodies were omitted. The reaction results were assessed by two independent experienced investigators without knowledge of the clinical and molecular virology data.

The immunopositive reaction resulted in the appearance of brown reaction products using the anti-CDKN2A/p16INK4a, anti-p53, anti-HPV16 E6 + HPV18 E6, and anti-HPV16 E7 antibodies. Specifically, solely nuclear, combined nuclear and cytoplasmic immunostaining was observed when detecting p53 and HPV16 E7 proteins, p16 protein and HPV16 E6 protein, respectively.

A cut-off at 50% positive tumor cells for the p16 immunostaining was used, as proposed by Hong et al. (2013) [35].

The assessment of immunostaining for p53 was performed semiquantitatively. We considered a sample to be p53-postive (p53+) when the criteria described by Halec et al. (2013) were met [36]. The p53 overexpression (upregulation) was considered when p53 positivity was confirmed in >50% of tumor cells with intensity = 2 or >25% of tumor cells with intensity = 3. All FFPE specimens that did not reach these criteria were considered p53-negative (p53-; downregulation).

As all HR-HPV-positive specimens contained HPV16 DNA, only those were used for the IHC detection of E6 and E7 proteins. The IHC reaction results for the E6 and E7 viral proteins were estimated semiquantitatively in 20 randomly selected visual fields of each sample including the tumor and the surface epithelium of the regions of interest. To achieve enough statistical power, we used the expression levels of E6 and E7 at <10% as negative and at ≥10% as positive.

2.8. Statistical Data Analysis

All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). A standard statistical analysis was performed to assess the data distribution. A nonparametric Spearman's correlation analysis was used to find any correlations between the groups [37]. A univariate survival analysis was performed using the Kaplan–Meier method; overall and disease-specific survivals (OS and DSS) were assessed. A multivariate survival analysis was performed using the Cox regression method. p values less than 0.05 ($p < 0.05$) were considered statistically significant.

3. Results

3.1. HPV DNA Analysis

We analyzed HNSCC samples for HPV DNA sequences and genotypes. HPV DNA was found in 92/106 (86.79%) of the HNSCC samples. More specifically, it was found in 29/34 (85.29%) of the OPSCC samples, 32/41 (78.05%) of the LSCC samples, and 31/31 (100%) of the HPSCC samples. The predominant genotype was HPV16, which was confirmed in 68/106 (65.09%) of the HNSCC samples. More precisely, it was found in 26/34 (76.47%) of the OPSCC samples, 22/41 (53.66%) of the LSCC samples, and 20/31 (64.52%) of the HPSCC samples. In 7/106 of the HNSCC samples, we detected HPV coinfections. In

addition to HPV16 DNA, we found HPV31 (2 of 7), 33 (1 of 7), 35 (1 of 7), and 56 (4 of 7). Our further analysis was therefore focused on HPV16 due to its high prevalence.

3.2. HPV16 E6/E7 mRNA Expression

HPV16-postive HNSCC samples were analyzed for the presence of HPV16 E6/E7 mRNA (E6/E7 mRNA+). We detected HPV16 E6/E7 mRNA in 15/26 (57.7%) of the OPSCC samples, 2/22 (9%) of the LSCC samples, and 0/20 of the HPSCC samples. A correlation analysis of the semiquantitative HPV16 viral load results and the presence of HPV16 E6/E7 mRNA showed a moderate positive correlation (Sr = 0.601, $p < 0.0001$). Moreover, a weak positive correlation was found between p16 overexpression and E6/E7 mRNA expression (Sr = 0.472, $p < 0.0001$). Simultaneously, no correlation between p53 downregulation (p53−) and E6/E7 mRNA expression was found.

3.3. IHC Expression of p16 in HNSCC

p16 overexpression (p16+; Figure 1A) was found in 24/106 (22.64%) of the HNSCC samples. More specifically, it was found in 16/34 (47.06%), 6/41 (14.63%), and 2/31 (6.45%) of the OPSCC, LSCC, and HPSCC samples, respectively. Simultaneously, when stratified by HPV16 positivity (HPV16+), p16 overexpression was confirmed in 15/26 (57.69%), 5/22 (22.73%), and 2/20 (10%) of the OPSCC, LSCC, and HPSCC samples, respectively.

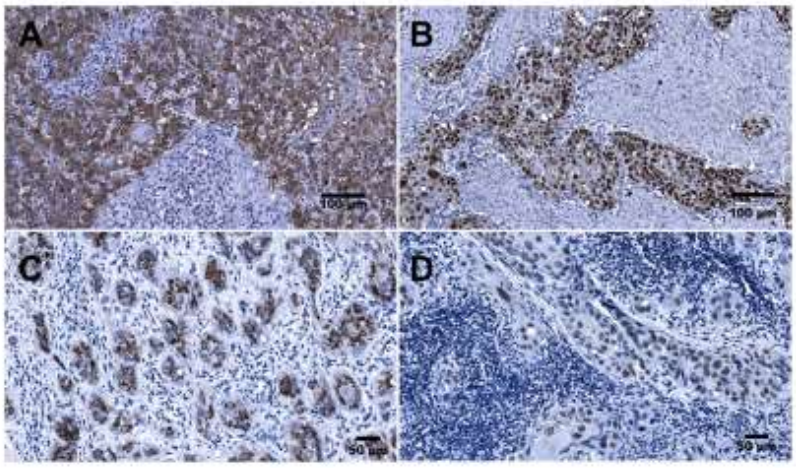

Figure 1. Immunohistochemical detection of p16, p53, HPV E6, and HPV E7 antigens in HNSCC: (**A**) OPSCC (palatine tonsil). Representative image from a case demonstrating >75% p16-positive tumor cells displaying mostly nuclear and cytoplasmic expression. (**B**) LSCC. Representative image of p53 overexpression demonstrating uniform strong nuclear staining of tumor cells. (**C**) OPSCC (palatine tonsil). Representative image demonstrating cytoplasmic expression of HPV16 E6 protein confirmed in tumor cells organized in cords. (**D**) OPSCC (palatine tonsil). Representative image demonstrating nuclear expression of HPV16 E7 protein confirmed in the tumor cells organized as nests and cords. Scale bars: 100 µm and 50 µm.

3.4. IHC Expression of p53 in HNSCC

p53 overexpression (p53+; Figure 1B) was confirmed in 49/106 (46.23%) of the HNSCC samples. More specifically, it was confirmed in 17/34 (50%), 21/41 (51.22%), and 11/31 (35.48%) of the OPSCC, LSCC, and HPSCC samples, respectively. An analysis of the HPV16+ samples showed p53 downregulation (p53−) in 15/26 (57.69%), 10/22 (45.45%), and 14/20 (70%) of the OPSCC, LSCC, and HPSCC samples, respectively. Furthermore, in the E6/E7 mRNA+ samples, p53 downregulation was found in 11/15 (73.33%) of the OPSCC samples and 1/2 (50%) of the LSCC samples.

3.5. IHC Expression of HPV16 E6 and E7 Proteins in HNSCC

Overexpression of HPV16 E6 protein (Figure 1C) was immunohistochemically confirmed in 44/106 (41.5%) of the HNSCC samples. More specifically, it was confirmed in 21/34 (61.8%), 14/41 (34.1%), and 9/31 (29.0%) of the OPSCC, LSCC, and HPSCC samples, respectively.

In turn, overexpression of HPV16 E7 protein (Figure 1D) was found in 39/106 (36.8%) of the HNSCC samples. More specifically, it was found in 19/34 (55.9%), 14/41 (24.1%), and 6/31 (19.4%) of the OPSCC, LSCC, and HPSCC samples, respectively.

3.6. Kaplan–Meier Survival Analysis

3.6.1. OS and DSS, Depending on HPV DNA (HR-HPV and LR-HPV)

The five-year OS and DSS were assessed in patients who were HPV-positive compared to HPV-negative, depending on the location of the primary tumor.

For the oropharynx, the OS rates were 26.82% and 0% for patients who were HPV-positive and HPV-negative, respectively, although this difference failed to reach statistical significance ($p = 0.077$; Figure 2A). The DSS rates were 27.78% and 0% for these groups of patients ($p < 0.05$; Figure 2B), respectively.

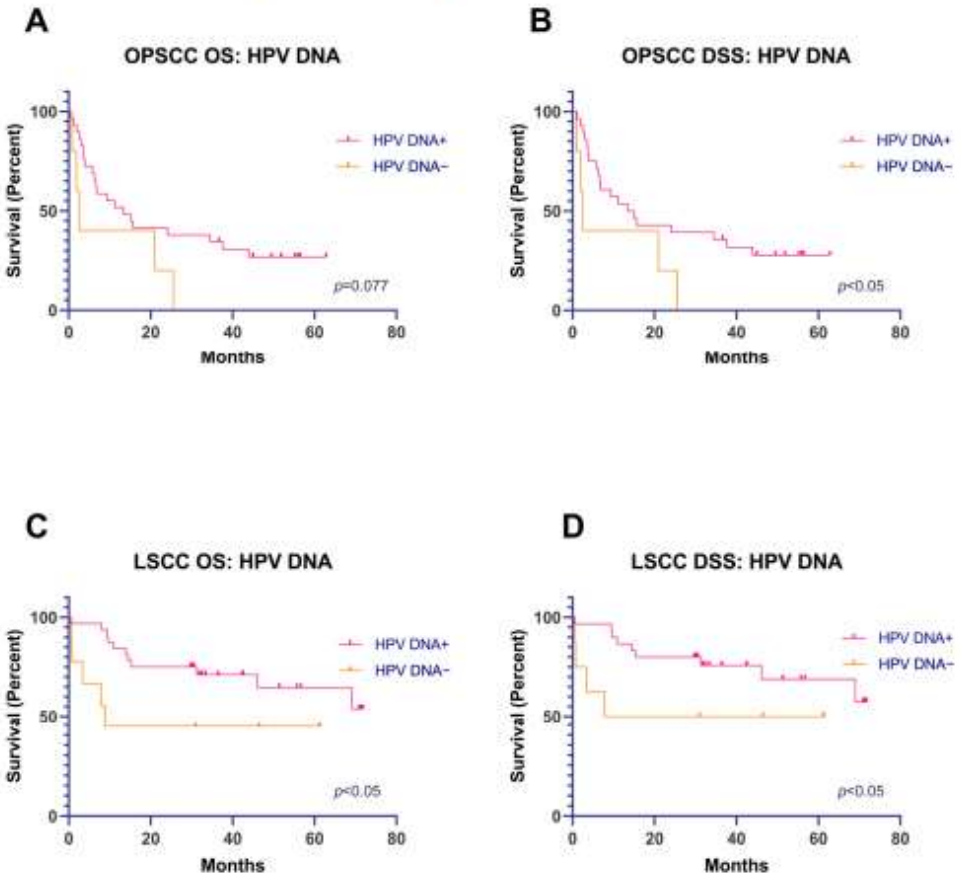

Figure 2. (**A**,**B**) OS and DSS analyses (Kaplan–Meier), depending on the presence of HPV DNA (HR and LR) in OPSCC. (**C**,**D**) OS and DSS analyses (Kaplan–Meier), depending on the presence of HPV DNA (HR and LR) in LSCC.

For patients with LSCC, the OS rates were 64.59% and 44.44% in patients who were HPV-positive and HPV-negative ($p < 0.05$; Figure 2C), respectively. The DSS rates were 68.90% and 50% for patients who were HPV-positive and HPV-negative ($p < 0.05$; Figure 2D), respectively.

As all HPSCC samples were HPV DNA+, a Kaplan–Meier survival analysis could not be performed.

3.6.2. OS and DSS, Depending on Immunohistochemical Expression of HPV16 DNA, HPV16 E6/E7 mRNA, and p16, p53, E6, and E7 Proteins

We performed a Kaplan–Meier survival analysis with a stratification of patients depending on the location of the primary tumor. The OS and DSS were calculated. For most variables, a univariate survival analysis with the Kaplan–Meier method failed to reach statistical significance.

There were borderline statistically significant differences ($p = 0.057$, Figure 3A,B) between p16+ and p16− OPSCC for OS and statistically significant differences for DSS.

A Kaplan–Meier survival analysis of p53+ and p53− HPSCC showed statistically significant differences in OS and DSS (Figure 3C,D).

The immunohistochemical overexpression of HPV16 E6 protein was associated with significantly better OS and DSS in patients with OPSCC (Figure 3E,F).

3.7. Multivariate Cox Regression Analysis

The age; sex; hazards; applied treatment; immunohistochemical expression of p16, p53, E6 protein, and E7 protein; and the presence of HPV16 DNA and E6/E7 mRNA were included in the Cox model. First, a multivariate survival analysis was performed for all patients with head and neck tumors. Second, each anatomical location of the head and neck cancers was analyzed separately: oropharynx, larynx, and hypopharynx.

3.7.1. All HNSCC

The results of the analysis of all patients with head and neck cancer are summarized in Table 2.

The Cox regression analysis suggested that the T1 stage was associated with a lower risk of early death. While the results for each T stage did not reach statistical significance, there was a trend towards a higher early death risk with a higher T stage; patients with HNSCC with a T4 tumor had a 2.68-fold higher probability of death. The analysis also showed that a higher N stage was associated with a higher risk of early death. The N1 stage (in reference to N0) was associated with a 4.98-fold greater risk of early death, and the risk notably increased in the N3 stage. A lower tumor differentiation grade (G) was associated with a higher risk of early death. Patients with G3 tumors (well-differentiated) had an 81% lower risk of early death than patients with G1 tumors (undifferentiated).

The effect on survival was also statistically significant for treatment. Patients who received a combined treatment (RT+ChT+/−OP) showed a lower risk of early death.

Immunohistochemical overexpression of HPV16 E6 protein (>10%) was associated with a lower hazard ratio (Exp(B) = 0.3492, $p = 0.0147$). Other variables did not show statistical significance.

3.7.2. OPSCC

This group encompassed 34 patients with 26 events (death). Two patients were excluded from the analysis due to missing values. Table 3 depicts the Cox regression results of the variables for OPSCC.

The Cox regression analysis showed that the immunohistochemical expression of p16, p53, and HPV16 proteins E6 and E7; the T grade; the applied treatment; and smoking significantly affected patients' survival.

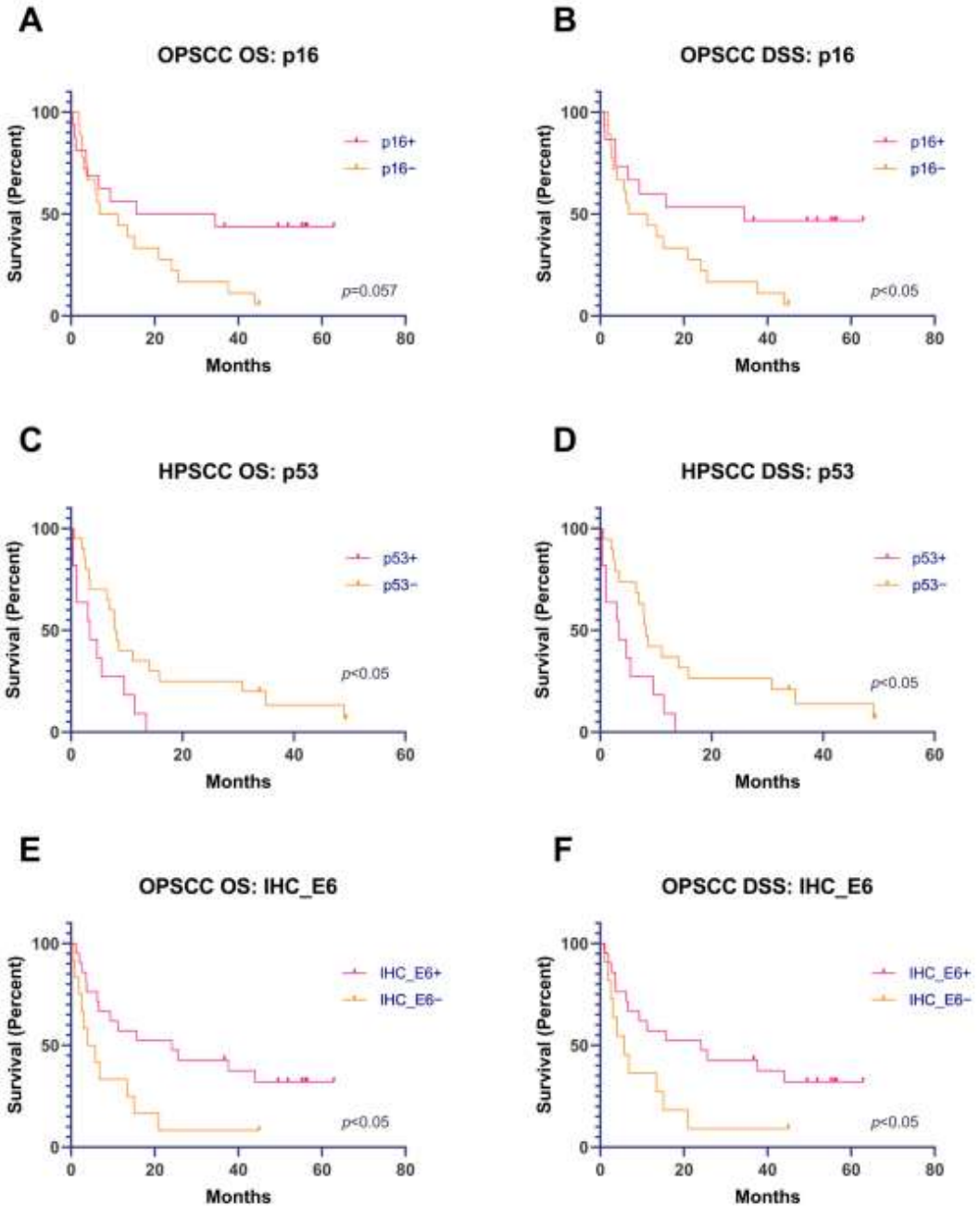

Figure 3. (**A**,**B**) OS and DSS analyses (Kaplan–Meier), depending on the result of the immunohistochemical expression of p16 in OPSCC. (**C**,**D**) OS and DSS analyses (Kaplan–Meier), depending on the results of the immunohistochemical expression of p53 in HPSCC. (**E**,**F**) OS and DSS (Kaplan–Meier), depending on the results of the immunohistochemical expression of HPV16 E6 protein in OPSCC.

Table 2. Cox regression survival analysis for all HNSCC.

Variables		N = 106 [$]	β	*p* [^]	Survival	
Name	**Groups** [*]				**Hazard Ratios (Exp(β))**	**95% CI**
Sex	Female [*]	11			(1)	
	Male	92	1.024	0.0627	2.785	0.9886 to 8.688
Age			0.01344	0.4200	1.014	0.9810 to 1.048
p16	Negative [*]	81			(1)	
	Positive	22	0.5351	0.2412	1.708	0.6693 to 4.081
p53	Negative [*]	55			(1)	
	Positive	48	0.4982	0.1658	1.646	0.8141 to 3.358
IHC_E6	<10% [*]	60			(1)	
	>10%	43	−1.052	**0.0147**	0.3492	0.1464 to 0.8037
IHC_E7	<10% [*]	65			(1)	
	>10%	38	0.4807	0.2956	1.617	0.6590 to 4.024
Hazards	None [*]	15			(1)	
	Smoking	57	0.5992	0.2624	1.821	0.6636 to 5.526
	Smoking and alcohol abuse	31	−0.1794	0.7552	0.8358	0.2768 to 2.700
Location	Oropharynx [*]	32			(1)	
	Larynx	40	−0.7745	0.3747	0.4609	0.08271 to 2.545
	Hypopharynx	31	−0.5893	0.3045	0.5547	0.1772 to 1.693
T	1 [*]	10			(1)	
	2	18	−0.2886	0.6946	0.7493	0.1843 to 3.473
	3	44	0.5419	0.4727	1.719	0.4207 to 8.411
	4	31	0.9882	0.1683	2.686	0.7107 to 12.33
N	0 [*]	41			(1)	
	1	33	1.607	**0.0011**	4.988	1.944 to 13.55
	2	22	1.372	**0.0182**	3.943	1.277 to 12.62
	3	7	2.208	**0.0036**	9.098	2.042 to 40.88
M	0 [*]	98			(1)	
	1	5	1.104	0.1662	3.015	0.5851 to 13.67
G	1 [*]	15			(1)	
	2	76	−0.9494	**0.0413**	0.387	0.1556 to 0.9791
	3	12	−1.658	**0.0058**	0.1906	0.05657 to 0.606
HPV16_DNA	Negative [*]	37			(1)	
	Positive	66	0.4515	0.2491	1.571	0.7248 to 3.395
HPV16_E6E7_RNA	Negative [*]	87			(1)	
	Positive	16	−0.9763	0.1399	0.3767	0.09878 to 1.335
Treatment	RT [*]	37			(1)	
	OP	9	−1.042	0.3186	0.3528	0.03495 to 2.325
	RT+OP	35	−0.6763	0.2432	0.5085	0.1548 to 1.506
	RT+ChT (Cetuximab) +/−OP	9	−2.089	**0.0163**	0.1239	0.01986 to 0.6441
	Symptomatic	13	0.8416	0.0635	2.32	0.9130 to 5.507

[*] group of reference. [$] Three were excluded due to missing values. [^] statistically significant values ($p < 0.05$) are highlighted in bold.

Table 3. Cox regression survival analysis for OPSCC.

Variables		N = 34 [§]	β	p [˜]	Survival	
Name	**Groups** *				**Hazard Ratios (Exp(β))**	**95% CI**
Sex	Female *	7			(1)	
	Male	25	−3.121	0.0810	0.04411	0.001033 to 1.742
Age		32	−0.02128	0.8114	0.9789	0.8275 to 1.177
p16	Negative *	18			(1)	
	Positive	14	−3.548	**0.0532**	0.02879	0.0005461 to 0.9340
p53	Negative *	16			(1)	
	Positive	16	−6.206	**0.0028**	0.002018	1.930×10^{-5} to 0.08535
IHC_E6	<10% *	12			(1)	
	>10%	20	−6.171	**0.0265**	0.002089	1.830×10^{-6} to 0.1431
IHC_E7	<10% *	14			(1)	
	>10%	18	6.154	**0.0355**	470.6	8.716 to 604,132
Hazards	None *	8			(1)	
	Smoking	8	8.18	**0.0323**	3568	3.203 to 10,181,954
	Smoking and alcohol abuse	16	5.424	0.0801	226.9	0.4392 to 139,247
T	1	6	−4.794	**0.0137**	0.008275	0.0001011 to 0.2757
	2	6	−7.933	**0.0010**	0.0003588	1.456×10^{-6} to 0.02453
	3	4	−5.286	**0.0480**	0.00506	5.478×10^{-6} to 0.2114
	4 *	16				
N	0	1	−29.16	>0.9999	2.166×10^{-13}	-
	1	13	0.5427	0.7926	1.721	0.02756 to 200.9
	2	12	−3.366	0.1093	0.03453	0.0001571 to 1.714
	3 *	6			(1)	
M	0	32	-	-	-	-
G	1 *	5			(1)	
	2	21	1.356	0.4788	3.882	0.08016 to 198.0
	3	6	−0.8802	0.6145	0.4147	0.007811 to 11.00
HPV16_DNA	Negative *	8			(1)	
	Positive	24	1.07	0.4826	2.914	0.1090 to 47.14
HPV16_E6E7_RNA	Negative *	18			(1)	
	Positive	14	−1.53	0.3384	0.2166	0.003954 to 4.418
Treatment	RT *	15			(1)	
	OP	0	-	-	-	-
	RT+OP	2	8.757	**0.0100**	6352	7.160 to 9,678,504
	RT+ChT (Cetuximab) +/− OP	9	1.005	0.6443	2.731	0.06476 to 538.3
	Symptomatic	6	9.218	**0.0003**	10072	154.8 to 6,028,349

* group of reference. [§] Two were excluded due to missing values. ˜ Statistically significant values ($p < 0.05$) are highlighted in bold.

The overexpression of p16, p53, and HPV16 E6 protein showed much lower hazard ratios and was associated with significantly improved survival. On the contrary, the overexpression of HPV16 E7 protein was associated with a high risk of early death. A graphical analysis showed that the overexpression of p16 (p16+) in a tumor was associated with better survival than that of patients with p16-negative tumors (Figure 4A). However, the overexpression of

HPV16 E7 protein was associated with decreased survival. Moreover, when combining the two markers (p16 and HPV16 E7 protein), E7 protein overexpression (E7+) decreased survival, even in patients with p16+ tumors (Figure 4A). Figure 4B shows that the best survival was seen in patients with p53-positive (p53+)/HPV16 E6 protein positive (E6+) tumors and that the worst was seen in patients with p53−/E6− tumors. There was no difference in survival between patients with p53−/E6+ and p53+/E6− tumors.

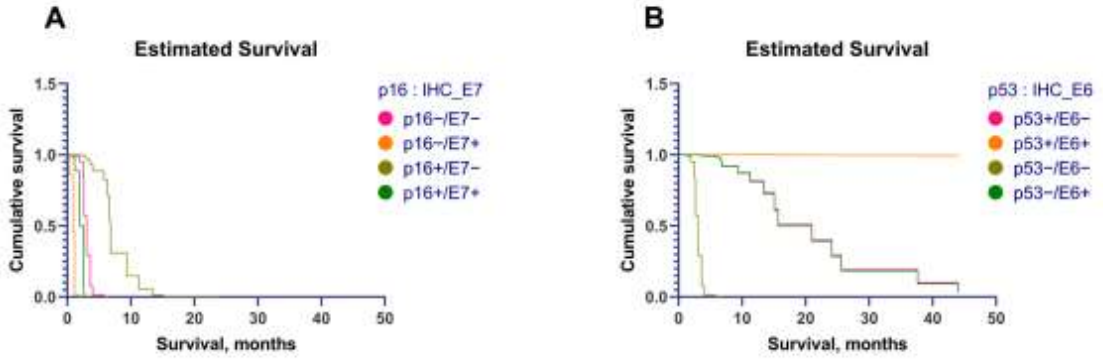

Figure 4. (**A**) Estimated survival, depending on the immunohistochemical expression of p16 and E7 protein. (**B**) Estimated survival, depending on the immunohistochemical expression of p53 and E6 protein.

A larger tumor size (T grade) negatively affected survival. An analysis showed a lower risk of early death for tumors with lower N grades; however, the difference was not statistically significant.

The patients who underwent radiotherapy had a significantly lower risk of early death than patients with other treatment modalities.

3.7.3. LSCC

This group encompassed 41 patients with 16 events (death). A Cox regression model with all included variables was statistically significant ($p < 0.001$). An analysis showed that no variable significantly affected survival.

3.7.4. HPSCC

This group encompassed 31 patients with 29 events (death). Table 4 depicts the Cox regression analysis for patients with HPSCC.

The Cox regression model showed that the expression of p16 and HPV16 E6 protein; the presence of HPV16 DNA; the hazards; and the T, N, and M grades statistically significantly affected survival. The effects of the other variables were not statistically significant.

The overexpression of p16 and HPV16 E6 protein was associated with an extremely low risk of early death (Figure 5A,C). By combining the p16 status and the HPV16 E7 protein status, we found that E7 protein expression did not affect survival (Figure 5B, overlaying of the curves). However, combining the p53 and HPV16 E6 protein statuses showed that patients with E6+ tumors had better survival and that p53 overexpression seems to increase survival even more in these patients (Figure 5D). The worst survival was in the group of patients with p53−/E6− tumors.

The Cox regression analysis revealed that larger primary tumors are associated with a higher risk of early death. Patients with T3 tumors had 87% less risk of early death than patients with T4. Moreover, a lower N grade was associated with lower hazard ratios. Lastly, the presence of distal metastases was associated with a 22-fold increase in the risk of death.

Table 4. Cox regression survival analysis for HPSCC.

Variables		N = 31	β	$p^{\wedge}$	Survival	
Name	Groups *				Hazard Ratios (Exp(β))	95% CI
Sex	Female *	2			(1)	
	Male	29	1.92	0.2873	6.823	0.2575 to 478.6
Age		31	0.0571	0.2702	1.059	0.9605 to 1.194
p16	Negative *	29			(1)	
	Positive	2	−6.638	**0.0049**	0.001309	5.631×10^{-6} to 0.08768
p53	Negative *	20			(1)	
	Positive	11	−1.099	0.2540	0.333	0.04332 to 2.109
IHC_E6	<10% *	22			(1)	
	>10%	9	−3.211	**0.0108**	0.04033	0.002158 to 0.3739
IHC_E7	<10% *	25			(1)	
	>10%	6	−0.04985	0.9711	0.9514	0.04166 to 10.39
Hazards	None *	3			(1)	
	Smoking	20	4.049	**0.0214**	57.36	2.263 to 3407
	Smoking and alcohol abuse	8	−0.8085	0.6127	0.4455	0.01394 to 9.424
T	1	0	-	-	-	-
	2	4	2.196	0.0950	8.986	0.7027 to 155.3
	3	16	−2.026	**0.0240**	0.1319	0.02240 to 0.7996
	4 *	11			(1)	
N	0 *	6			(1)	
	1	16	−2.825	**0.0421**	0.05932	0.003106 to 0.8054
	2	8	−2.719	**0.0235**	0.06597	0.005426 to 0.6552
	3	1	4.872	**0.0108**	130.6	2.628 to 7490
M	0 *	27			(1)	
	1	4	3.091	**0.0274**	21.99	1.535 to 460.4
G	1 *	6			(1)	
	2	21	−2.035	0.0912	0.1307	0.01189 to 1.553
	3	4	−2.087	0.1338	0.124	0.006553 to 1.883
HPV16_DNA	Negative *	11			(1)	
	Positive	20	2.205	**0.0194**	9.071	1.578 to 70.34
HPV16_E6E7_RNA	Negative *	31	-	-	-	-
	Positive	0	-	-	-	-
Treatment	RT *	21			(1)	
	OP	0	-	-	-	-
	RT+OP	4	1.563	0.1378	4.771	0.5985 to 42.89
	RT+ChT (Cetuximab) +/− OP	0	-	-	-	-
	Symptomatic	6	0.17	0.8610	1.185	0.1367 to 7.244

* group of reference. ^ Statistically significant values ($p < 0.05$) are highlighted in bold.

Smoking patients had a 57-fold increase in the risk of early death in comparison to non-smokers/non-drinkers.

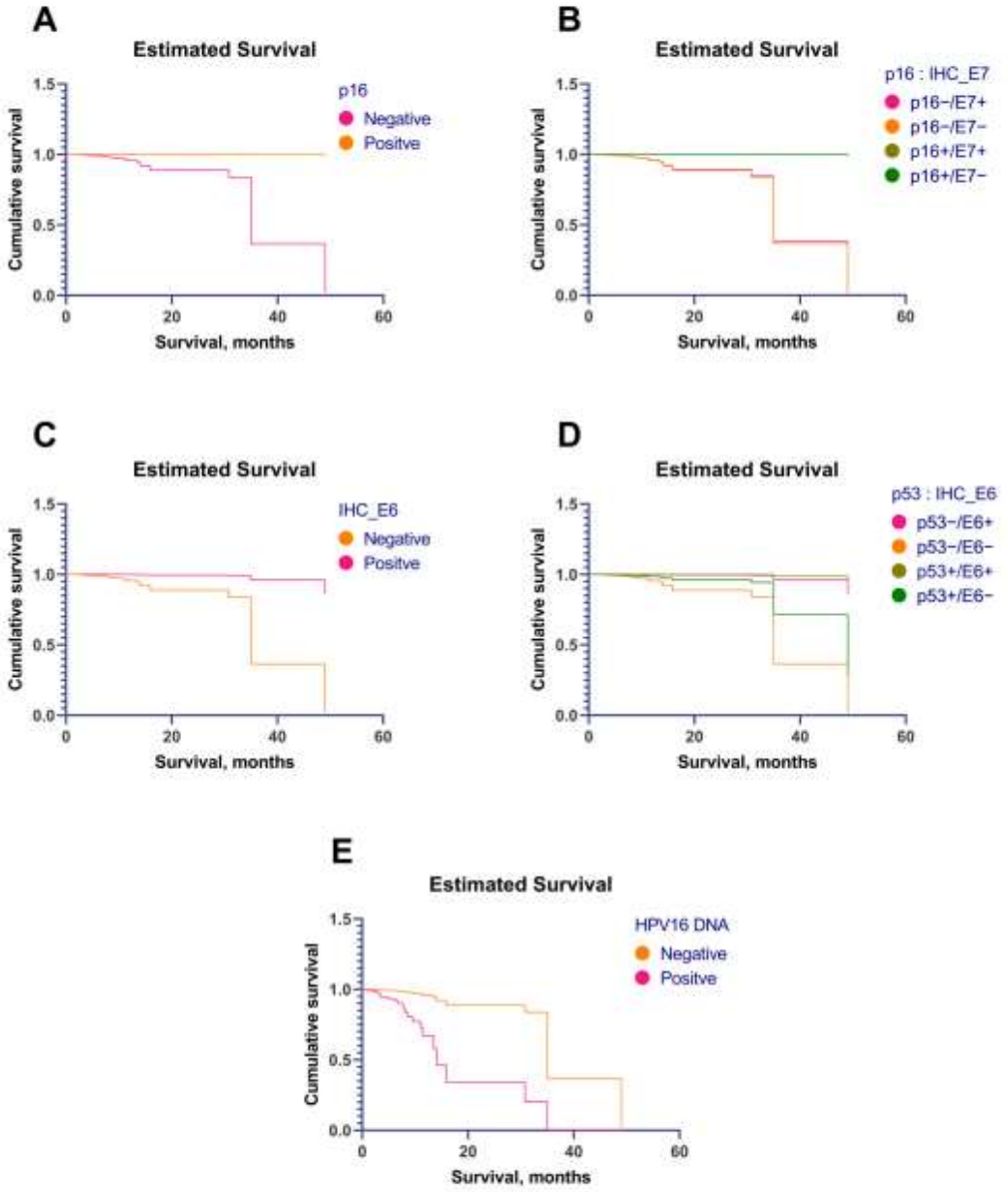

Figure 5. (**A**) Estimated survival (Cox regression), depending on the immunohistochemical expression of p16. (**B**) Estimated survival (Cox regression) depending on the immunohistochemical expression of p16 and HPV16 E7 protein. (**C**) Estimated survival (Cox regression), depending on the immunohistochemical expression of HPV16 E6 protein. (**D**) Estimated survival (Cox regression), depending on the immunohistochemical expression of p53 and HPV16 E6 protein. (**E**) Estimated survival (Cox regression), depending on the presence of HPV16 DNA.

4. Discussion

The current study aimed to assess the roles of HPV infection and associated markers such as p16, p53, HPV16 E6/E7 oncoproteins, the presence of HPV DNA, and E6/E7 mRNA in survival.

An initial univariate survival analysis (Kaplan–Meier) shows the potential role of not only HR-HPV but also LR-HPV infection in the survival of patients with OPSCC and LSCC, as 1/3 of the patients have a probability of LR-HPV infection. The study results suggest that patients with HPV-DNA-positive OPSCC and LSCC have a better 5-year OS and DSS. These results agree with other studies where patients with HNSCC and patients with tonsil cancer also had better survival rates if the tumors were positive for HPV DNA [38,39]. This is probably due to better radiosensitivity of HPV+ tumors, which means patients could benefit from the "softer" treatment applied to HPV-positive tumors and increases positive outcomes for the patients [38]. On the other hand, HPV-infected cells could be more visible to the host's immune system, allowing for easier identification as well as the destruction of virus-related tumor tissues. In that case, a deeper investigation of the HPV activity in patients with HNSCC and the interaction with their immune systems would be required.

It is well documented that patients with HPV-positive OPSCC have higher 3- and 5-year survival rates than patients who are HPV-negative [40], but the consensus is made for HR-HPV (mostly HPV16 and 18). For LSCC, many studies have shown no significant survival increase for HPV-positive tumors [7,41,42]. However, in recent years, there have been studies with results similar to ours, with better survival in patients with HPV-positive LSCC [9,43].

On the other hand, in our study, the stratification of patients with HNSCC by the tumor location and the identification of specific HPV types showed that the presence of HPV16 DNA in hypopharyngeal squamous cell carcinoma cases substantially decreased the survival rates of patients. This indicates that HPV16 may play a significant role in HPSCC development. Additionally, the immunological aspects should be considered. The presence of viral antigens could promote anti-tumor immunity and lead to better survival of the patients [44–46].

Head and neck cancers encompass a multitude of subsites for cancer development. Sometimes studies analyzing the effects of HPV on the survival of head and neck cancers can be confusing in that they unify the survival analysis without stratifying the primary tumors by location, especially hypopharyngeal and laryngeal cancers, which are sometimes combined in non-oropharyngeal cancers [14,47]. In our view, this could lead to incorrect conclusions. The oropharynx, larynx, and hypopharynx are three distinct locations with different prognoses based on lymphatic drainage alone. In our study, an analysis of all HNSCC in a Cox regression did not show p16, p53, or other variables to be significant factors affecting the survival of the patients. This indicates that patients should preferably be stratified by the primary location of the tumor to obtain a more comprehensive view of the potential risk factors.

This study reaffirmed the predictive role of p16 overexpression in OPSCC (univariate survival analysis), confirming better survival in patients with p16+ tumors [48,49]. This trend continued in the Cox regression analysis, with statistical significance further confirming its role as a distinct predictive marker for OPSCC. However, for HPSCC and LSCC, this could not be confirmed in the univariate survival analysis. The Cox regression analysis showed better survival and a lower risk of death for patients with p16+ HPSCC, suggesting that there might be a reason to consider it as a predictive marker. Several studies have shown similar findings [50,51]. There is also a question of p16's association with HPV activity in non-oropharyngeal squamous cell carcinoma, whether it can be used as a surrogate marker for HPV infection, and whether it serves as a suitable prognostic factor of survival. Several studies have shown that p16 often does not correspond to the HPV status in non-oropharyngeal cancers; however, it has a prognostic value for survival [52–54].

The lack of significance for many analyzed variables in OPSCC (univariate survival analysis) of our study could be due to the relatively small patient number in this subgroup,

which could affect the statistical power of analysis. Additionally, the high number of smokers and alcohol abusers could also affect the significance of the results. This is accounted for in the Cox regression model.

The univariate survival analysis of p53 immunohistochemical expression showed significantly better OS and DSS in p53+ HPSCC. The trend persisted in the Cox regression, although without statistical significance. Similar findings were present for OPSCC in the Cox regression analysis; p53 overexpression (p53+) was associated with a significantly lower risk of death. This could be due to the tumor-suppressing properties of p53. However, there was a considerable number of HPV16-positive samples and even more HPV16 E6/E7 mRNA-positive OPSCC samples. A logical picture would be that in HPV-driven cancer, p53 is suppressed, resulting in a p53-negative result that is confirmed using immunohistochemistry. Published data suggest that HPV-driven tumors show p53 downregulation [55–57]. On the contrary, Hasegawa et al. [58] reported that p53 overexpression correlates with a better response to chemotherapy and is thus associated with better survival. Similar results were demonstrated by Sun et al. [59]. In these studies, however, the HPV status was not studied. Initially, in HPV-driven cancers, there could be p53 overexpression due to the degradation of pRb by E7 oncoprotein and increased stabilization of p53 [60]. A meta-analysis of oral tongue squamous cell carcinoma showed that p53 could not be used as a prognostic biomarker for these tumors [61]. Similar conclusions were made by Halec et al. for LSCC [36]. Unfortunately, our study did not include an assessment of TP53 gene mutations, which could have clarified some questions about the previously mentioned points [62,63]. Additionally, there is a possibility that p53 overexpression is unrelated to HPV infection, especially considering the high number of smokers in our study. Additional studies are needed to study the prognostic role of p53 in HNSCC, especially in OPSCC and HPSCC.

To the best of our knowledge, very few studies have been focusing on HPV oncoprotein E6/E7 immunohistochemical expression and its role in survival or prognostic values. As E6 and E7 are considered to be the main driving forces of HPV-mediated carcinogenesis, we found it interesting to study the role of these proteins in survival using immunohistochemistry. In the cases of both OPSCC and HPSCC, the immunohistochemistry results of HPV16 E6 protein expression showed that patients with positive staining in their tumor samples had a better survival rate. However, a high expression of either p16 or p53 was simultaneously found with E6, which could be considered a positive outcome marker for the patient. Moreover, there is a possibility that at a certain stage of viral activity, this oncogene (E6) did not have time to disrupt the cell cycle. For example, E6 initiates proteasome-dependent p53 degradation by recruiting the ubiquitin ligase E6AP. Furthermore, only the combined complex of E6 and E6AP is reactive with p53. This means that the expression of a single HPV16 E6 protein cannot affect p53 degradation (detection could be less informative for a patient's outcome prognosis) [64]. Unfortunately, E6AP activity was not studied in this research. A prospective study (of the dynamics with several time points) might better reveal HPV oncogenes' roles in the progression of an HNSCC tumor, as a persistent HPV infection is a major factor for carcinogenesis [65]. With this study, it is difficult to distinguish persistent from non-persistent HPV infections (sampling was performed only a single time). However, in patients with HPSCC, E6 protein was detected using only immunostaining, while E6 mRNA was not detected, and HPV16 DNA was still detectable. This could indirectly indicate the presence of a persistent HPV16 infection, which could be one of the reasons why the presence of HPV16 DNA in the samples of patients with HPSCC showed worse outcomes.

E7 is recognized as the major transforming protein of high-risk HPVs due to mutational analyses in transformation assays [66]. In addition, it was shown that E7 precisely drives early tumorigenesis [67]. The present study shows that the IHC overexpression of HPV16 E7 protein in OPSCC is associated with a poorer prognosis (Cox regression). However, in HPV-associated tumors, the E7 protein should be the driving factor for p16 overexpression, which is associated with better survival. On the other hand, some studies

report that the overexpression of p16 has consistently and repeatedly been shown to be associated with a better response to therapy and a favorable clinical outcome in OPSCC, and not all cases of p16 overexpression could be related to HPV's oncogenic activity [68,69]. This suggests the presence of additional mechanisms of E7-protein-associated carcinogenesis. Several studies have shown that E7 induced the upregulation of several types of matrix metalloproteinases [70,71]. This process has been linked to the promotion of the invasiveness the tumors [72]. Additionally, the protein function of HR-HPV E7 has been associated with a more stable mitotic function that is needed for viral genome maintenance and replication [73,74]. These processes could lead to an invasive and potentially metastatic phenotype of cancer, and this could explain the poorer prognosis in OPSCC with IHC HPV16 E7 protein overexpression [66]. Oton-Gonzalez et al. [75] showed that patients with OPSCC with detectable HPV16 E7 protein in their serum had poorer relapse-free survival and OS. The authors also showed a correlation between E7 protein in serum and E7 mRNA expression. Thus, they concluded that the source of the E7 protein must have been HPV16-positive cancer, more specifically circulating tumor cells, suggestive of the metastatic process. It is worth noting that not all tumors are HPV-related, and it was shown that virus-induced oncogenesis takes a long time to develop and that some patients with HNSCC can have a concomitant HPV infection [66].

One limitation of our study is the relatively small number of patients for each region (oropharynx, larynx, and hypopharynx), which could result in insufficient statistical power and limit the conclusions drawn for some markers, especially if they did not reach statistical significance. However, it is hard to deny the observed trends of the studied markers and their effects on survival. The other limitation is that almost all HPSCC samples were FFPE due to possible genetic material degradation, especially that of RNA. On the other hand, all samples were viable for analysis based on the intrinsic control of the kit that was used (mRNA detection) or β-globin detection (DNA quality).

5. Conclusions

HPV infection plays a significant role in the tumorigenesis of HNSCC, especially OPSCC. It should be noted that not only HR-HPV but also LR-HPV could affect survival prognosis. The immunohistochemical assessment of HPV16 E6 protein expression should be interpreted as a useful prognostic marker for OPSCC and HPSCC.

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Data Availability Statement: The results related to the immunohistochemical assessment of p16, viral proteins HPV16 E6 and E7, and HPV DNA testing in the samples obtained from patients with hypopharyngeal and laryngeal cancer were published in Viruses (https://doi.org/10.3390/v13061008). The aforementioned article aimed to estimate the prevalence of aforementioned markers in laryngeal and hypopharyngeal cancers.

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