HUMAN PAPILLOMAVIRUS AND OROPHARYNGEAL CANCER

IN BRITISH COLUMBIA

by

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Abstract

Oropharyngeal squamous cell carcinoma (OpSCC) patients have improved survival when tested positive for high-risk human papillomavirus (HR-HPV). However, tissue assessment of HPV status is currently not standardized and additional factors may influence survival among HPV-positive patients. The main objectives were to evaluate HPV detection methods and to identify possible factors that impact survival of OpSCC patients in British Columbia.

We retrospectively analyzed 972 primary OpSCC patients diagnosed between 2000-2008 and referred to the BC Cancer Agency for treatment with curative intents of radiotherapy with or without concurrent chemotherapy. Patient charts were reviewed and collected information for demographics, smoking history, clinical assessments, treatment received, and outcomes. We analyzed two cohorts of Study Cohort, 244 cases with enough formalin-fixed, paraffin-embedded (FFPE) tissues for experiment, and General Cohort, 728 cases without tissues available. Experimental procedures included *in situ* hybridization (ISH) to detect DNA and RNA HPV and immunohistochemistry (IHC) to detect p16, p53, the retinoblastoma protein (pRB), cyclin D1, and Ki67. We used polymerase chain reaction (PCR) to detect type-specific HPV from cases with enough FFPE tissues for DNA extraction (n=41). Cox proportional hazard (Cox-PH) and Kaplan-Meier (KM) survival analysis were conducted to identify potential clinical and biological factors impacting on 5-year overall survival (OS), disease-specific survival (DSS), and development of loco-regional recurrence (LRR).

The incidence rates of males increased from 3.2 to 7.6 per 100,000 whereas females declined from 1.1 to 0.8 per 100,000. The Study Cohort was relatively representative of the General Cohort. The Study Cohort of patients classified as ever-smokers, had tumours staged at T3/4, and received radiotherapy only had poorer 5-year OS, DSS, and LRR (p<0.05). HPV was detected in 77.6% of patients. Using PCR as standard, DNA/RNA ISH to detect HPV was more specific than IHC p16. Stratification of patients by HPV status showed that HPV-positive/p53-positive and HPV-negative/cyclin D1-positive patients had significantly poorer DSS (p=0.03) and 5-year OS (p=0.02), respectively.

In conclusion, both HPV burden and its prognostic significance were found in BC. ISH assessment may be used to determine HPV status. IHC assessments of p53 or cyclin D1 status may be prognostic indicators to guide treatment.

Lay Summary

The human papillomavirus (HPV) can induce the development of cancers in the oropharynx. Paradoxically, HPV detection is associated with improved survival among oropharyngeal cancer patients. HPV can be detected indirectly for the p16 protein or directly for the virus itself. However, the detection method for HPV status has not been standardized in the clinic and additional factors may affect survival among HPV-positive patients. The main part of the study was analyzing a cohort of oropharyngeal cancer patients and to identify important clinical and biological factors that may explain the outcomes. Different methods were also evaluated in detecting HPV.

Patients with a smoking history, had advanced stages of tumour, and received radiotherapy only had poorer clinical outcomes. The direct detection of HPV was more reliable than detecting p16. The expressions of p53 and cyclin D1 may be important to guide treatment.

Preface

This manuscript of the thesis is an original work by the author of Xian Jun David Lu. The author contributed to all data collection, data analysis, statistical analysis, and produced all the figures and tables.

All the clinical and outcome data collected were from the British Columbia Cancer Agency/Research Centre in Vancouver, British Columbia, Canada. Patient charts were reviewed by the author. The study has been approved by the British Columbia Cancer Agency/University of British Columbia Research Ethics Board: H10-03153.

The experiments of DNA and RNA *in situ* hybridization and immunohistochemistry staining of p16, p53, pRB, cyclin D1, and Ki67 were performed by the author and Dr. Sarah Zhu. All slides were reviewed and scored by an oral pathologist, Dr. Catherine Poh and the author. DNA extraction for the HPV PCR was carried out by Dr. Sarah Zhu. PCR experiments were performed and the results were analyzed by the author.

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List of Abbreviations

AJCC   American Joint Committee on Cancer

APC   Annual percent change

BCCA   British Columbia Cancer Agency

Cox-PH   Cox proportional hazard

CDK4/6   Cyclin-dependent kinases 4 and 6

CRT   Concurrent chemoradiotherapy

CT   Computed tomography

DAB   3,3-diaminobenzidine

DM   Distant metastasis

DSS   Disease-specific survival

EGFR   Epidermal growth factor receptor

FCE   Fluorescence capillary electrophoresis

FFPE   Formalin-fixed, paraffin embedded

HIVID   High-throughput viral integration detection

HNSCC   Head and neck squamous cell carcinoma

HR-HPV   High-risk human papillomavirus

HRP   Horse radish peroxidase

HSPG   Heparan sulfate proteoglycan

IHC   Immunohistochemistry

ISH   *In situ* hybridization

KM   Kaplan-Meier

LR   Local recurrence

LR-HPV   Low-risk human papillomavirus

LRR	Loco-regional recurrence
MRI	Magnetic resonance imaging
OpSCC	Oropharyngeal squamous cell carcinoma
OS	Overall survival
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFS	Progression free survival
pRB	Retinoblastoma protein
qPCR	Quantitative polymerase chain reaction
RFU	Relative fluorescent units
RNA-seq	RNA sequencing
RPA	Recursive partitioning analysis
RR	Regional recurrence
RT	Radiotherapy
TMA	Tissue microarray
VLP	Virus-like particle
WGS	Whole genome sequencing

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For my family and friends

I.   Introduction

I.1.   Oropharyngeal cancer

The head and neck anatomical classification includes the regions of the oral cavity, pharynx, and larynx. Cancers that arise from these regions are often from squamous cells, thus these cancers are collectively referred to as head and neck squamous cell carcinomas (HNSCCs).[1] Genetic mutations such as *CCND1* amplification or *CDKN2A* deletion can induce HNSCC development.[2] Alternatively, cancers could be virally induced. The oropharynx is a sub-anatomical region that encompasses the tonsils, base of tongue, soft palate, vallecula, uvula, and the epiglottis. The susceptibility for infection by the human papillomavirus (HPV) at the oropharynx has gained much interest for the development of oropharyngeal squamous cell carcinoma (OpSCC).

I.1.1.   Epidemiology of oropharyngeal cancer

The annual worldwide incidence and mortality counts of pharyngeal cancer, excluding the nasopharynx, were estimated at 142,000 and 97,000, respectively, in 2012.[3] A study using data from 23 countries of worldwide cancer registries found that between 1983 and 2002, incidence rates of OpSCC increased more significantly in countries defined to be economically developed.[4] Incidence rates were 2-17 times higher among men than women and higher among relatively younger individuals, i.e., less than 60 years of age. The study suggested that relatively younger men were more susceptible for OpSCC development.

Tobacco is commonly associated with cancer development, though the worldwide consumption of tobacco has decreased.[5] In Canada, tobacco consumption has decreased over time, but not all head and neck subsites have collectively decreased in their cancer incidence.[6] Between the years 1992 and 2006, the incidence rate of oral cavity cancers among men had declined at a rate of 2.1% per year, but OpSCC had increased at a rate of 1.5% per year.[7] A similar trend was observed in women, but the change in incidence rates were lower such that oral cavity cancer decreased by 0.4% per year and OpSCC increased by 0.8% per year.[7] A more recent analysis of Canadian data from 2000 to 2012 found that approximately 3600 patients were diagnosed with OpSCC based on five cancer centres that participated in the study from across the nation.[8] The average number of OpSCC patients

diagnosed ranged between 1.13 to 16.5 per year and the variability was attributed to the difference in the time periods of data contributed.[8] However, the overall analysis showed that more males were diagnosed with OpSCC and patients averaged 60.4 years of age, thus paralleling the worldwide trend.[4,8]

I.1.2.   Survival of oropharyngeal cancer patients

Although the incidence rate has been increasing in Canada, the survival rate among OpSCC patients has also improved. According to the latest annual publication of the Canadian Cancer Statistics (2016), the 5-year age-standardized disease-specific survival (DSS, or net survival as defined by the publication) has increased from 43% to 58% between the period of 1992-1996 and 2004-2008.[9] Additionally, between the years of 1992 and 2012, the mortality rates have declined from 2.8 to 1.7 and from 0.8 to 0.5 per 100,000 males and females, respectively. To compare to the United States, one study analyzed data collected by the Surveillance, Epidemiology, and End Results Program between the years of 1982 and 2006.[10] The analysis indicated that the 5-year overall survival (OS) of patients with cancers of the tongue, tonsil, and oropharynx have significantly increased when comparing between the years of 1992-1996 (50.5%, 47.6%, and 33.3%, respectively) and 2002-2006 (64.9%, 69.8%, and 42.2%, respectively). Of note, the study grouped all tongue cancers into a single category, including other oral cavity subsites. In another study, the authors reviewed HNSCC prospective trials that investigated radiation and chemotherapy treatments and categorized the patients into the oropharynx and non-oropharynx subsites.[11] Upon further separation of the trial studies by time periods of 1993-1998, 1999-2003, and 2004-2010, the review found that the 5-year OS rates of OpSCC patients were 42.3%, 72.5%, and 78.4%, respectively. However, the 5-year OS rates among non-oropharyngeal patients were 51.0%, 58.8%, and 66.3%, respectively for the time periods. The increase in survival of OpSCC could also be observed outside of North America. For example, a study of the Danish population investigated for the population adjusted 5-year OS rate, as defined by the ratio of observed survival among OpSCC patients and the expected survival rates of the population in Denmark as compared by the age, sex, and calendar year.[12] An increase from 33.1% to 58.5% was found when comparing between the years of 1980-1984 and 2010-2014.

I.1.3.   Diagnosing and staging oropharyngeal cancer

The diagnoses for OpSCC can be challenging and often late due to the difficulty in locating the primary tumour.[13] Clinical symptoms that can lead to a diagnosis include enlarged neck mass, sore throat, and dysphagia.[13] Patients should be thoroughly examined with physical, imaging, and pathological assessments. Physical examinations include palpating for lymph nodes and using an endoscope to look inside the oropharynx. Imaging examinations include computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), chest X-rays, and ultrasound. CT and MRI scans are used to assess bone and soft tissue, respectively, and both techniques produce cross sectional images to help detect nodal disease.[14] PET scans use a radioactive tracer to assess for metabolic activities in which tumour cells are more metabolically active compared to normal cells.[14] Chest X-rays can assess whether the lungs have tumour involvement either metastatic from the primary lesion or is a separate diagnosis altogether. Ultrasound examinations can be useful for patients that present with an enlarged neck mass, but unknown primary tumour. The technique can be used to guide the process of a fine needle aspiration (FNA) for pathological analysis.[15] Pathology remains the gold standard for cancer diagnoses; however, the process is limited by the patients' compliance to undergo biopsies, the clinicians' expertise to obtain representative samples, and the sizes of biopsy tissues.

Collectively, the assessments are used to accurately stage the cancer for treatment planning. The 8th edition of the American Joint Committee on Cancer (AJCC) Staging Manual was implemented in January 2018.[16] However, the treatment for patients remains to be guided through the staging by the 7th edition of the AJCC Staging Manual.[17] The three areas assessed are the primary tumour (T), the regional lymph nodes (N), and distant metastasis (M) (**Table 1**). By definition, the T stage describes the size of the primary tumour and the extent of involvement of nearby sub-anatomical structures, the N stage describes the size, number, and laterality of any involved lymph nodes, and the M stage indicates the presence of distant metastasis. For HNSCC in general, patient prognoses have been associated with tumour and nodal stages.[13,18-20] Collectively, the TNM stages for each patient are then assigned a clinical staging of I to IV (**Table 2**).[17] Stages I/II describe patients with small primary tumours with no nodal involvement while stages III/IV describe patients with large primary tumours and/or nodal involvement and/or presence of metastasis. Patients at

Stage IV can be further classified into IVA (moderately advanced disease), IVB (very advanced disease), or IVC (metastatic disease). Stage IVA implies that patients may be suitable for curative surgical treatment. Stage IVB implies that patients are not suitable for surgical treatment, but may benefit from curative systemic therapy. Stage IVC implies that patients may only benefit from palliative treatment.[20]

Table 1. 7th edition TNM staging system for oropharyngeal cancer[17]

Stage	Description
Primary tumour (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour ≤2 cm in greatest dimension
T2	Tumour >2 cm, but ≤4 cm in greatest dimension
T3	Tumour >4 cm in greatest dimension or extension to lingual surface of epiglottis
T4a	Moderately advanced local disease Tumour invades the larynx, extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible
T4b	Very advanced local disease Tumour invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases carotid artery
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node, >3 cm, but ≤6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none >6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none >6 cm in greatest dimension
N3	Metastasis in a single ipsilateral lymph node, >6 cm in greatest dimension
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

Table 2. 7th Edition AJCC staging for oropharyngeal cancer[17]

AJCC Stage	TNM stage		
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1/2/3	N1	M0
Stage IVA	T4a	N0/1	M0
	T1/2/3/4a	N2	M0
Stage IVB	T4b	Any N	M0
	Any T	N3	M0
Stage IVC	Any T	Any N	M1

I.1.4. Treatment for oropharyngeal cancer

The available options for treatment can be either single or multi-modality. The intent of treatment can be curative or palliative and the timing of treatment delivery indicates if the treatment is the primary form of treatment, neoadjuvant or adjuvant to the primary treatment.

Traditionally, treatment either consists of surgery with or without adjuvant radiotherapy (RT) or RT only as the primary, if the patient is not suitable for surgery.[21] For surgery with curative intent, the location, size, and depth of invasion of the primary tumours are all factors for consideration. The surgical procedure may also include a neck dissection if the lymph nodes are involved. HNSCC patients that were treated with surgery and received adjuvant RT with concurrent administration of chemotherapy (CRT) have been found to increase OS, disease-free survival (DFS), and local and regional control, as compared to adjuvant radiotherapy only.[22,23] For radiotherapy with curative intent, the radiation dosage given over the course of treatment to the primary tumour can be in the total of 50-70 Gy.[24] The involved neck lymph nodes may also receive radiation. Post-radiation patients may also undergo salvage surgery with or without neck dissection if residual disease is present with or without palpable lymph nodes.[25]

CRT is found to increase 5-year OS by 8.1% and this bimodal treatment has been recommended for patients who can tolerate this treatment.[26,27] The treatment regimen of RT (total dose of 70 Gy) with concurrent high-dose cisplatin (three doses of 100 mg/m^2 that was given every three weeks) was developed from a pilot study of HNSCC patients.[28] A clinical trial that consisted of 295 HNSCC patients with unresectable disease compared the efficacy of RT only (2 Gy per day for 70 Gy total) and RT with concurrent high-dose cisplatin (same radiotherapy protocol with 100 mg/m^2 cisplatin given on days 1, 22, and 43 of treatment).[29] The results showed that CRT was significantly associated with improved 3-year OS compared to the radiotherapy only cohort (37% vs. 23%, p=0.014). The alternative chemotherapy agents of carboplatin with infusional 5-fluorouracil (5-FU) have also been investigated in patients with Stages III/IV OpSCC for CRT.[30] In this study, carboplatin (70 mg/m^2) and 5-FU (600 mg/m^2) were given over a period of four days for three cycles and RT remained at 70 Gy total. As compared to the RT only group, concurrent chemoradiotherapy resulted in improved rates for 3-year OS (51% vs. 31%), DFS (42% vs. 20%), and loco-

regional control (LRC) (66% vs. 42%). The efficacy of RT with concurrent administration of either cisplatin or carboplatin with 5-FU has been retrospectively analyzed among OpSCC patients.[31] No statistical significance was found between the different chemotherapy agents for rates of 3-year OS, 3-year DFS, and 3-year LRC. On the other hand, neoadjuvant chemotherapy is not recommended over concurrent chemotherapy with radiotherapy, as OS and LRC were significantly better among patients treated with CRT.[27,32,33]

I.1.5.   Recurrences in oropharyngeal cancer

Cancer could recur in the same site (local recurrence, LR), metastasized to the lymph nodes (regional recurrence, RR), or metastasized to other organs (distant metastasis, DM). There are currently no predictive biomarkers for chances of recurrence, thus post-treatment monitoring is important.[34] A monitoring schedule may consist of close follow-up times for the first five years post-treatment.[35] More specifically, follow-up visits should occur every 1-3 months during the first year, every 2-6 months during the second year, every 4-8 months during the third to fifth years, and finally, annual visits after five years.

While imaging scans can help clinicians to determine the development of recurrence, scans that are taken too early post treatment can have high false positive and negative rates.[36] Recurrences are associated with poor prognoses;[37] therefore the identification of recurrences is critical for patients that are eligible for additional treatments. Depending on the initial curative treatment prescribed, patients may be eligible for surgery and/or RT. An early meta-analysis of OpSCC patients with recurrences treated with salvage surgery found a 5-year OS rate of 26%.[25] More recently, another meta-analysis compared surgical and non-surgical approaches for treating recurrences in OpSCC patients.[38] The study found a 5-year OS rate of 23% and patients that were treated surgically had significantly better 5-year OS, compared to non-surgically treated patients (26% vs. 16%). However, more surgical studies were available for analysis compared to non-surgical studies (11 vs. 4). The study also showed that if the surgically treated studies were divided based on year of patient recruitments, recruited before 2000, recruited before and after 2000, recruited after 2000, an increasing trend of 5-year OS was observed for 20%, 35%, and 51%, respectively. However, the meta-analysis did note a lack of uniformity between studies, which was evident when the total number of patients for the three groups were 127, 281, and 70, respectively.

I.2.   Etiology of OpSCC

The development of OpSCC can be attributed to risk factors such as tobacco and the human papillomavirus (HPV). Through many years of research, tobacco has undeniably become a well-known carcinogen for development of cancers in general.[39] With changes in sexual practices, an increasing number of individuals are being infected with HPV, which has been recognized to virally induce OpSCC development.[40] The following sections discuss the risk factors of tobacco and HPV in contributing to OpSCC development. Important to note, however, is that having the risk factors does not necessitate carcinogenesis.

I.2.1.   Tobacco

The relative risk for development of OpSCC has been found to be 6.8 more times among smokers compared to non-smokers.[41] Quitting smoking for more than ten years can reduce the relative risk for developing OpSCC.[39] Compared to non-smokers, former and current smokers were 2-4 and 10-14 times more likely, respectively, to develop OpSCC.

Patients with a smoking history are associated with worse prognosis. A prospective study of HNSCC patients, prior to receiving treatment, were surveyed for their smoking history.[42] In comparison to non-smokers, patients that were identified as current and former smokers had significantly increased risk for OS and DSS. Interestingly, the study also found that the timing of quitting smoking, either within 10 years or more than 10 years before the cancer diagnosis, was not statistically significant between OS, recurrence-free survival (RFS), and DSS. In another study of HNSCC patients that received CRT, smokers were 1.5 times more likely than non-smokers to have poor 5-year OS.[43] For OpSCC in particular, a decline of LRC rates of 92%, 88%, and 75% have been reported for patients identified as non-smokers, former smokers, and current smokers, respectively.[19] The patients that continue to smoke during RT are also associated with poorer survival and lower response rate to treatment.[44]

I.2.2.   The human papillomavirus

Over 150 genotypes of the virus have been identified and are categorized as low and high-risk.[45] Low-risk HPV (LR-HPV), such as HPV 6 and 11, are associated with warts, whereas high-risk HPV (HR-HPV), such as HPV 16 and 18, are associated with cancer development.[40] The link between HPV and development of cervical cancer was identified by Dr. Harald zur Hausen in the 1970s in which he was later awarded the Nobel Prize in Physiology and Medicine in 2008.[46,47] The discovery led to the development of the HPV vaccine that is now available in Canada and elsewhere.[48] HPV could also induce the development of anal, penile, and oral and oropharyngeal cancers.[40]

I.2.2.1. Prevalence rate of oral HPV infections

According to a systemic review of 18 studies published between 1997 and 2009, the worldwide prevalence rates of HPV for any type and HPV 16 are 4.5% and 1.3%, respectively.[49] The prevalence rates of HPV are influenced by factors such as the economic status of a country. Compared to developed nations, developing nations were found to have higher prevalence for all HPV types (7.3% vs. 3.6%) and HPV 16 (4.3% vs. 0.7%). However, the review may have contained a regional bias as 5/18 studies were conducted in the United States. The review may have also included a gender bias as more females than males were analyzed (70.6% vs. 29.4%).

In Canada, the numbers of HPV infections are not reported and a national study has yet to be conducted, but an estimated 75% of sexually active Canadians are or have been infected with HPV.[48] To gain an idea of the HPV burden in Canada, the statistical figures from the United States is discussed.

The oral prevalence rate of HPV in the United States was collected as part of the 2009-2010 National Health and Nutrition Examination Survey (NHANES).[50] The study consisted of 5,579 individuals between the ages of 14 and 69 and an even distribution of men (49.3%) and women (50.7%). Each participant provided an oral rinse for analysis of 37 different HPV types. The oral prevalence rates of HPV of any type and of HPV 16 were found to be 6.9% and 1.0%, respectively. HPV prevalence rates were found to be associated with the factors of age, biological sex, number of lifetime sexual partners, and smoking habits. In particular, higher oral prevalence rates of HPV were found among the 60-64 age

group (11.4%), men (10.1%), individuals with ≥21 lifetime sex partners (20.5%), and smokers with ≥20 cigarettes per day (20.7%).

The higher prevalence rate of oral HPV among men was investigated with a follow-up study by combining two cycles of NHANES, 2009-2010 and 2011-2012, to identify risk factors.[51] Participants similarly provided an oral rinse for analysis of 37 different HPV types. In comparison to the 2009-2010 cycle, the prevalence rate of oral HPV in men increased (10.3% vs. 10.8%), but decreased in women (3.3% vs. 2.9%).[52] HPV 16 continued to be the most prevalent genotype detected in men and women. Sexual behaviour was a factor affecting the oral prevalence rates among men and women. A statistical model of the oral HPV prevalence rate was analyzed with respect to the number of sex partners for different types of sex. The model showed that comparing to women, men has a higher oral HPV prevalence with the increase of number of sex partners in either vaginal or oral sex. In both men and women, the follow-up study continued to show that age, smoking habits, and number of lifetime partners are factors to affect HPV oral prevalence rates. The estimated individuals with oral HPV infections between 2009 and 2012 were 7.07 million and 1.54 million for men and women, respectively, thereby highlighting the higher susceptibility of oral HPV infections in men.

I.2.2.2. Oral HPV clearance rate

The clearance rate of oral HPV has been prospectively studied in the HPV Infections in Men (HIM) cohort in which participants attended six months follow-up visits for up to four years total with collection of oral rinses at each follow-up (n=1626).[53] The original intent of recruiting the study cohort was to use a standardized protocol for sample collection and determine whether the prevalence of penile HPV genotypes differed among different age groups and between three countries of Brazil, Mexico, and the United States.[54] Upon recognition that the prevalence of oral HPV was increasing, oral rinses were collected.[55] The clearance rate of 'incident' oral HPV (n=115) was investigated in participants that were originally tested HPV-negative but HPV-positive during the follow-up. The study found that 39% (n=45) of 'incident' oral HPV were cleared within a year from infection, the median duration of infection was 6.9 months, and the clearance for HPV 16 were slightly longer than any other oncogenic HPV infections (7.3 vs. 6.3 months).[53]

Another prospective cohort based in the United States, The Persistent Oral Human Papillomavirus Study (POPS), included both males and females to investigate the clearance rate of oral HPV.[56] The participants were followed every 6 months for 3 years total and provided oral rinses to detect 37 HPV types. Prevalent oral HPV infection was defined by the detection of any HPV type at initial visit. Incident oral HPV infection was defined by the negative detection of HPV at initial visit, but positive HPV detection at a follow-up visit. For clearance rates, the study analyzed both definitions of requiring only one visit of HPV-negative detection and requiring two consecutive visits of HPV-negative detections. The majority of oral HPV infections (83%) were cleared within a year for incident detections, as determined by one HPV-negative HPV detection, but approximately half of the prevalent oral HPV infections (51%) were cleared within the one-year timeframe. Clearance rates were lower for both incident and prevalent oral HPV infections when defined by two consecutive visits of HPV-negative detections. Interestingly, the estimated one-year clearance rates were higher among women than men (70% vs. 59%, p=0.002). Smoking was a factor in reducing clearance rates among women and increasing age negatively correlated with clearance rates for HPV 16.

Collectively, studies found that oral HPV infections were generally cleared within one year and women were more likely than men to clear their infections. In terms of factors impacting on the clearance rate, age was associated in both men and women, whereas factors of smoking and sexual behaviours were less definitive. In comparison to other HPV genotypes, the relatively higher persistence of HPV 16 may well explain its carcinogenic capability.

I.3. HPV-related oropharyngeal cancer

An early retrospective study by Gillison *et al.* suggested a causal association between HPV and OpSCC.[57] Tissue samples consisted of 253 HNSCC from anatomical regions of the oral cavity, oropharynx, nasopharynx, larynx, and hypopharynx. The study found that 22% of samples (n=55/253) were HPV-positive and 62% of the HPV-positive samples (n=34/55) were from the oropharynx. In comparison to the oral cavity, the odds ratio of the oropharynx to be HPV-positive was 9.7 suggesting that HPV was more likely to cause cancer in the oropharynx. The association between HPV infections and OpSCC was further supported from a case-control study.[58] With an increasing number of epidemiological studies and more knowledge regarding the biological properties of the viral oncoproteins produced by HPV, the virus has been recognized as a Group 1 carcinogen by the International Agency for Research on Cancer.[59]

With the recognition that HPV causes OpSCC and the rising prevalence of HPV in the United States, Chaturvedi *et al.* addressed the issue of whether the rising incidence of OpSCC was in fact due to HPV.[60] The study retrospectively analyzed 271 OpSCC tumours collected between the years of 1984 and 2004. Samples were tested for the detection of 28 HPV types, including HPV 16. An increasing percentage of HPV-positive OpSCC samples were observed across the calendar years. Using the Surveillance, Epidemiology, and End Results population data between 1988 and 2004, the incidence rates of HPV-positive and HPV-negative OpSCCs were calculated. An overall incidence rate of OpSCCs increased by 28%. The incidence rate for HPV-negative OpSCCs declined by 50%, but an increase of 225% for HPV-positive OpSCC effectively offset the reduction. Future projections estimated that by the year 2030, the incidence rate for OpSCCs would exceed that of cervical cancer and the incidence rate of OpSCC in men would continue to increase. OpSCCs were also estimated to encompass 47% of all HNSCCs cases by 2030.

The increase in incidence rate of OpSCC was projected in another study based in England.[61] As opposed to detecting HPV among tissue samples, the researchers analyzed population data from cancer registries between the years of 1995 and 2011. HPV-related HNSCC was defined as tumours from the tonsils, base of tongue, and the oropharynx, i.e., OpSCC, whereas other anatomical sites were considered non-HPV-related. The study found

an overall increase for incidence rates of HNSCCs and the annual percent change (APC) for males and females were 2.2% and 2.4%, respectively. However, the largest increase in APC was due to OpSCC, which was 7.3% and 6.5% for males and females, respectively. Future projections predicted that between 2011 and 2025, rates of HNSCCs would increase by 34.8% and 48.9% for males and females, respectively, and OpSCCs were predicted to encompass 35% of all HNSCC cases. To compare the increasing trend of OpSCC with HPV incidences, the study analyzed the diagnoses of two STIs of anogenital warts and genital herpes. As expected, increased diagnoses of the STIs paralleled the increase in OpSCC between 1995 and 2011 for both males and females.

As a result of the incidence of HPV, both studies suggested that the burden of OpSCC is projected to increase in more men than women. Infection of the virus does not guarantee carcinogenesis, as the majority of individuals could clear the virus. To better understand the disease, the molecular structure of the virus and its biological mechanism to infection and integration will be discussed.

I.3.1. Susceptibility of the oropharynx to HPV infection

The oral cavity is anatomically the first site of contact, but HPV has been detected at lower rates compared to the oropharynx (3.9% vs. 18.3%).[62] The detection for HPV in HNSCC found that among tissue samples from the larynx, oral cavity, hypopharynx, pharynx, tonsils, tongue, and floor of mouth, the highest positive detection rate for HPV was from the tonsils (60%, n=9/15).[63] Other studies have observed that among HPV-positive OpSCC samples, the majority of cases were from the lingual and palatine tonsils.[57,61] The tonsils are part of the sub-anatomical region, known as the Waldheyer's ring, which serves as a primary defence against foreign antigens.[1,64] Crypts have been observed from the microscopic analysis of the palatine tonsils, which function to increase surface area of the tonsillar epithelium.[65] The epithelial linings of the crypts also contain discontinuous cell layers, i.e., reticulated, and immune cells such as lymphocytes, macrophages, and plasma cells can be observed on the epithelial surface.[66] Ironically, while the micro-anatomical structure facilitates the transport and presentation of foreign antigens to elicit immune responses, the increased surface area due to crypts may allow viral particles to be more easily deposited.[1] Kim *et al.* has suggested that HPV-related OpSCC initiates with the integration

of the virus in tonsillar crypts.[67] This hypothesis may explain the finding that HPV 16 has been detected among 30% of patients (n=3/10) presented with a metastatic nodal disease, but unknown primary tumour.[68]

I.3.2.   Molecular structure of HPV

The family of papillomaviruses have non-enveloped capsids, have circular double-stranded DNA genome, and are known to cause infections on epithelial surfaces, i.e., epitheliotropic.[40] HPV is classified under the alpha-papillomaviruses genus and include similar features of humans and primates being host species and low-risk and high-risk classification categories for causing malignancy.[69] HPV has a genome of approximately 8000 base pairs and contains three regions of early (E1-7) genes, late (L1-2) genes, and a non-coding, long control region involved in replication and transcription processes.[40] The nomenclature of early and late genes refers to their relative timing of expressions in the viral life cycles. E1-7 encode for proteins that are involved in viral DNA replication and L1-2 encode for proteins that form and assemble structure of new viruses.[70] L1 could also self assemble into empty capsids, i.e., virus-like particles (VLP), which is the basis for vaccine development.[71,72] Please see **Table 3** for the overview of HPV proteins and their functions.

I.3.3.   Mechanism of HPV infection

The heparan sulfate proteoglycans (HSPGs) on the surface of epithelial cells act as receptors for signaling pathways.[73] Viruses, including HPV, can initiate infection through attachment to HSPGs.[74] After HPV attaches to the host cell surface, the L2 protein undergoes a conformational change to expose a consensus sequence in its N-terminus domain that can be cleaved by a furin protease.[75] The cleavage is hypothesized to expose a secondary receptor binding site on L1 that would otherwise be obstructed by the L1-2 complex.[74] The purpose for the cleavage has not been clearly defined, but the process is necessary for infection.[75]

Table 3. Functions and descriptions of HPV proteins

Protein	Functions and characteristics	Ref
E1	- Has helicase activity for unwinding viral DNA - Involved in viral DNA replication cycle	76
E2	- Involved in recruiting E1 to site of replication - Negatively regulate transcription; low levels can activate, but elevated levels repress transcription	77
E4	- Cause host cells to arrest in G2 phase and may serve to inhibit competing host DNA synthesis to facilitate viral DNA replication - Bind to mitochondria and lead to host cell apoptosis for releasing virus	78-81
E5	- Upregulate the activity of epidermal growth factor receptor (EGFR) to activate signaling pathways and prevent cellular apoptosis - Increase cellular proliferation for tumour growth	82-84
E6	- Bind to p53 directly or form a complex with an E3 ubiquitin ligase (E6-associated protein) to target p53 for ubiquitination to prevent apoptosis - Associate with the human telomerase reverse transcriptase to upregulate telomerase activity for cell immortalization	85-89
E7	- Cause ubiquitination of the retinoblastoma protein (pRB) by associating pRB and the cullin 2 ubiquitin ligase complex - Binding of pRB also prevents the association between pRB and the E2F transcription factor to lead to cellular proliferation	90,91
L1	- Major component of the capsid - Forms a complex with L2 - Contain genetic region of conserved sequences.	92
L2	- Minor component of the capsid - Forms a complex with L1 - Involved in the infection of host cells	93-95

The internalization process of the virus into the host cell is still under debate. Endocytic pathways of clathrin-mediated, caveolin-mediated, and neither clathrin nor caveolin mediated pathways have all been suggested.[93,96,97] Regardless of the pathways under study, Schiller *et al.* cautioned the interpretation of results.[74] Primary endocytic pathways were investigated through the uses of inhibitors, thus alternative pathways may activate to circumvent the inhibition.[74] Early endosome is known to convert into the late endosome upon internalizing particles. After HPV has internalized into the endosomes, the virus then undergoes endosomal escape in which the viral genome has been observed to form a complex with L2 and may be trafficked to the nucleus through the actions of microtubules.[93,98] Active transport of the viral genome into the host nucleus has been previously suggested.[99] Alternatively, the observation that HPV infection requires cell-cycle progression led to the suggestion that the viral genome may enter the nucleus during mitosis when the nuclear membrane is disassembled.[100]

I.3.4.   Mechanism of HPV integration

Viral DNA exists as episomes upon entering the host cell nucleus. Integration of viral DNA into the host genome is considered necessary for malignancy as evident from higher grades of cervical cancer and HNSCC.[101,102] However, episomal forms of the virus have been observed among cervical cancers, albeit at a smaller percentage, and the potential for integration may be related to the particular HPV genotype.[103] Double stranded breaks in host DNA may promote integration of HPV 16 DNA from a study of using a cervical keratinocyte cell line.[104] The purpose of integration may be to disrupt the expression of the HPV E2 gene to inhibit repressions for transcribing viral genes such as HPV E6/E7,[105] thereby resulting in continuous cell growth and promotion of malignancy.

To gain some insights for the consequences of integration, Akagi *et al.* analyzed seven HPV-positive cervical and head and neck cancer cell lines, three HPV-negative cancer cell lines, and two HPV-positive primary head and neck tumours.[106] Molecular techniques of whole genome sequencing (WGS), RNA sequencing (RNA-seq), and spectral karyotyping were used for analysis. By seeking the HPV 16 genome in the host genome, the study found that the HPV E2 sequence was disrupted in four of the seven HPV-positive cancer cell lines. Contrastingly, HPV E6 and E7 genes were retained in all HPV-positive cancer cell lines and

the tumour samples. Approximately 110 integration breakpoints were observed and integration was found to induce rearrangement, amplification, and deletion of host genomic segments. Based on the observations, the study proposed a looping model to explain the process of altering the host genetic profile. The model describes a nicking of the host genome, integration of the viral genome, formation of a circular host-viral DNA structure that undergoes a rolling circle amplification, to result in concatemers with viral-host breakpoints.

In another study of HPV integration, Hu *et al.* analyzed 135 HPV-positive samples of cervical intraepithelial neoplasias, cervical carcinomas, and cell lines.[107] The study used WGS and high-throughput viral integration detection (HIVID), a combined next-generation sequencing and computational approach. Initial analysis of two HPV-positive cell lines of SiHa and HeLa and two cervical carcinomas found that while only 11 integration breakpoints were reported using WGS, a total of 145 integration breakpoints were reported from HIVID. This was to show the higher sensitivity of HIVID compared to WGS. Complete analysis of all samples using the HIVID approach discovered approximately 3600 breakpoints that were all validated through Sanger sequencing and RNA-seq. Interestingly, breakpoints were observed throughout the entire genome of HPV 16, which included the E6 and E7 genes. An enrichment of the same short sequences of genes, i.e., microhomologies, between the host and viral genomes were discovered and a model of integration was proposed. Host genomic elements were believed to be unstable during HPV infection and form DNA breaks to result in the activation of a microhomology-mediated DNA repair pathway. Then HPV seizes the repair mechanism, fuses to the broken segment, and integrates itself into the host genome.

Understanding the process of HPV integration is still underway and although molecular techniques have advanced drastically, sensitivity of detection remains an issue. In addition, molecular detection for HPV primarily targets HPV E6/E7 and as Hu *et al.* has observed this region could also be disrupted from integration of the virus, an underestimation of HPV prevalence may exist.[107] The discussed models for HPV integration should also be further investigated to determine whether the process is mediated by the virus or the host.[106,107]

I.3.5.   Consequences of HPV integration

The tumour suppressor proteins of p53 and the retinoblastoma protein (pRB) have been widely studied in cancer development. The role of p53 is to act as a G1 phase checkpoint marker and regulates for cell cycle progression.[108] Similarly, pRB is also involved in cell cycle progression through controlling the cell cycle to enter into the S-phase.[109] The innate function of pRB is to bind directly to E2F transcription factors and repress transcription of E2F-dependent promoters.[110] For cells to proceed into the S-phase, the dissociation of pRB from E2F is necessary and occurs through phosphorylation by the cyclin-dependent kinases 4 and 6 (CDK4/6)-cyclin D1 complex.[111] To further control for cell-cycle progression, p16 acts as an inhibitor by forming a complex with CDK4/6 and prevent the interaction of the kinase with cyclin D1.[111,112] Interestingly, p16 could also bind to the CDK4/6-cyclin D1 complex to inhibit the activity of the kinase.[113]

It is known that the development of HPV-related OpSCC is due to the viral oncoproteins of E6 and E7 (**Figure 1**). Co-precipitation studies found that HPV E7 is associated with pRB and the cullin 2 ubiquitin ligase complex to result in downregulation of pRB through ubiquitination, i.e., degradation.[90] Compared to LR-HPV, E7 from HR-HPV binds to pRB at a higher affinity and prevents the association of pRB and E2F.[91] The inhibited activity of pRB through either ubiquitination or associating with E7 could lead to uncontrolled E2F activity to promote cell proliferation. A role of pRB is to negatively regulate p16 transcription, thus downregulation of pRB also leads to an overexpression of p16.[112] Furthermore, the overexpressed p16 would preferentially bind to CDK4/6, in which freely unbound cyclin D1 could be degraded, thus explaining the accompaniment of p16 overexpression with underexpressed cyclin D1.[114-116] The affected pathway of pRB could lead to an upregulation of p53 to control cell cycle progression.[117] However, HPV E6 directly bind to p53 to inhibit its function or form a complex with an E3 ubiquitin ligase (E6-associated protein) and target p53 for ubiquitination.[86,88,89] The loss of p53 activity could lead to an accumulation of DNA damage and genomic instability from uncontrolled cell growth.[118]

Of note, the alterations of these molecules are not specific to HPV integration. For HNSCC development, the gene that encodes for p53 (*TP53*) can also be inactivated through somatic mutations and chromosomal loss at 17p13.[119] Additional genetic alterations could occur at the pRB/cyclin D1/p16 pathway. The mutation and chromosomal loss of 9p21 (*CDKN2A*), resulting in downregulation of p16, and the chromosomal gain of 11q13 (*CCND1*), resulting in the upregulation of cyclin D1.[119,120]

I.3.6.   Impact on clinical outcomes

There is an increasing trend of HPV testing through p16 for newly diagnosed OpSCC patients.[8] HPV status does not currently guide treatment planning, but HPV-related OpSCC patients have better clinical outcomes. OS was found to have a longer median time of 15 months (91 vs. 76 months of HPV-positive and HPV-negative, respectively).[57] Kaplan-Meier (KM) survival analysis of 5-year OS was found to be approximately 75-90% for HPV-positive compared to 50-65% for HPV-negative.[57,121,122] The similar comparison was also observed for DSS. KM survival analysis for 5-year DSS was found to be approximately 50-90% and 30-65% for HPV-positive and HPV-negative OpSCC patients, respectively.[123,124] Interestingly, the rate of developing loco-regional recurrence (LRR) was also estimated to be reduced among HPV-positive OpSCC patients (3-year LRR rate of 13.6% vs. 35.1%).[121] Longer median times to develop LRR (20.9 vs. 9.7 months) and DM (18.0 vs. 11.2 months) were also observed when comparing between HPV-positive and HPV-negative OpSCC patients.[125] This further highlights the prognostic importance of HPV status because the treatment option and survival time is reduced upon development of LRR in HNSCC.[126]
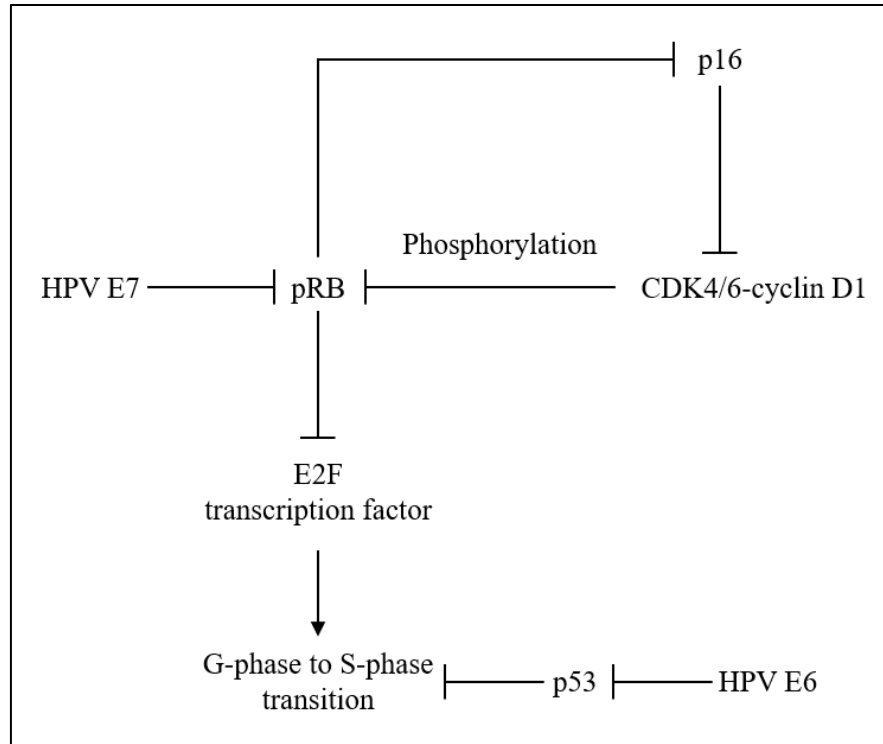
Figure 1. Biological consequence of HPV integration for relevant proteins

Cell cycle progression is tightly controlled by the retinoblastoma protein (pRB) and the checkpoint protein, p53. HPV E6 and E7 viral oncoproteins target p53 and pRB for degradation, respectively. The loss of pRB results in p16 overexpression and acting as the surrogate marker for HPV. Overexpressed p16 could lead to increased inhibition of cyclin-dependent kinase 4 and 6 (CDK4/6) activity and resulting cyclin D1 degradation. Line with arrow head represents progression and line with flat end represents inhibition.

HPV has been established as an independent prognostic factor that is largely restricted to the oropharynx.[62,121,127] HPV-related OpSCC is associated with higher nodal stage and classifies the overall diagnoses as advanced-stage cancer.[13,128] However, the paradoxical finding of improved clinical outcomes led to the proposal that the staging system should be revised.[129] One proposed staging system used recursive partitioning analysis (RPA) to derive new stages based on OS and tumour and nodal staging.[130] This proposed staging system suggested to use information such as tumour size (T4 or not), nodal status (≤N2c) with incorporation of age (70 years), and tobacco usage (20 pack year). Attempts to validate the new model was unsuccessful due to the lack of statistical significance between groups for OS and a modification was proposed.[131] By replacing the nodal staging criteria of OpSCC, the RPA-based staging was found to better separate patients based on OS.[131] Regardless of the proposed model, validation of any proposed model is necessary and crucial for managing the burden of HPV-related OpSCC.[128]

I.3.7.   Ethical concerns for HPV testing

With the increase in burden of HPV-related cancers, efforts should be made on increasing the knowledge of HPV in the general population. Ragin *et al.* conducted a survey that focused on the knowledge of HPV from participants that resided in Pittsburgh, Pennsylvania and Hampton, Virginia.[132] The majority of participants (94%) had heard of the virus, but only 74% indicated that they were aware HPV is sexually transmitted. To compare the knowledge between countries, Marlow *et al.* recruited participants from the United States, the United Kingdom, and Australia.[133] Participants were given an online survey that assessed for awareness and knowledge of HPV. The study found that collectively, 61.1% of all participants were aware of HPV. Relatively more women than men were aware of HPV and the highest awareness rates were in the United States (87.7% vs. 63.6%), followed by Australia (71.8% vs. 41.4%), and lastly in the United Kingdom (61.6% vs. 39.2%). The participants who indicated to being aware of HPV were further given a 15-item assessment for their knowledge. Similar rates of men (70.0-74.4%) and women (72.9-76.1%) knew that HPV was sexually transmitted. The majority of men (76.9-79.4%) and women (85.1-92.6%) knew that HPV could induce cervical cancer.

The knowledge of the general population regarding HPV-related OpSCC has also been assessed with participants from Madison, Illinois.[134] The study used two questionnaires to assess participants regarding their knowledge of HPV-related OpSCC and HPV risk perception. Compared to men, women were found to be more knowledgeable on HPV-related OpSCC. Race and education levels were associated with both knowledge for HPV-related OpSCC and HPV risk perception; Whites, in comparison to Blacks, and individuals with at least a college degree, compared to individuals with a high school diploma or less, scored higher on both questionnaires.

Efforts have been made to understand the impact of HPV among HPV-related OpSCC patients from participating in surveys regarding their own disease. Milbury *et al.* found from 62 patients that participated, only 66% of these patients self-declared to have an HPV-positive tumour and 35% indicated their disease was caused by HPV.[135] A minority of patients (14%) intended to keep their HPV status to themselves and 3% of patients withheld their HPV status to their partners. Furthermore, patients indicated that embarrassment and stigma were some of the reasons to keep their HPV status a secret. A qualitative study has been conducted to better understand the experiences of HPV-related OpSCC.[136] Patients reported that more emphasis was placed on the cancer itself rather than HPV when discussing with their physicians and patients perceived some discomfort regarding the topic of HPV either from the patient, their spouse, or their physician. Some patients mentioned stigma and embarrassment when diagnosed with a sexually transmitted infection and some patients expressed feelings of sadness and anger from the belief that their past behaviour led to their cancer development. Taberna *et al.* surveyed patients and their partners to assess whether the quality of life for OpSCC patients differed base on their HPV status.[137] The study found that both HPV-positive and HPV-negative patients reported a similar decline in frequency of sexual activities after the diagnosis of OpSCC. Thus, the changes in sexual behaviour may be related to the overall cancer diagnosis as opposed to HPV status. For HPV-positive patients, both patients and their partners reported positive changes in their relationships.

The prognostic factor of HPV status indicates that newly diagnosed OpSCC patients should be screened for the presence of the virus. HPV-positive patients could potentially benefit from receiving a de-intensified treatment and have reduced side effects.[138] However,

sexually transmitted infections can be associated with stigma and negative connotations such as promiscuity and embarrassment.[139] Although the majority of patients reported their relationships remained the same or had positive changes, issues of infidelity have been mentioned among the patients that felt negative changes in their relationships.[135,137] Physicians could explain to patients the purpose of HPV testing, mode of viral transmission, and/or providing patients with trusted sources for further information.[136] Health agencies could better educate the general public regarding the prevalence rate of HPV as a method to reduce the feelings of shame.[140] The initial cancer diagnosis is already a stress inducing event thus, care providers should have due diligence when inquiring further into the HPV status of patients.

I.3.8.   HPV vaccine

Prophylactic vaccines against HPV provide preventative measures for development of HPV-related cancers. Three types are currently approved for use in Canada: 1) Cervarix (HPV2), protects against HPV 16 and 18; 2) Gardasil (HPV4), protects against HPV 6, 11, 16, and 18; 3) Gardasil 9 (HPV9), protects against HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58.[48] The National Advisory Committee on Immunization of Canada has recommended HPV vaccine for females between the ages of 9 and 26 in 2007 and included males between the ages of 9 and 26 in 2012. The original immunisation schedule for the vaccines was three separate doses of 0.5 mL each. For healthy, immunocompetent individuals between 9 and 14 years of age, the recommended schedule has been modified to be two doses of 0.5 mL each for HPV2, HPV4, and HPV9 since the years of 2014, 2015, and 2016, respectively.

Therapeutic HPV vaccines are currently being tested for their efficacy. The goal for therapeutic vaccines is to target HPV E6 and E7 and to activate the cell-mediated immunity following HPV infection.[141] Results from clinical trials show reduction in tumour size of cervical cancer,[142] reduction in size of high-grade vulvar or vaginal intraepithelial lesions,[143] clearance of anogenital intraepithelial lesions among men and women,[144] and histological regressions from high-grade to low-grade cervical intraepithelial neoplasia.[145,146] The efficacy of therapeutic HPV vaccines in head and neck cancers is still being explored in ongoing clinical trials.[141] With continued investigations, therapeutic HPV vaccines may become a treatment for HPV-related cancers.

I.4. Methods to detect HPV

Detection of the virus can use numerous methods, but no standardized protocol currently exists in the clinical setting. Approaches to detecting the virus can be direct or indirect in which the factors of sensitivity and specificity, time, and cost need to be considered. Discrepancies between approaches do arise, thus each method has its own advantages and disadvantages. HPV can be detected through molecular techniques such as polymerase chain reaction (PCR) or through staining of tissue sections such as *in situ* hybridization (ISH). Types of samples that have been analyzed include tissues (fresh, frozen, or formalin-fixed, paraffin-embedded (FFPE)) and body fluids (saliva, blood).

I.4.1. Polymerase chain reaction – DNA

PCR has high sensitivity to detect low levels of HPV DNA.[147] Two types of HPV DNA PCR assays have been described: consensus and type-specific. Consensus PCR use primers that target the L1 region of HPV and requires downstream analysis for distinguishing the HPV genotypes.[148] Consensus PCR is advantageous in detecting numerous types of HPV, but a high false positive rate, 38.7% specificity, has been reported.[148] Type-specific PCR targets the unique regions of E6/E7 in HPV and genotypes can be resolved through fluorescence capillary electrophoresis (FCE).[149] However, samples screened as HPV-negative may be further tested with another method to rule out the possibility of a false negative from the exclusion of a specific genotype in the original assay. Ironically, the high sensitivity of PCR can be a limiting factor as contaminating species could also be amplified. Therefore, efforts to limit chances of cross contamination may require isolated processing of specimens which may add to the cost.[147] In addition, positive detection for HPV DNA does not indicate the virus has been integrated into the host genome as samples could be HPV DNA positive, but RNA negative.[150]

I.4.2. Polymerase chain reaction – RNA

The current gold standard for determining HPV status is the detection of HPV E6/E7 mRNA transcripts, which is commonly used as the benchmark for determining the sensitivity and specificity of new methods.[147] The premise is that integrated HPV can transcribe mRNA for the potential to be tumourigenic, but more importantly, presence of mRNA HPV transcripts correlated to a better survival among a sample of HPV-positive OpSCCs.[151] The

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method is specific and sensitive, but impractical in a clinical setting. The detection of mRNA uses quantitative PCR (qPCR) and a sufficient quality and quantity of RNA is necessary. However, RNA is notorious for its ease of degradation. Having a designated laboratory space for working with RNA is recommended, but may not always be available. Alternative methods may be used to increase the turnover rate for analysis of patient samples.

I.4.3. *In situ* hybridization – DNA

HPV DNA ISH is a tissue staining technique that uses labeled DNA probes to complementary bind to viral DNA. The probes can be fluorescently labeled, i.e., fluorescent *in situ* hybridization, or non-fluorescently labeled, i.e., chromogenic *in situ* hybridization, to omit the need for a specialized fluorescent microscope.[152] Probes can consist of a cocktail of HPV types as a wide screening panel for HPV, which can help to save time and cost, but the particular HPV genotype cannot be determined. Alternatively, HPV 16 has been detected in >90% of OpSCC samples tested through DNA ISH[121,147] which reduces the cost of probes, but samples negative for HPV 16 may need to be further analyzed for other HPV types. A trained specialist could also distinguish between episomal and integrated HPV DNA based on the staining patterns of diffuse or punctate nuclear signals, respectively.[153] The sensitivity of DNA ISH does depend on the viral load and sensitivity values of 18-94% have been reported.[154-156]

I.4.4. *In situ* hybridization – RNA

The principle of HPV RNA ISH is similar to HPV DNA ISH by using probes that are designed to complementarily bind to target sequences of HPV E6/E7 mRNA. RNAscope is an emerging, commercially available RNA ISH technique that can currently detect up to 18 types of HR-HPV.[157] Genotyping purposes does require further downstream analysis, but the technique omits the need for RNA extraction. The tissue fixation process can be rigorous to induce false positive and negative results thus, the use of control samples can minimize these outcomes. Schache *et al.* used a positive control sample of Ubiquitin C (*UBC*) to assess for RNA quality and a negative control of *dapB* to assess non-specific staining.[154] Patient samples were detected for HPV using a cocktail of seven HR-HPV probes. However, samples were only considered for scoring if *dapB* was negatively or weakly stained. After the first pass with *dapB*, the patient sample would then be scored as HPV-positive when both

UBC was positively stained and the sample shows a punctate signal pattern. Using the outlined algorithm and qPCR as reference, RNAscope was calculated to have sensitivity and specificity values of 97% and 93%, respectively. The algorithm had been subsequently validated using a cocktail of 18 HR-HPV probes and reported similar sensitivity and specificity values of 93% and 94%, respectively.[158]

I.4.5.   Immunohistochemistry

Immunohistochemistry (IHC) is a tissue staining technique that detects for proteins of interest through the use of antibodies. Advantages of IHC include its feasibility with FFPE tissues and samples can be processed at high turnover rates with autostainers. However, interpreting the percentage of positive signals require specialized training.

The detection of p16 overexpression through IHC has been used as the surrogate marker for HPV. The method was originally developed to distinguish between dysplastic and neoplastic cervical samples and complement the Papanicolaou's smear test.[159,160] A strong correlation between p16 overexpression and HPV status has been found in OpSCC analyzed through p16 IHC.[161] By using qPCR as the reference, p16 IHC was calculated to have sensitivity and specificity values to range between 94-100% and 82-93%, respectively.[154,158,162,163] A comparison between HPV DNA ISH and p16 status has found a Cohen's Kappa coefficient of 0.80.[121] Unfortunately, the scoring system has not yet reached a consensus. A summary of different studies has scored p16 IHC as positive with the criteria of strong, diffuse, nuclear, and cytoplasmic staining in 1-80% of cells.[164] The discrepancy of scoring methods cautions for careful interpretation of p16 staining results.[165]

I.4.6.   Detecting HPV antibody in blood samples

Antibodies are produced in response to HPV infections. One method to detect HPV antibodies is through the enzyme-linked immunosorbent assay.[166] A 96-well plate could be pre-coated with HPV VLPs that were generated in another host organism. By using plasma or serum samples, the HPV antibodies produced in the individual then bind to the VLPs to elicit positive signals for detection. Studies have found that approximately 60% of women had detectable levels of HPV antibodies within 12-18 months from incident infection of genital HPV 16, as determined through HPV DNA PCR.[167,168] In men, however, the detection of HPV antibodies is less prevalent. Giuliano *et al.* found that within 36 months of

HPV 16 detection in anatomical sites of either genital, anal, or oral, only 3.6% of men (n=8/223) had detectable levels of HPV antibodies.[169] While the lower prevalence rate of producing HPV antibodies in men compared to women suggest a risk factor for HPV-related OPSCC development, the results also indicate that the HPV status of patients should not be determined through detection of HPV antibodies. Rather, the detection of HPV antibodies can be used to screen the general population for HPV burden and to refine policies regarding vaccination.[170]

I.4.7. Strategy for testing HPV

Using one method for testing HPV may save cost and time, but an incorrect assessment may negatively impact treatment guidance. Combinations of methods for IHC, PCR, and/or ISH have been proposed and HPV testing could follow an algorithm.[147,171,172] The sequence of testing may delay the turnover rate for treatment planning, but necessary to acquire accurate results. An example of using the overexpression of p16, unrestricted to any particular HPV types, can act as a wide panel screening method. However, the misclassification rate of p16 to HPV status has been reported as 7-12%[121,172,173] therefore, approximately 10% of the misclassified patients would either be HPV-positive and p16-negative or vice versa. Thus, a suitable protocol for HPV testing would require substantial level of evidence and consideration to support.

I.5.   Prognostic values of cell cycle related biomarkers in HPV

Differences in survival rates of HPV-related OpSCC patients have been observed. Clinical factors such as smoking history and radiation dosage could affect the outcome, but the biological factors from the patients themselves may also be used to predict for survival. As HPV integration results in regulatory changes of cell cycle related proteins, studies have investigated whether some of the proteins could serve as prognostic biomarkers. With the relative ease of obtaining and working with FFPE tissue and the lack of requiring high-end instruments, IHC has been an attractive method for evaluating biomarkers.

I.5.1.   p16

The overexpression of p16 as a proxy for any type of HPV established the importance of this marker. However, interpretations of results need to be carefully taken due to the scoring system. For example, 14% (n=4/29) of HPV-negative tumours were considered p16-positive when using a 5% cut-off.[116] Interpretations can be further complicated since control tonsillar samples show p16-positive staining.[174] A 91% concordance rate has been reported between p16 and HPV status,[175] but one must be critically aware of the difference as p16 can be overexpressed through alternative pathways such as copy number alterations.[2]

Positive p16 staining is associated with improved clinical outcomes among OpSCC patients as analyzed by KM survival analysis. The 5-year OS rates of 60-87% vs. 21-59% for p16-positive and p16-negative patients, respectively, have been reported.[121,175-180] Higher rates of progression-free survival (PFS)[121,178] and DFS[177,180] and lower rates of recurrences[180,181] were found to be associated with p16-positive expression. Among OpSCC patients treated with CRT, p16-positive patients also had lower rates for loco-regional failure.[182]

Combined testing of p16 and HPV status is a reasonable approach compared to using a singular test.[183] Samples positive for both HPV and p16 suggest transcriptionally active HPV, whereas discordant cases suggest an alternative pathway for p16 overexpression, i.e., HPV-negative/p16-positive, or inactive virus, i.e., HPV-positive/p16-negative. However, some studies suggest that dual modality of testing may not be necessary. Lewis *et al.* reported comparable clinical outcomes of OS, DFS, and DSS between HPV-positive/p16-positive and HPV-negative/p16-positive patients and to further suggest that that p16 testing

alone is adequate to stratify the risk.[184] Similarly, Hong *et al.* reported no differences between HPV-positive/p16-negative and HPV-negative/p16-negative patients for rates of LRR, DSS, and OS.[185] On the other hand, Liu *et al.* reported higher median OS among patients tested HPV-positive/p16-negative compared to HPV-negative/p16-positive (62 vs. 47 months), but the numbers of patients per subgroup were limited.[186] The evaluation of singular and dual modality of testing may be more conclusive when using larger sets of discordant cases for HPV and p16 statuses.

I.5.2. Tumour suppressor p53

Genetic mutations of p53 can cause the protein to become inactivated and result in cancer development.[187] Compared to wild-type p53, higher risk of death is associated among OpSCC patients with p53 mutations.[188] As mentioned previously, the HPV E6 oncoprotein can promote the degradation of p53 through ubiquitination and cause the protein to become inactivated. Interestingly, HPV-positive status is more associated with absence of p53 mutation.[57,189-192] The improved clinical outcomes for HPV-positive OpSCC patients may be due to the retainment of wild-type p53 and for cells to be sensitive for RT.[193]

Consensus for low/high-levels, or alternatively negative/positive, p53 expression has not been reached. Studies have interpreted p53-positive expression as between 10-50% of stained tumour cells.[116,179,194-199] Wild-type p53 also has a short half-life.[200] The half-life can increase, i.e., accumulation of the protein, as a response to DNA damage for wild-type p53[201] or due to a mutation in the ubiquitin binding site for p53.[202] A combination of investigating the expression level of p53 through IHC and conducting DNA sequencing to determine mutant p53 may be necessary to truly define the p53 status. Interestingly, Yemelyanova *et al.* was able to associate IHC staining patterns with p53 mutation status in ovarian carcinoma.[203] However, associations between p53 IHC staining patterns and p53 mutation status were not statistically significant among HNSCC samples.[204]

Whether p53 has prognostic value in HPV-related OpSCC remains unclear. Based on individual assessment of p53 expression in OpSCC, p53 was found to either have no prognostic value[196,199] or associated with better OS at low expression levels (<50%).[194] When paired with HPV status, better rates of OS were observed among HPV-positive/wild-type p53 patients suggesting the importance of wild-type p53.[205] However, the lowest risks of

death have been observed among HPV-positive patients regardless of p53 mutational status to suggest that HPV more so than p53 can influence the outcome.[188] When survival was assessed using p16 and p53, patients with p16-positive/p53-negative status were observed to have 5-year OS and DSS rates of 96% each and were significantly higher compared to patients that were either p16-negative or p53-positive with 5-year OS and DSS rates of 48% and 63%, respectively.[179]

I.5.3.   Retinoblastoma protein

As discussed earlier, the hypophosphorylated form of pRB acts to control cell cycle progression by binding to E2F transcription factor. Upon phosphorylation by the CDK4/6-cyclin D1 complex, pRB loses its affinity for E2F to allow cell cycle to progress.[109] With respect to HPV, the viral oncoprotein of HPV E7 acts to ubiquitinate pRB for degradation and additionally, HPV E7 could also bind to E2F for uncontrollable cell cycle progression.[206] Previous IHC studies with OpSCC have interpreted 5-25% of positively stained cells as pRB-positive.[116,207-209] Low pRB expression is associated with less risk of death and higher rates of OS, but no differences in outcome have been reported when analyzed in conjunction to HPV status.[209,210] Interestingly, among HPV 16-positive OpSCC samples, p16-positive, but not p16-negative, were associated with low expression of pRB.[211] The results suggest that predictions for pRB status may require testing for both HPV and p16 status.

I.5.4.   Cyclin D1

The amplification of *CCND1* is a known event that occurs in HNSCC and resulting in cyclin D1 overexpression. Contrastingly among HPV-positive OpSCC, cyclin D1 is observed to have decreased level of expression. In OpSCC, between 5-50% of positive staining has been interpreted as cyclin D1-positive.[175,185,195,196,209,212,213] Cylin-D1 negative expression was associated with better outcomes. KM survival analysis found lower rates of LRR,[209] better rates of OS,[175,209,213] and better rates for DFS.[212,213] Compared to cyclin D1-negative, cyclin D1-positive expression is associated with relatively higher risk for LRR[209] and death.[175,209,210] In conjunction with HPV status, the highest rates of death and developing LRR were HPV-positive/cyclin D1-negative patients.[209] Cyclin D1 shows prognostic value among OpSCC, but clinical uses of the biomarker would require strict guidelines and agreement for minimum protein expression to be regarded as positive or negative expression.

I.5.5. Ki67

The expression levels of Ki67 correlate to its stage in cell cycle progression and the expression is absent during G0 phase.[214] The protein has been associated with different breast cancer subtypes[215] and lower expression levels confer better survival in non-Hodgkin's lymphoma.[216] Among HNSCCs, elevated expressional levels of Ki67 are generally associated with poorer prognoses[217] however, the biomarker has not been extensively studied with regards to HPV status and OpSCC. Based on the limited number of studies, 25-50% of stained tumour cells have been interpreted as Ki67-positive expression.[196,199,218] KM survival analysis of Ki67 by itself suggested that Ki67-positive expression is associated with poorer OS among OpSCC patients.[199] However, no increased risk for recurrences or deaths were observed with respect to Ki67 expression when analyzed by Cox proportional hazards (Cox-PH) regression.[196] In relation to HPV status, HPV-positive was more likely than HPV-negative OpSCC samples to be Ki67-positive (56% vs. 33%) and although the statistical significance was not reached (p=0.14), the best OS was observed among HPV-positive samples, regardless of Ki67 expression.[218] Interestingly, the risk of developing LRR was lowest among HPV-positive/Ki67-positive samples as analyzed by both KM survival and Cox-PH analysis, which suggests the prognostic value of Ki67.[218] More studies would need to be conducted to further establish the prognostic value and cut-offs for Ki67 expression in conjunction to HPV status in OpSCC.

II.   Questions to Address

HPV-related OpSCC have now been accepted as a clinically different entity.[219] There is a growing number of clinical trials that aim to de-intensify and/or explore alternative treatments for radiotherapy, chemotherapy, and surgery. In order for these patients to receive optimal care, numerous issues need to be addressed. One issue is regarding the epidemiology data of HPV-related OpSCC burden in British Columbia (B.C.). Although Habbous *et al.* included B.C. in their study of the HPV burden in Canada, the testing was conducted through p16 IHC staining.[8] As discussed earlier, detection of p16 serves as an indirect assessment for HPV status. Thus, using a more direct approach for determining HPV status in OpSCC patients among B.C. needs to be revisited. Another issue is that determining HPV status has not reached a consensus. In the clinical setting, p16 IHC staining is a high-throughput approach, but alternative pathways can affect the expression of p16. Molecular and histological techniques for direct HPV detection have their respective advantages and disadvantages. An algorithm for HPV detection consisting of multiple techniques is a reasonable approach and needs to be further defined. Finally, while HPV status is an independent prognostic factor, the variable alone cannot fully explain and stratify patients' outcomes. There is a need to investigate additional biomarkers and clinical factors for their prognostic values. The guiding questions that this study are:

1. What are the incidences of HPV-related oropharyngeal squamous cell carcinoma cases in British Columbia?
2. What is a clinically feasible method or algorithm for detecting HPV in oropharyngeal squamous cell carcinoma patients?
3. In addition to HPV status, do cell cycle related proteins and clinical factors provide more information for patient outcome?

III.   Hypothesis

The study Hypotheses are:

1. The number of newly diagnosed OpSCC cases is the same from 2000 to 2008 in B.C.
   H₁: The number of newly diagnosed OpSCC cases is significantly increased from 2000 to 2008 in B.C.

2. There is no association between HPV status and p16 expression.

   H₁: There is an association between HPV status and p16 expression.

3. There is no association of HPV status and the expression of p53, pRB, cyclin D1, or Ki67.
   H₁: There is an association of HPV status and the expression of p53, pRB, cyclin D1, or Ki67.

4. HPV status is not associated with demographics, clinical, pathological, treatment, or clinical outcomes.
   H₁: HPV status is associated with demographics, clinical, pathological, treatment, or clinical outcomes.

5. No clinical factors or any of the biomarkers examined is associated with patient outcomes for recurrences and survival.

   H₁: There is identifiable variable to be associated with patient outcomes for recurrences and survival.

IV. Objectives

The overall goal of this study is to identify prognostic biomarkers for OpSCC patients in the B.C. population.

1. To identify OpSCC patients who were diagnosed between 2000 and 2008 and referred to the BC Cancer Agency.

2. To identify a subset of OpSCC patients with enough FFPE tissues for detecting the HPV status and p16 expression.

3. To use immunohistochemistry to investigate the protein expression levels of p53, pRB, cyclin D1, and Ki67.

4. To acquire information of demographics, clinical, pathological, treatment, and clinical outcomes.

5. To associate clinical factors, treatment, and molecular markers with patient outcomes for recurrences and survival.

V.   Methodology

V.1.   Clinical data collection

To address Hypothesis #1, OpSCC patients diagnosed between 2000 and 2008 were retrospectively analyzed. This time interval with adequate follow-up time allows to informatively determine the clinical outcome of survival and recurrences post-treatment. Only patients that were referred to the BC Cancer Agency (BCCA) were analyzed. The advantage of including patients referred to the BCCA would ensure that most, if not all, patient charts would contain detailed and full records including pathology reports, consultation reports, and the treatment prescribed. The homogeneous collection of data can better produce accurate and representative results. Of note, the patients that received surgery only were often not referred to the BCCA and therefore, were not analyzed in this study.

V.1.1.   Inclusion criteria

The inclusion criteria included the anatomical site and diagnosis. The primary lesions were restricted only to sub-anatomical regions of the oropharynx as defined by the topography codes of the 3rd edition of the International Classification of Diseases for Oncology (ICD-O-3) in which we included C01.9, C02.4, C05.1, C05.2, C09.0, C09.1, C0.9.8, C09.9, C10.0-10.4, C10.8, and C10.9 (Appendix A).[220] In addition, the ICD-O-3 morphological codes were also used to further include patients that were pathologically diagnosed as carcinomas and squamous cell carcinomas in which we included 80103, 80203, 80213, 80523, 80703, 80713, 80723, 80733, 80743, and 80763 (Appendix B).

V.1.2.   Patient chart reviews

We identified a total of 1259 OpSCC patients that was referred to the BCCA between 2000 and 2008. Each electronic chart was reviewed to collect information for anatomical site of primary lesion, year of diagnosis of primary lesion, age at diagnosis, sex, smoking history, and the intent and the type of primary treatment received. Patients that were former or current smokers were classified as ever-smokers and patients that never smoked were classified as never-smokers. For patients that were alive, at the time of chart review and as indicated by the database, the last contact date was recorded as the date of the last follow-up appointment. Otherwise, the death date was used as the last contact date and the cause of

death was also recorded. The cause of death was also categorized as death due to disease or death due to other causes.

To address Hypotheses #2-5, cases with enough biopsy FFPE tissues were retrieved (n=254). In-depth information was collected among these patients. The information included TNM staging, AJCC staging, adjuvant treatments (if any), and recurrences. Diagnosis dates for the primary lesions were confirmed through checking pathology reports. The TNM staging was checked for accuracy through pathology reports, imaging results, and clinical assessment notes. For recurrences, information regarding local, regional, and/or distant metastasis were also collected. Event dates were recorded from, in descending order of priority, pathology reports, imaging results, or clinical assessments.

V.2.   Experimental data collection

FFPE blocks (n=254) were retrieved from various pathology departments in B. C. All hematoxylin-eosin stained slides were reviewed to confirm the pathological diagnoses and adequacy of the study material by an oral pathologist. We assembled tissue microarray (TMA) blocks using two 6-mm cores taken from 212 cases. The remaining 42 cases were analyzed as whole sections. Ten consecutive 5-µm sections were prepared for each block. All slides were deparaffinized by baking in a 60 °C oven followed by xylene immersion and rehydrated with serial ethanol and distilled water prior to experiments describe below.

V.2.1. HPV DNA *in situ* hybridization

The detection of HPV DNA using the ISH method was conducted with the GenPoint HPV Biotinylated DNA probe (Dako, USA) that consisted of a cocktail of HR-HPV types of 16/18/31/33/35/39/45/51/52/56/58/59/68. Briefly, following deparaffinization, tissue sections were submerged in sodium citrate target retrieval solution (pH 6; Dako, USA) at 97 °C, then enzymatically digested with 0.1% pepsin diluted in 0.2 N hydrochloric acid at 37 °C. The background was then blocked with 0.3% H_2O_2 diluted in absolute methanol. Following the application of the HPV DNA probe, the tissue sections were left to hybridize with the probes overnight in a 37 °C humidifying oven. The following day, slides were washed in stringency wash buffer at 48 °C to remove unspecific probe binding. Then primary streptavidin-horse radish peroxidase (HRP) was applied, followed by biotinyl tyramide, and secondary streptavidin-HRP. The signals were visualized with 3,3-diaminobenzidine (DAB) and counterstained with Carazzi's hematoxylin.

V.2.2. HPV RNA *in situ* hybridization

The detection of HPV E6/E7 mRNA for types 16/18 using the ISH method was conducted with the RNAscope 2.0 HD Detection Kit (Advanced Cell Diagnostics, USA), following the manufacture's instruction. Briefly, following deparaffinization, tissue sections were pretreated with solutions for target retrieval and protease digestion, as included in the kit. The probes were applied to the tissue sections and left to hybridize at 40 °C in the HybEZ™ Oven (Advanced Cell Diagnostics, USA) for 2 hours. Following a series of amplification steps with reagents also included in the kit, the signals were visualized with DAB and counterstained with Gill's hematoxylin I.

V.2.3. Immunohistochemistry

Following deparaffinization, tissue sections were submerged in sodium citrate antigen retrieval solution (pH 6, Dako, USA) at 95 °C for 20 minutes, then the background was blocked with 3% H_2O_2. For p16 staining, tissue sections were incubated with the E6H4 clone (mouse monoclonal primary antibody, ready-to-use; Ventana Medical Systems, Inc., USA) for 30 minutes at room temperature. For p53, pRB, cyclin D1, and Ki67 staining, tissue sections were incubated overnight at 4 °C with the DO7 clone (mouse monoclonal primary antibody, 1:25 dilution; Cell Signaling Technology Inc., USA), the 92G2 clone (rabbit monoclonal primary antibody, 1:35 dilution; Cell Signaling Technology Inc., USA), the D20B12 clone (rabbit monoclonal primary antibody, 1:300 dilution; Cell Signaling Technology Inc., USA), and the MIB-1 clone (mouse monoclonal primary antibody, 1:75 dilution; Dako, USA), respectively. Tissue sections were further incubated with streptavidin-HRP labelled, anti-mouse or anti-rabbit secondary antibody (Dako, USA), visualized with DAB, and counterstained with Carazzi's hematoxylin.

V.2.4. HPV type-specific DNA polymerase chain reaction

Type-specific HPV DNA PCR was conducted to detect for HR-HPV types 16/18/31/33/35/39/45/51/52/56/58/59/66/68 by targeting the E6/E7 regions using previously published primer sequences.[149] The results from PCR also served as a benchmark to assess the sensitivity and specificity for ISH and p16 IHC data. A total of 42 cases had enough tissues and were prepared as described below for DNA extraction using the QIAamp FFPE DNA kit (QIAGEN, Germany). The kit uses silica-based spin columns to extract DNA.

Following deparaffinization, 5-10 tissue sections from each case were stained with 0.2%, pH 6 methyl green. A fine point needle was used to manually collect the tumour area under a light microscope. Tissues were enzymatically digested with Proteinase K and ATL buffer, as supplied in the kit, by incubating in a 56 °C water bath for 72 hours. Following complete digestion of tissues, samples received 1 hour of heat treatment at 90 °C and RNase A treatment (QIAGEN, Germany) before transferring to spin columns. After a series of wash and centrifuging steps, DNA was eluted from spin columns using elution buffer supplied in the kit. The concentrations of DNA samples were quantified using the Nanodrop 2000 (ThermoFisher Scientific, USA).

As the first step to ensure the quality of DNA, two sets of primers were designed to amplify stable regions of carnitine palmitoyltransferase II (*CPT2,* 210bp) and human beta-globin (*β-globin*, 268bp). Reverse primers were fluorescently labeled with FAM or HEX fluorophores for multiplexing. Each reaction consisted of 7.5 µL of QIAGEN Multiplex PCR Kit, final concentrations of primers at 0.2 µM, 5-10 ng of DNA template, and enough volume of RNase-free water for a total volume of 15 µL. If samples passed the quality check (see scoring section, **V.2.5**), the sample will proceed with the following step for HPV typing (see an example in **Figure 2**).

PCR reactions to analyze HPV were setup for each sample and each reaction consisted of 25 µL of QIAGEN Multiplex PCR Kit (QIAGEN, Germany), a cocktail of HPV primers, 50 ng of DNA template, and enough volume of RNase-free water for a total of 50 µL. The final concentrations for primers detecting HPV types 16/39/66/68 were 0.3 µM and the remaining ten HPV types were 0.2 µM. Primer sequences of forward primers were fluorescently labeled with FAM, HEX, or NED fluorophores for multiplexing.[149]

Both sets of reactions followed the same thermocycling protocol that consisted of 29 cycles.[149] The Taq polymerase was initially activated at 95 °C for 15 minutes, then followed by a series of annealing (65 °C, 90 seconds), elongation (72 °C, 45 seconds), and denaturing (95 °C, 30 seconds) steps. The first eight annealing temperatures decreased by 1 °C for each subsequent cycle. The 21 additional cycles used the annealing temperature of 57.5 °C and the same elongation and denaturing steps as before. The last step was final elongation at 68 °C for 15 minutes.

To detect for fluorescent signals, the PCR products were analyzed by FCE through the ABI3130*xl* Genetic Analyzer (Applied Biosystems, USA). Briefly, 1 µL of cleaned up PCR product was loaded into a mixture of 8.5 µL of HiDi Formamide (Applied Biosystems, USA) and 0.5 µL of ROX400HD (Applied Biosystems, USA). Following a short heat denature step, the mixture was loaded onto the FCE that was filled with POP-7 Polymer (Applied Biosystems, USA) in its capillaries. Sample separation protocol used the default settings with adjustment of runtime of 800 seconds.
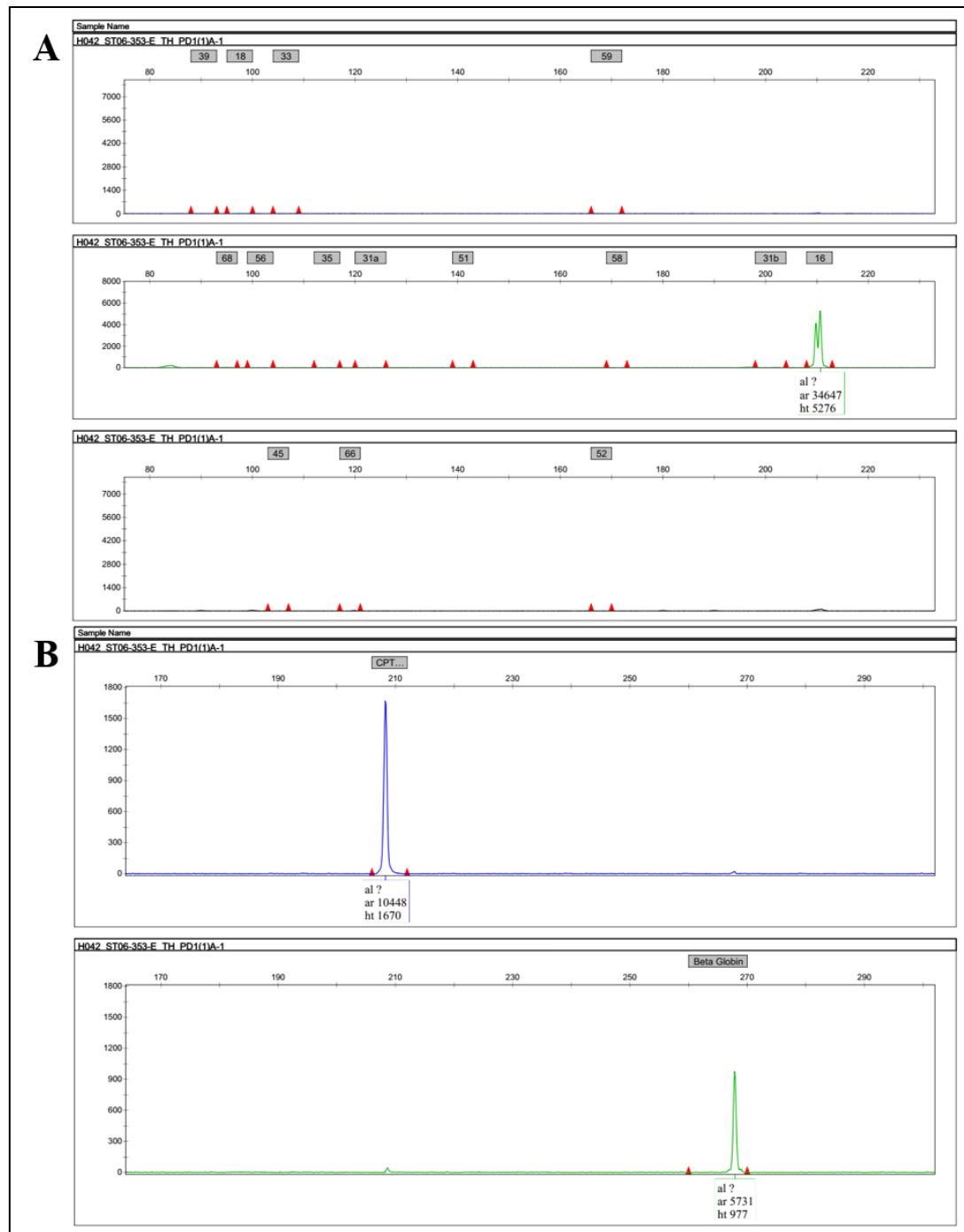
Figure 2. An example of type-specific detection for HR-HPV DNA using multiplex PCR

This sample showed positive detection for HPV 16 (green peak at the middle panel of A) and good quality of DNA template as determined by *CPT2* (blue peak at the upper panel of B) and *β-globin* (green peak at the lower panel of B).

V.2.5. Scoring

All tissue sections for HPV ISH experiments were scored at 200-400x magnification by an oral pathologist. For RNA ISH, the manufacturer's scoring guidelines were followed, but simplified such that samples were scored as positive if there was at least 1 positive signal per cell in tumour regions (**Figure 3B**). For DNA ISH, samples were scored as positive if punctate patterns were observed within nuclei of the tumour region (**Figure 3C**). For both RNA and DNA ISH, episomal cytoplasmic localization, i.e., not integrated, was considered HPV-negative (**Figure 3D**). The intensity of staining was not considered and ISH results were binarized as positive or negative for HPV. Samples were considered HPV-positive if either DNA ISH or RNA ISH or both were scored positive. Samples were considered HPV-negative only if both RNA and DNA ISH were scored as negative.

Tissue sections for IHC experiments were also scored at 200-400x magnification by an oral pathologist. The five biomarkers of p16, p53, pRB, cyclin D1, and Ki67 were all considered positive if nuclear staining was observed in ≥50% of tumour areas. Please see **Figure 4** for examples of positive and negative staining of each marker.

For HPV type-specific PCRs, raw data obtained from FCE were analyzed with the GeneMapper v4.1 software (Applied Biosystems, USA). Signal peaks with >500 relative fluorescent units (RFU) were considered positive. Results of the *β-globin* and *CPT2* signals were used to determine the quality of the DNA templates. Samples were only considered HPV-negative if at least one of the two genes met the minimum threshold signal. Samples with negative signals for both *β-globin* and *CPT2* were reanalyzed with double the initial amount of DNA template. If signals for both targets still did not meet the minimum threshold, then the sample was considered to have poor quality of DNA. If using twice the amount of DNA template resulted in at least one target meeting the minimum threshold signal, then the sample was reanalyzed for HPV with double the amount of DNA template to confirm the HPV status.
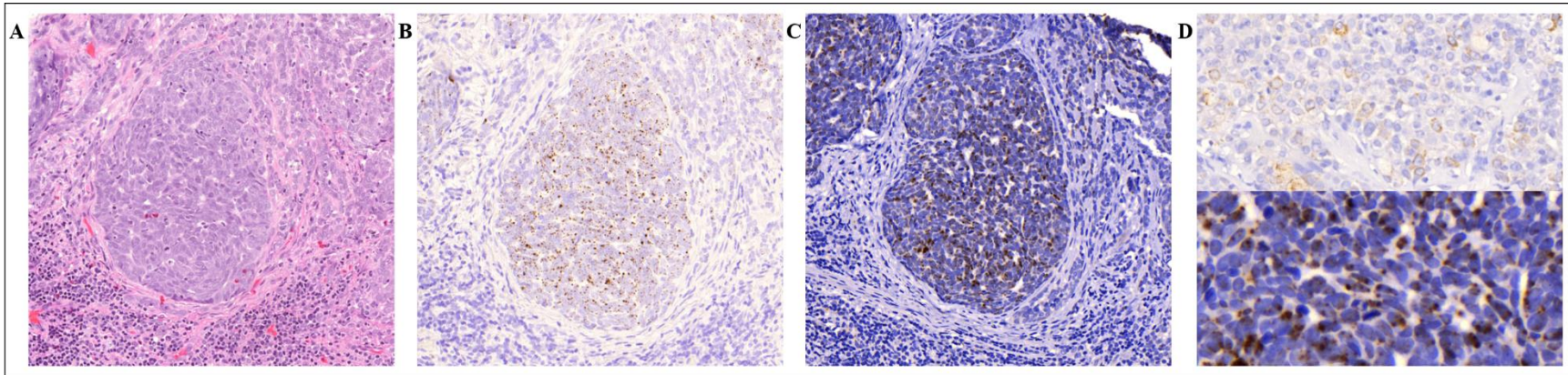
Figure 3. Examples of *in situ* hybridization for HR-HPV DNA and HPV16/18 RNA

An example of OpSCC (A, purple tumour nest under hematoxylin and eosin staining) showing positive results for RNA *in situ* hybridization for HPV 16/18 (B, nuclear brown dots) and DNA *in situ* hybridization for HPV-HR (C, nuclear brown dots). Examples of episomal localization of HPV signals (D, top, brown intracytoplasmic signals) and integrated localization of HPV signals (D, bottom, brown punctate intranuclear signals). B-D, ISH signals were visualized with 3,3-diaminobenzidine (brown color) and counterstained using Carazzi's hematoxylin (blue-purple nuclei).
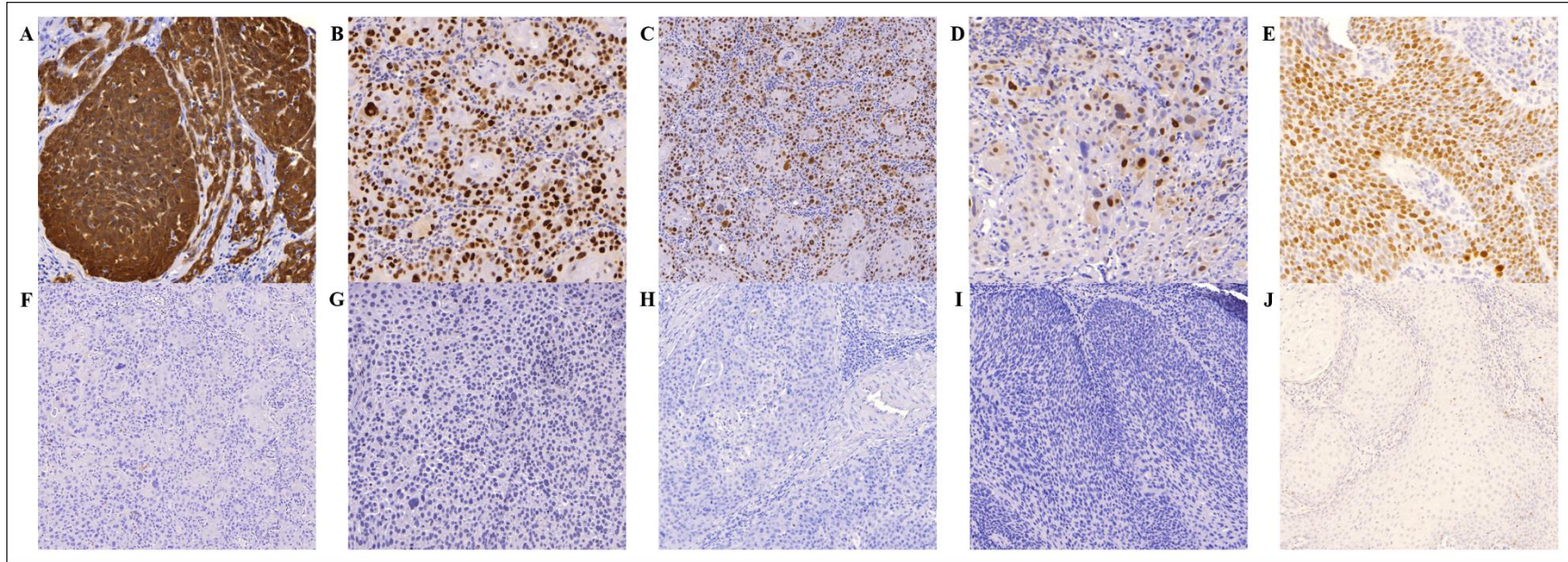
Figure 4. Examples of immunohistochemistry (IHC) stainings for p16, p53, pRB, cyclin D1, and Ki67

Formalin-Fixed, Paraffin-Embedded sections showing examples of positive (top panel) and negative (bottom panel) IHC results for p16 (A, F), p53 (B, G), pRB (C, H), cyclin D1 (D, I), and Ki67 (E, J). IHC signals were visualized with 3,3-diaminobenzidine and counterstained using Carazzi's hematoxylin (blue-purple nuclei).

V.3.   Statistical analysis

For time-to-outcome events, the date of diagnosis was used as the initial timepoint. All statistical analysis was conducted using the R software (ver. 3.3.3). Results were considered statistically significant at $p < 0.05$.

V.3.1. Representativeness of subset cohort

The subset cohort of patients of 254 OpSCC cases with FFPE tissues was compared to the remaining population of patients (n=1005) during the period for its representativeness. The variables for comparison included age at diagnosis, biological sex, anatomical site of primary lesion, smoking history, type of primary treatment received, and 5-year survival. We grouped the sub-anatomical sites that did not originate from the tonsils or base of tongue as "other oropharynx". The continuous variable of age was analyzed with Student's *t*-test and the remaining categorical variables were analyzed with Fisher's exact test.

V.3.2. Association of factors with HPV status

The sensitivity and specificity of detecting HPV status using the combined results of DNA and RNA ISH were initially determined using PCR results as a benchmark. The Study Cohort of patients was investigated for the associations with HPV status and other variables, including age at diagnosis, biological sex, anatomical site of primary lesion, smoking history, tumour stage, nodal stage, AJCC staging, the type of primary treatment received, local recurrence, regional recurrence, distant metastasis, 5-year survival, and biomarkers of p16, p53, pRB, cyclin D1, and Ki67. The continuous variable of age was analyzed with Student's *t*-test and the remaining categorical variables were analyzed with Fisher's exact test.

V.3.3. Cox proportional hazard and Kaplan-Meier survival analysis

Cox-PH analysis with Wald test was used to determine the relative risk of variables and the corresponding 95% confidence intervals (95% CI) and KM survival analysis for estimating survival rates for 5-year OS, DSS, and LRR. All clinical and biological variables were analyzed. Statistical analysis used the *survival* package from the R software.

VI. Results

VI.1. Total, Study and General Cohorts

VI.1.1. Clinical data summary for Total Cohort

We identified 1259 OpSCC patients that were referred to the BCCA between 2000 and 2008 (**Figure 5**). After the initial chart review, some patients were excluded for further analysis. The patients were excluded were mainly ones that did not receive curative treatment or no record of any treatment (n=197) or were not suitable for radiotherapy with or without concurrent chemotherapy (n=65). A few patients did not receive the primary treatment at the BCCA, but attended follow-up appointments (n=3). Unfortunately, some patients had to discontinue treatment due to health or personal related issues (n=8) or passed away during their treatment period (n=14).

A total of 972 patients were included for analysis. We compared the number of OpSCC patients to the population of British Columbia (18 years and older) to determine the trends (**Figure 6**). More females were in the B.C. population, but more males than females were diagnosed with OpSCC. Between 2000 and 2008, the incidence rates of males increased from 3.2 to 7.6 per 100,000 whereas females declined from 1.1 to 0.8 per 100,000. This demonstrated that the increase in OpSCC burden was independent from the increase in the B.C. population. Please see **Table 4** for the summary of clinical data. The majority of patients were males (77.8%), ever-smokers (79.9%), and had cancer in the tonsils (51.9%). The average age was 59.4±10.3 years. More patients received RT only (58.3%) with a median radiation dosage of 66 Gy. We observed that 63.7% of patients were alive 5-years after initial diagnosis and 22.7% patients died of disease.
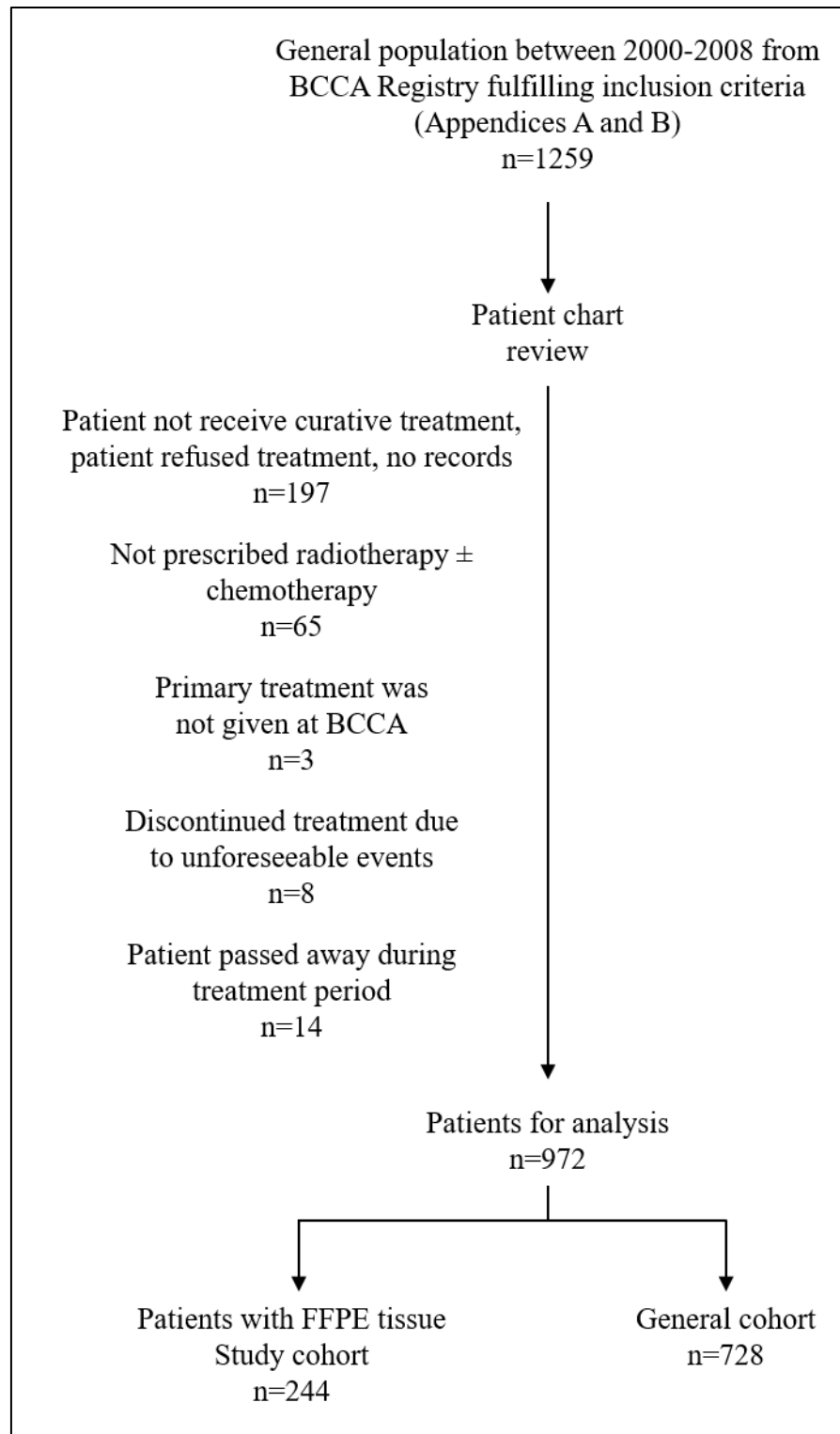
Figure 5. Breakdown of OpSCC patients diagnosed between 2000 and 2008 for analysis
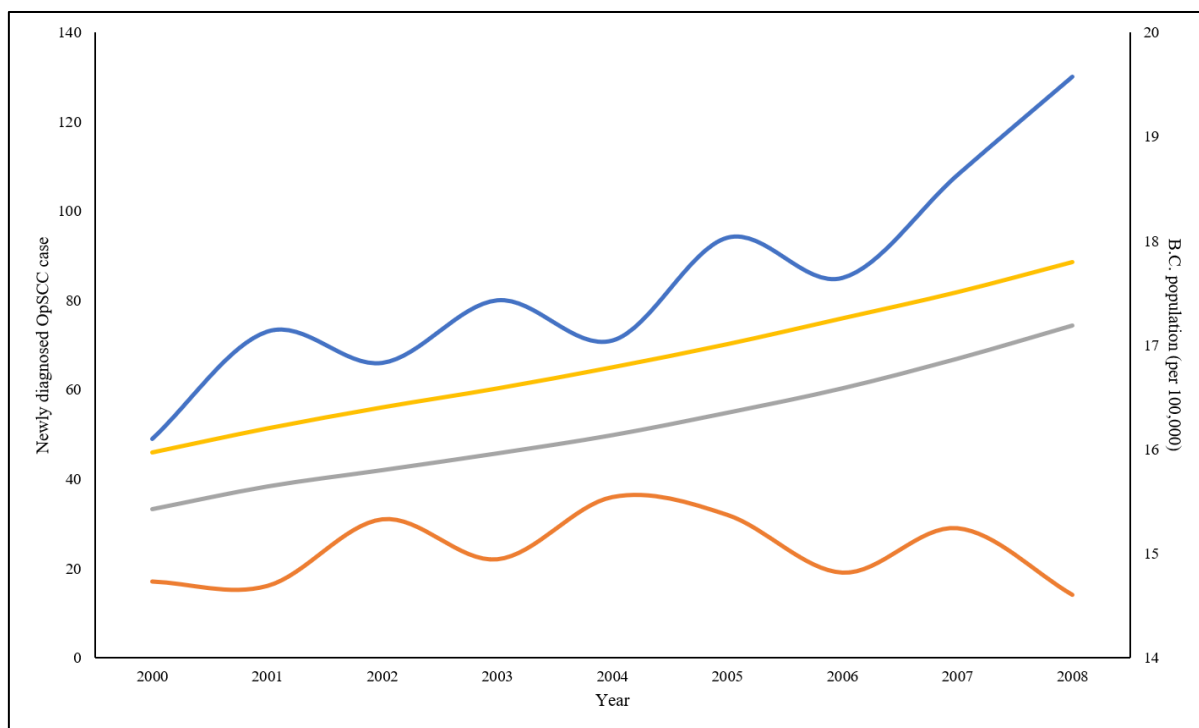
Figure 6. Newly diagnosed OpSCC cases compared to population of British Columbia (18 years and older) between 2000 and 2008

At the population level, more females (yellow line) than males (grey line) were observed. A higher number of newly diagnosed OpSCC cases occurred in males (blue line) than in females (orange line).

Table 4. Comparison of demographics and clinical factors between General and Study Cohorts

Variables, no. (%)	Total (n=972)	General Cohort (n=728)	Study Cohort (n=244)	*p*-value
Age, years (Mean±SD)	59.4±10.3	60.1±10.4	57.3±9.4	**<0.01**
Sex				0.53
Male	756 (77.8)	570 (78.3)	186 (76.2)	
Female	216 (22.2)	158 (21.7)	58 (23.8)	
Anatomical site				**<0.01**
Tonsil	504 (51.9)	314 (43.1)	190 (77.9)	
Base of tongue	360 (37.0)	312 (42.9)	48 (19.7)	
Other oropharynx[a]	103 (10.6)	98 (13.5)	5 (2.0)	
Unknown primary	5 (0.5)	4 (0.5)	1 (0.4)	
Smoking history				0.22
Never-smoker	191 (19.7)	137 (18.8)	54 (22.1)	
Ever-smoker	777 (79.9)	591 (81.2)	186 (76.2)	
Unknown	4 (0.4)	0	4 (1.6)	
Primary treatment				0.37
RT	567 (58.3)	431 (59.2)	136 (55.7)	
CRT	405 (41.7)	297 (40.8)	108 (44.3)	
5-year survival				0.06
Alive	619 (63.7)	451 (62.0)	168 (68.9)	
Died of disease	221 (22.7)	168 (23.1)	53 (21.7)	
Died of other cause	132 (13.6)	109 (15.0)	23 (9.4)	

[a]Includes: Soft palate (n=48), Oropharyngeal wall (n=32), Vallecula (n=14), Uvula (n=8), Anterior surface of epiglottis (n=1)

Abbreviations: RT: Radiotherapy; CRT: Concurrent chemoradiotherapy

VI.1.2. Comparison between General and Study Cohort

The Study cohort was selected on the basis of the availability of FFPE tissues. Among the Total cohort (n=972), 244 (25.1%) cases had FFPE samples available for study and will be referred to as the "Study Cohort". The remaining patients (n=728) will be referred to as the "General Cohort". Summary of the General and Study Cohorts were compared to determine the representativeness of the Study Cohort (**Table 4**). Among the variables that were not statistically different in both cohorts, we observed more males, majority of patients were ever-smokers, more patients received RT only, and majority of patients were alive after 5-years from date of diagnosis. For the patients that received CRT, cisplatin was the most commonly administered agent in both the General Cohort (66.9%, n=200) and the Study Cohort (78.7%, n=85). We found that average age was statistically different, in which the Study Cohort was relatively younger (p<0.01). Interestingly, while approximately equal number of patients in the General Cohort had tonsillar and base of tongue cancers, the majority of our Study Cohort was diagnosed with tonsillar cancer.

VI.1.3. The Study Cohort

After conducting further in-depth chart reviews, the Study Cohort is summarized in **Table 5**. Adding to the previous results, 18.4% of patients (n=45) received tonsillectomies for diagnoses of their cancer. We observed that majority of patients were staged at T2 or higher (74.2%) and N2 or higher (64.8%). In accordance to the AJCC (7th edition) criteria, majority of patients were staged at III/IV (88.9%). As mentioned earlier, among the patients that received CRT, cisplatin was the most commonly administered agent and the remaining patients received carboplatin with 5-FU infusion (21.3%, n=23). On the basis of suspected residual disease or recurrences, 21.7% of patients (n=53) received adjuvant surgery with a median time of 0.61 years after the initial treatment of RT or CRT. Further review of surgical pathology reports and dictations from clinicians, 23 patients (43.4%) were negative for malignancy from adjuvant surgery. Given the relatively small percentages of patients that received adjuvant surgery, the variable of treatment was analyzed only based on primary treatment using RT or CRT.

Based on pathology reports, imaging results, or clinical assessments, we found that the majority of patients did not have any recurrences 5-years after the date of diagnoses. We observed that less than 10% of patients developed distant metastasis thus, the outcome was not further analyzed. By combining local and regional recurrences into a single category of loco-regional recurrences (LRR), we found that approximately one-fifth of the Study Cohort developed LRR. We observed that 47 patients developed second primary lesions, in which the majority of cases were lung cancer (34.0%, n=16), head and neck cancer (17.0%, n=8), and skin cancer (10.6%, n=5). Among the 21 patients that developed distant metastasis, the most common metastatic site were the lungs (57.1%, n=12).

Table 5. Demographics, clinical characteristics, outcomes and HPV status of the Study Cohort

Variables, n (%)	Total (n=244)	HPV-positive (n=190)	HPV-negative (n=54)	*p*-value
Age, years (Mean±SD)	57.3±9.4	55.8±8.9	62.3±9.1	**<0.01**
Sex				**<0.01**
Male	186 (76.2)	155 (81.6)	31 (57.4)	
Female	58 (23.8)	35 (18.4)	23 (42.6)	
Anatomical site				**<0.01**
Tonsil	190 (77.9)	155 (81.6)	35 (64.8)	
Base of tongue	48 (19.7)	33 (17.4)	15 (27.8)	
Other oropharynx[a]	5 (2.0)	1 (0.5)	4 (7.4)	
Unknown primary	1 (0.4)	1 (0.5)	0	
Smoking history				**<0.01**
Never-smoker	54 (22.1)	50 (26.3)	4 (7.4)	
Ever-smoker	186 (76.2)	137 (72.1)	49 (90.7)	
Unknown smoking	4 (1.6)	3 (1.6)	1 (1.9)	
Tumour stage				**0.02**
TX	2 (0.8)	2 (1.1)	0	
T0/1	61 (25.0)	51 (26.8)	10 (18.5)	
T2	102 (41.8)	85 (44.7)	17 (31.5)	
T3	56 (23.0)	37 (19.5)	19 (35.2)	
T4	23 (9.4)	15 (7.9)	8 (14.8)	
Nodal stage				0.05
N0	41 (16.8)	26 (13.7)	15 (27.8)	
N1	45 (18.4)	34 (17.9)	11 (20.4)	
N2	135 (55.3)	109 (57.4)	26 (48.1)	
N3	23 (9.4)	21 (11.1)	2 (3.7)	
AJCC staging				0.63
I/II	27 (11.1)	20 (10.5)	7 (13.0)	
III/IV	217 (88.9)	170 (89.5)	47 (87.0)	
Primary treatment				0.16
RT	136 (55.7)	101 (53.2)	35 (64.8)	
CRT	108 (44.3)	89 (46.8)	19 (35.2)	
Overall treatment				0.50
RT	99 (40.6)	74 (38.9)	25 (46.3)	
RT+Sx	37 (15.0)	27 (14.2)	10 (18.5)	
CRT	92 (37.4)	76 (40.0)	16 (29.6)	
CRT+Sx	16 (6.5)	13 (6.8)	3 (5.6)	

Variables, n (%)	Total (n=244)	HPV-positive (n=190)	HPV-negative (n=54)	p-value
Recurrence				0.05
None	180 (73.8)	146 (76.8)	34 (63.0)	
LR only	23 (9.4)	13 (6.8)	10 (18.5)	
RR only	10 (4.1)	8 (4.2)	2 (3.7)	
DM only	12 (4.9)	12 (6.3)	0	
LR+RR	10 (4.1)	6 (3.2)	4 (7.4)	
LR+DM	3 (1.2)	2 (1.1)	1 (1.9)	
RR+DM	6 (2.5)	3 (1.6)	3 (5.6)	
LRR				**<0.01**
No	192 (78.7)	158 (83.2)	34 (63.0)	
Yes	52 (21.3)	32 (16.8)	20 (37.0)	
5-year survival				**<0.01**
Alive	168 (68.9)	146 (76.8)	22 (40.7)	
Died of disease	53 (21.7)	36 (18.9)	17 (31.5)	
Died of other cause	23 (9.4)	8 (4.2)	15 (27.8)	

[a]Includes: Soft palate (n=2), Oropharyngeal wall (n=2), Uvula (n=1)

Abbreviations: HPV, Human papillomavirus; AJCC, American Joint Committee on Cancer (7th ed.); RT: Radiotherapy; CRT: Concurrent chemoradiotherapy; Sx: Adjuvant surgery; LR, Local recurrence; RR, Regional recurrence; DM, Distant metastasis; LRR: Loco-regional recurrence

VI.2. Experimental data summary

VI.2.1. Detection of HPV Using DNA/RNA ISH

All 244 cases were analyzed for HPV DNA and RNA using ISH. Using DNA and RNA ISH, we observed HPV-positive for 71.3% and 75.4% of samples, respectively, and the agreement between DNA and RNA ISH results were 91.0% (**Table 6**). When combining both DNA and RNA ISH results, we observed that 77.9% (n=190) of our samples were HPV-positive.

Table 6. Comparison between DNA and RNA in situ hybridization for detecting HPV

	HR-HPV DNA ISH		Agreement
	Positive	Negative	
HPV 16/18 RNA ISH			
Positive	168	16	91.0%
Negative	6	54	

Abbreviations: HR-HPV: High-risk human papillomavirus; ISH: *In situ* hybridization

VI.2.2. Detection of HPV Using DNA PCR

      A total of 42 cases had enough FFPE tissues for HR-HPV detection using PCR. **Figure 2** showed examples of negative and positive results as well as the control reactions to ensure the DNA quality and prevent false negative due to poor or lower amount of amplifiable DNA. We did not observe any unexpected fluorescence signals to suggest that there were any cross-interactions between primers. The PCR reactions for two cases required double the amount of DNA from initial setup due to poor DNA quality. Only one of the two cases met the minimum RFU threshold for quality assessment thus, the total number of cases for PCR analysis was 41.

      We observed 82.9% (n=34) of our samples were positive for HPV. Among the HPV types analyzed, HPV 16 was the most common (97.1%), one sample was positive for HPV 33. None of our samples had coinfection of multiple HPV types.

      When using the 41 cases analyzed by DNA PCR as benchmark, the specificity and sensitivity of DNA ISH were both 100% but the HPV16/18 for RNA ISH were 100.0% and 91.7%, respectively. The latter is because HPV16/18 RNA-ISH failed to detect HPV 33 infection (**Table 7**).

Table 7. Comparison of *in situ* hybridization to polymerase chain reaction for detection of HPV

	HR-HPV DNA PCR		Agreement	Sensitivity	Specificity
	Positive	Negative			
HR-HPV DNA ISH					
Positive	34	0	100.0%	100.0%	100.0%
Negative	0	7			
HPV 16/18 RNA ISH					
Positive	33	0	97.6%	91.7%	100.0%
Negative	1[a]	7			
DNA/RNA ISH					
Positive	34	0	100.0%	100.0%	100.0%
Negative	0	7			

[a]HPV type 33 from HPV-PCR testing.
Abbreviations: HR-HPV: High-risk human papillomavirus; ISH: *In situ* hybridization; PCR: Polymerase chain reaction

VI.2.3. Detection of HPV – p16 IHC

All 244 cases were stained for p16 using IHC (**Table 9**). We observed 77.5% (n=189) of samples positive for p16 IHC. Using DNA PCR as benchmark (n=41), the sensitivity and specificity for p16 IHC were 91.2% and 71.4%, respectively (**Table 8**). When using combined DNA/RNA ISH as benchmark (n=244), the sensitivity and specificity for p16 IHC were 91.0% and 67.3%, respectively.

Table 8. Comparison of p16 IHC to PCR and combined DNA/RNA ISH for detecting HPV

	p16 IHC		Agreement	Sensitivity	Specificity
	Positive	Negative			
HR-HPV DNA PCR					
Positive	31	3	87.8%	91.2%	71.4%
Negative	2	5			
DNA/RNA ISH					
Positive	172	18	85.7%	91.0%	67.3%
Negative	17	37			

Abbreviations: IHC: Immunohistochemistry; HR-HPV: High-risk human papillomavirus; ISH: *In situ* hybridization; PCR: Polymerase chain reaction

VI.2.4. Summary of p53, pRB, cyclin D1, Ki67 IHC results

Table 9 summarizes the IHC results of the four biomarkers. Some TMA cores fell off during the experimental process or contained no tumour cells. Number of cases were available for analysis for p53, pRB, cyclin D1, and Ki67 were 234, 238, 240, and 239, respectively. Using the 50% cut-off for all four biomarkers, we found 29.1%, 33.6%, 25.4% and 69.9% positivity of p53, pRB, cyclin D1, and Ki67, respectively.

Table 9. Summary of biomarkers as detected using immunohistochemistry

Markers, no. (%)[a]	Total (n=244)	HPV-positive (n=190)	HPV-negative (n=54)	*p*-value
p16 status				**<0.01**
Negative	55 (22.5)	18 (9.5)	37 (68.5)	
Positive	189 (77.5)	172 (90.5)	17 (31.5)	
p53 status				**0.02**
Negative	166 (70.9)	140 (74.9)	26 (55.3)	
Positive	68 (29.1)	47 (25.1)	21 (44.7)	
pRB status				0.74
Negative	158 (66.4)	123 (65.8)	35 (68.6)	
Positive	80 (33.6)	64 (34.2)	16 (31.4)	
Cyclin D1 status				**<0.01**
Negative	179 (74.6)	162 (85.7)	17 (33.3)	
Positive	61 (25.4)	27 (14.3)	34 (66.7)	
Ki67 status				**<0.01**
Negative	72 (30.1)	48 (25.3)	24 (49.0)	
Positive	167 (69.9)	142 (74.7)	25 (51.0)	

[a]Missing: p53, n=10; pRB, n=6; Cyclin D1, n=4; Ki67, n=5
Abbreviations: HPV: Human papillomavirus; pRB: Retinoblastoma protein

VI.3.   Analysis of Study Cohort

VI.3.1. Associations of clinical factors with HPV status

Table 5 summarizes HPV status, as defined by combined DNA/RNA ISH, and clinic-pathological variables. We observed that compared to HPV-negative patients, HPV-positive patients tend to be younger ($p<0.01$), more likely to be males ($p<0.01$), more likely to be never-smokers ($p<0.01$), relatively more likely to have tonsils as the primary site ($p<0.01$), and less likely for tumour stage to be T2 or higher ($p=0.02$). When analyzing for associations between HPV status and the development of any recurrences, i.e., LR, RR, or DM, statistical significance was not reached ($p=0.05$). However, statistical significance ($p<0.01$) was found upon classifying patients based on LRR in which lower percentages of HPV-positive patients developed LRR. HPV-positive patients were also found to have had a higher 5-year OS ($p<0.01$). Among the patients who died, relatively less HPV-positive patients had died of disease, compared to HPV-negative (18.9% vs. 31.5%). Based on HPV status, we did not observe statistical differences for AJCC staging, primary treatment received, and nodal staging.

VI.3.2. Downstream biological effects of HPV status

Table 9 summarized the IHC results and HPV status. We observed that compared to HPV-negative samples, HPV-positive patients were more likely to be p53-negative, pRB-negative, cyclin D1-negative, and Ki67-positive. However, only p53, cyclin D1, and Ki67 were statistically significant with respect to HPV status.

It is known that HPV oncoproteins E6 and E7 may interact with tumour suppressors and their downstream proteins (**Figure 1**). We considered the negative results of p53 and pRB to be the consequence of degradation by HPV E6 and E7, respectively. Although HPV status was not associated with pRB status, we were interested to determine whether the samples in our Study Cohort followed the pRB/p16/cyclin D1 pathway. By considering the samples with complete results for pRB, p16, and cyclin D1 (n=237), we found that the majority of cases (45.1%, n=107/237) had the characteristics of HPV-positive, pRB-negative, p16-positive, and cyclin D1-negative (**Table 10**).

Table 10. Relation of pRB/p16/cyclin D1 pathway to HPV status

HPV		pRB[a]		p16		Cyclin D1[a]	
Status	No. (%)	Status	No. (%)	Status	No. (%)	Status	No. (%)
Neg	50 (21.1)	Neg	34 (14.3)	Neg	19 (8.0)	Neg	7 (3.0)
						Pos	12 (5.1)
				Pos	15 (6.3)	Neg	8 (3.4)
						Pos	7 (3.0)
		Pos	16 (6.8)	Neg	15 (6.3)	Neg	1 (0.4)
						Pos	14 (5.9)
				Pos	1 (0.4)	Neg	0
						Pos	1 (0.4)
Pos	**187 (78.9)**	**Neg**	**123 (51.9)**	Neg	10 (4.2)	Neg	7 (3.0)
						Pos	3 (1.3)
				Pos	**113 (47.7)**	**Neg**	**107 (45.1)**
						Pos	6 (2.5)
		Pos	64 (27.0)	Neg	8 (3.4)	Neg	2 (0.8)
						Pos	6 (2.5)
				Pos	56 (23.6)	Neg	44 (18.6)
						Pos	12 (5.1)

[a]Missing data: pRB, n=6; Cyclin D1, n=1
Abbreviations: HPV: Human papillomavirus; pRB: Retinoblastoma protein; Pos: Positive; Neg: Negative

VI.3.3. Overall outcome analysis

Univariate and multivariate analysis using Cox-PH showed that smoking history, tumour staging, and treatment received were independently associated with OS, DSS, and LRR (Appendices C, F, I). Significantly worse outcomes for OS, DSS, and LRR were observed among patients who were grouped as ever-smokers, with tumours staged T3/4, and received RT only. KM survival analysis of the three clinical factors were also observed to be associated with worse time to outcomes (OS, DSS, and LRR) for patients that were ever-smokers, staged at tumour T3/4, and receiving RT only (Appendices D, G, J).

Among biological factors, univariate analysis found that HPV-positive, p16-positive, and cyclin D1-negative statuses were associated with relatively lower risks of deaths and recurrences for all three outcomes (Appendices C, F, I). However, no associations were found among any biological factors using multivariate analysis. KM survival analysis showed significantly better time to outcomes (OS, DSS, and LRR) for those HPV positive, p16 overexpressed, and cyclin D1 negative groups (Appendices E, H, K).

We established that independent clinical and biological factors have prognostic significance in our Study Cohort. To better address the guiding question of whether clinical and biological factors in addition to HPV status can further contribute to prognoses, we divided the Study Cohort based on HPV status and then reanalyzed the clinical and biological factors to outcomes.

VI.3.4. Outcome analysis of HPV-positive patients

Univariate and multivariate analysis showed that smoking status was an independent factor for DSS and LRR. Compared to never-smokers, the patients classified as ever-smokers were at higher risk for disease-specific deaths (HR=4.7; 95% CI, 1.4-15.7; p=0.01) and developing LRR (HR=4.1; 95% CI, 1.3-13.6; p=0.02) (**Table 11**). Univariate analysis for 5-year OS also showed smoking status to be the only significant factor (HR=3.9; 95% CI, 1.4-11.0; p<0.01). Poorer prognoses of ever-smokers were also observed from KM survival analysis for rates of 5-year OS (70.3% vs. 91.7%; p<0.01), DSS (75.6% vs. 93.7%; p<0.01), and not developing LRR (78.7% vs. 93.6%; p=0.02) (**Figure 7A, C, E**).

Tumour staging was an independent factor for both univariate and multivariate analysis when assessed for DSS. Patients with tumours staged at T3/4 were associated with relatively higher risk of disease-specific deaths (HR=3.2; 95% CI, 1.6-6.4; p<0.01). Advanced tumour stage trended toward an increased risk for 5-year overall death and development of LRR, but the results were not statistically significant (**Table 11**). Similarly, KM analysis showed worse outcomes among patients with tumours staged at T3/4, but only DSS was statistically significant (68.1% vs. 84.6%; p=0.01; **Figure 8C**).

Both univariate and multivariate analysis indicated that the primary treatment received was statistically significant for DSS and LRR. Compared to patients that received RT only, CRT was associated with lower risk of death for DSS (HR=0.4; 95% CI, 0.2-0.8; p=0.01) and lower risk of developing LRR (HR=0.4; 95% CI, 0.2-0.8; p=0.02). KM survival analysis also estimated higher rates of DSS (86.5% vs. 74.8%; p=0.04; **Figure 9C**) and higher rates of not developing LRR (89.0% vs. 76.7%; p=0.02; **Figure 9E**). No statistical significance was observed from 5-year OS in both Cox-PH and KM survival analysis.

Our Study Cohort indicated that p53 status was associated with DSS for univariate analysis. Compared to p53-negative group, p53-positive group had 2-time higher risk (HR=2.0; 95% CI, 1.0-4.0; p=0.04) for the disease specific death. Using KM analysis, it also showed significantly less favourable DSS rates (68.6% vs. 83.7%; p=0.03; **Figure 10C**). Patients with p53-positive status trended toward poorer 5-year OS, but the result was not statistically significant (**Figure 10A**). On the other hand, no separation was observed based on p53 status for the development of LRR (**Figure 10E**).

Both Cox-PH analysis (**Table 11**) and KM survival analysis did not find any associations with clinical outcomes for the other biomarkers of p16 (Appendix T), pRB (Appendix U), Ki67 (Appendix V), and cyclin D1 (**Figure 11**). Please refer to Appendices M and O-S for the remaining variables not discussed.

VI.3.5. Outcome analysis of HPV-negative patients

A total of 54 patients were HPV-negative. Similar analysis was conducted as those in HPV-positive group. To avoid over interpreting the results, the subgroups with less than 10 patients were not included in the analysis, i.e., patients with primary tumours as other oropharynx (n=4), classified as never-smokers (n=4), and with nodal staging at N3 (n=2). Only cyclin-D1 positive patients showed significantly worse rate of OS (HR=2.9; 95% CI, 1.2-7.3; p=0.02) (**Table 12**). KM survival analysis showed ~40% reduction in 5-year OS for cyclin D1-positive patients compared to cyclin D1-negative (47.7% vs. 76.5%, p=0.02; **Figure 11**). A trend of poorer DSS and LRR were observed for cyclin D1-positive status, but the results were not statistically significant. Please refer to Appendices N-S and U for the analyses of other factors.

Table 11. Cox-PH analysis of clinical outcomes among HPV-positive patients

Variables[a,b]	5-year OS HR (95% CI)	p-value	DSS HR (95% CI)	p-value	LRR HR (95% CI)	p-value
Univariate analysis						
Smoking history						
Never-smoker	1.0		1.0		1.0	
Ever-smoker	3.9 (1.4-11.0)	**<0.01**	4.3 (1.3-14.0)	**0.02**	3.8 (1.2-12.6)	**0.03**
Tumour stage						
T0/1/2	1.0		1.0		1.0	
T3/4	1.8 (1.0-3.3)	0.06	2.3 (1.2-4.4)	**0.01**	1.8 (0.9-3.6)	0.11
Primary treatment						
RT	1.0		1.0		1.0	
CRT	0.6 (0.3-1.0)	0.07	0.5 (0.2-1.0)	**0.04**	0.4 (0.2-0.9)	**0.02**
p16 status						
Negative	1.0		1.0		1.0	
Positive	0.6 (0.3-1.5)	0.27	0.5 (0.2-1.2)	0.11	0.7 (0.2-1.9)	0.44
p53 status						
Negative	1.0		1.0		1.0	
Positive	1.5 (0.8-2.8)	0.21	2.0 (1.0-4.0)	**0.04**	1.1 (0.5-2.5)	0.77
pRB status						
Negative	1.0		1.0		1.0	
Positive	1.1 (0.6-2.1)	0.72	1.2 (0.6-2.5)	0.54	1.2 (0.6-2.6)	0.61
Cyclin D1 status						
Negative	1.0		1.0		1.0	
Positive	1.1 (0.5-2.6)	0.81	1.4 (0.6-3.5)	0.42	1.6 (0.7-4.0)	0.28
Ki67 status						
Negative	1.0		1.0		1.0	
Positive	0.9 (0.5-1.7)	0.73	0.9 (0.4-1.8)	0.71	0.6 (0.3-1.3)	0.24

Variables[a,b]	5-year OS HR (95% CI)	p-value	DSS HR (95% CI)	p-value	LRR HR (95% CI)	p-value
Multivariate analysis						
Smoking history						
Never-smoker			1.0		1.0	
Ever-smoker			4.7 (1.4-15.7)	**0.01**	4.1 (1.3-13.6)	**0.02**
Tumour stage						
T0/1/2			1.0			
T3/4			3.2 (1.6-6.4)	**<0.01**		
Primary treatment						
RT			1.0		1.0	
CRT			0.4 (0.2-0.8)	**0.01**	0.4 (0.2-0.8)	**0.02**
p53 status						
Negative			1.0			
Positive			2.0 (1.0-4.0)	0.05		

[a]Missing data: Smoking history, n=3; Tumour stage, n=2; p53, n=3; Cyclin D1, n=1

[b]Please see Appendix M for remaining variables

Abbreviations: OS: Overall survival; DSS: Disease-specific survival; LRR: Loco-regional recurrence; HR: Hazard ratio; RT: Radiotherapy; CRT: Concurrent chemoradiotherapy; pRB: Retinoblastoma protein

Table 12. Cox-PH analysis of clinical outcomes among HPV-negative patients

Variables[a,b,c]	5-year OS HR (95% CI)	p-value	DSS HR (95% CI)	p-value	LRR HR (95% CI)	p-value
Univariate analysis						
Tumour stage						
T0/1/2	1.0		1.0		1.0	
T3/4	1.8 (0.9-3.7)	0.09	1.8 (0.7-4.6)	0.26	1.4 (0.6-3.4)	0.43
Primary treatment						
RT	1.0		1.0		1.0	
CRT	0.5 (0.2-1.1)	0.08	0.6 (0.2-1.8)	0.40	0.5 (0.2-1.4)	0.20
p16 status						
Negative	1.0		1.0		1.0	
Positive	0.7 (0.3-1.5)	0.35	1.0 (0.4-2.6)	0.96	0.6 (0.2-1.7)	0.36
p53 status						
Negative	1.0		1.0		1.0	
Positive	0.8 (0.4-1.6)	0.50	0.8 (0.3-2.4)	0.75	0.5 (0.2-1.4)	0.18
pRB status						
Negative	1.0		1.0		1.0	
Positive	1.8 (0.8-3.8)	0.13	1.9 (0.7-5.4)	0.23	2.3 (0.9-5.7)	0.08
Cyclin D1 status						
Negative	1.0		1.0		1.0	
Positive	2.9 (1.2-7.3)	**0.02**	2.0 (0.6-6.3)	0.21	2.4 (0.8-7.3)	0.12
Ki67 status						
Negative	1.0		1.0		1.0	
Positive	1.0 (0.5-2.0)	0.90	0.8 (0.3-2.3)	0.75	0.9 (0.4-2.2)	0.83

[a]Missing data: Tobacco use, n=1; p53, n=7; pRB, n=3; Cyclin D1, n=3; Ki67, n=5

[b]Not analyzed: Smoking history

[c]Please see Appendix N for the remaining variables

Abbreviations: OS: Overall survival; DSS: Disease-specific survival; LRR: Loco-regional recurrence; HR: Hazard ratio; RT: Radiotherapy; CRT: Concurrent chemoradiotherapy; pRB: Retinoblastoma protein
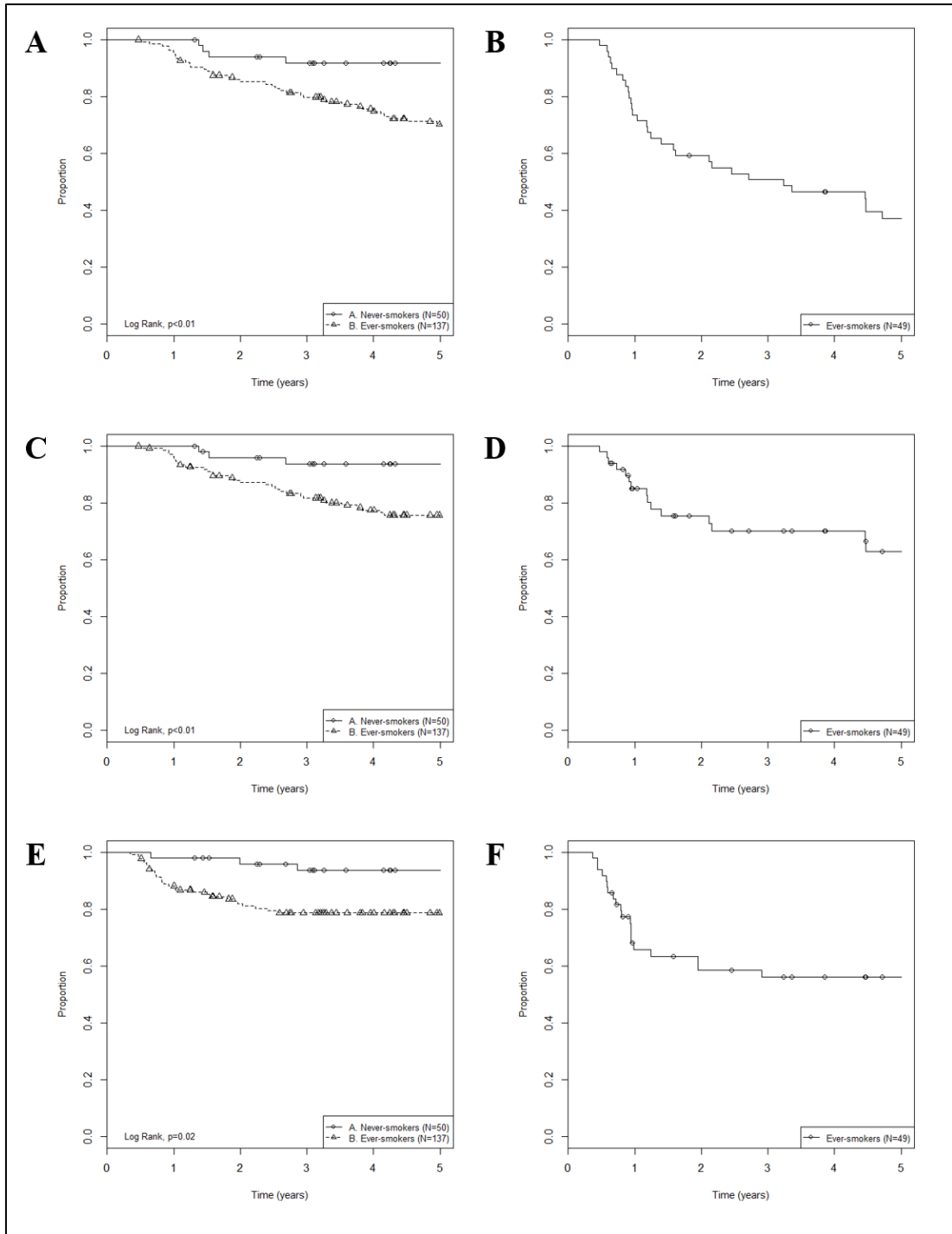
Figure 7. KM survival analysis of smoking history

Patients were separated into HPV-positive (left) and HPV-negative (right) status. Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F). Please refer to Appendices W and X for survival rates.

Figure 8. KM survival analysis of tumour staging

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status.

Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F).

Please refer to Appendices W and X for survival rates.

Figure 9. KM survival analysis of treatment received

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status.

Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F).

Please refer to Appendices W and X for survival rates.

Figure 10. KM survival analysis of p53 status

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status.
Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F).
Please refer to Appendices W and X for survival rates.
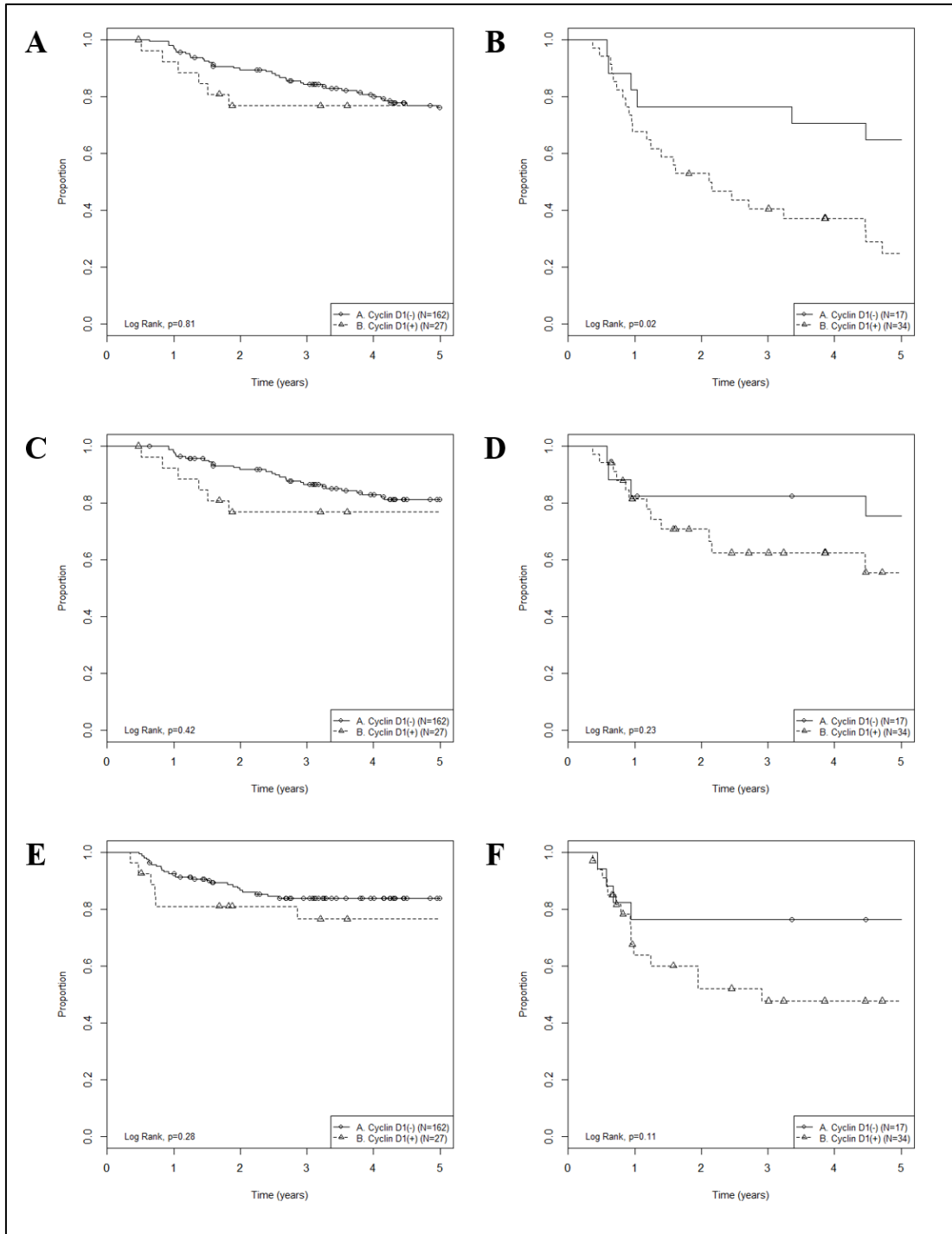
Figure 11. KM survival analysis of cyclin D1 status

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status.

Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F).

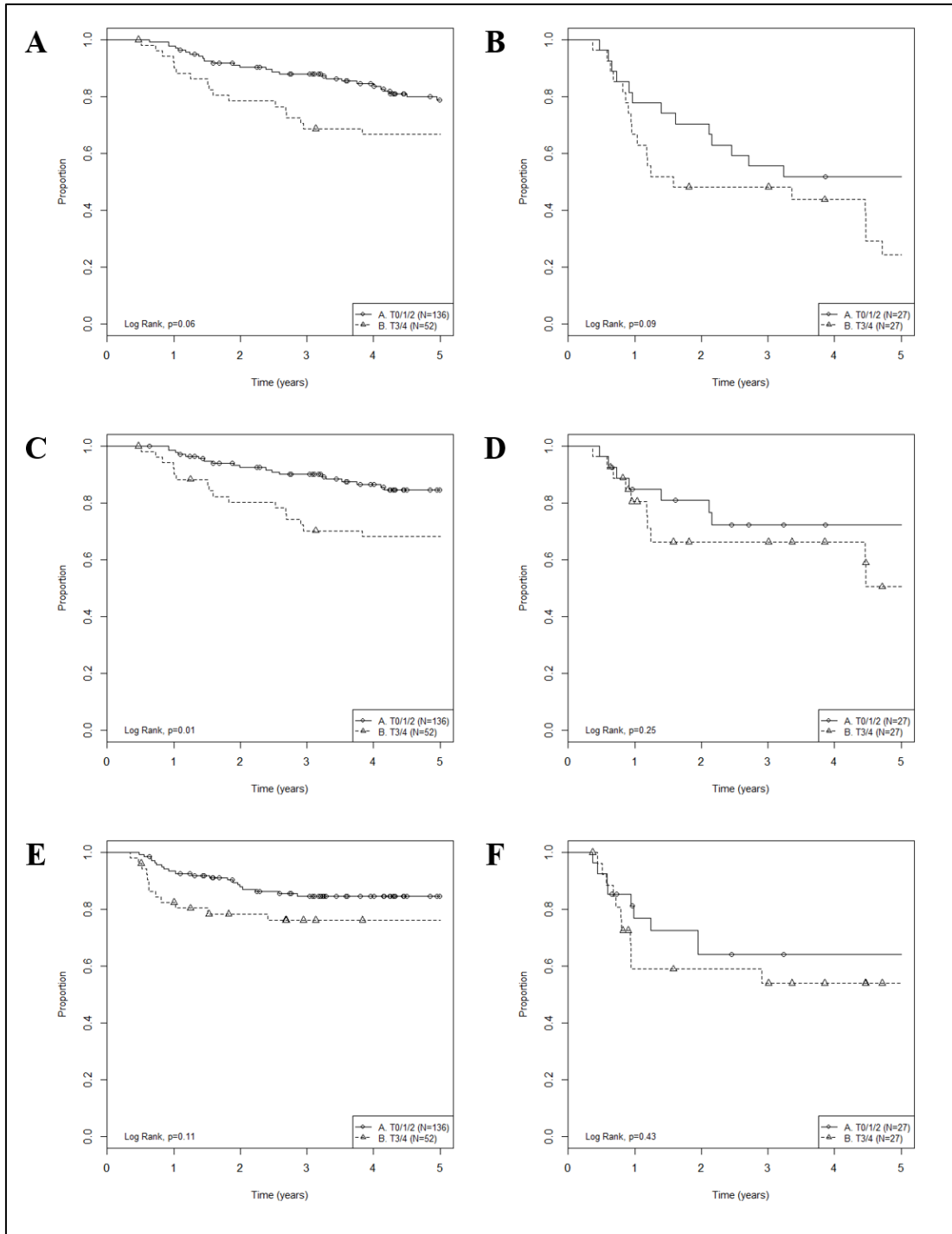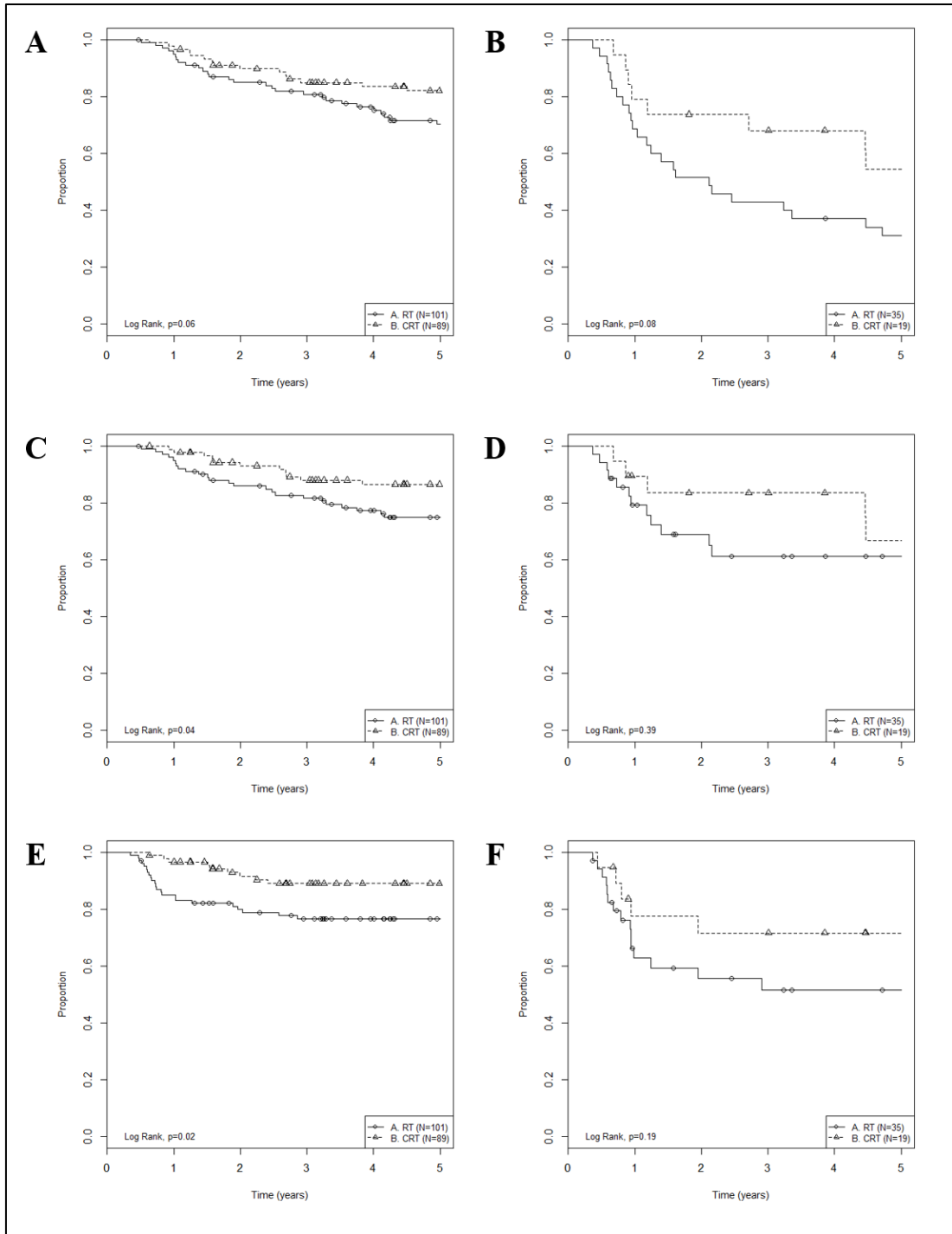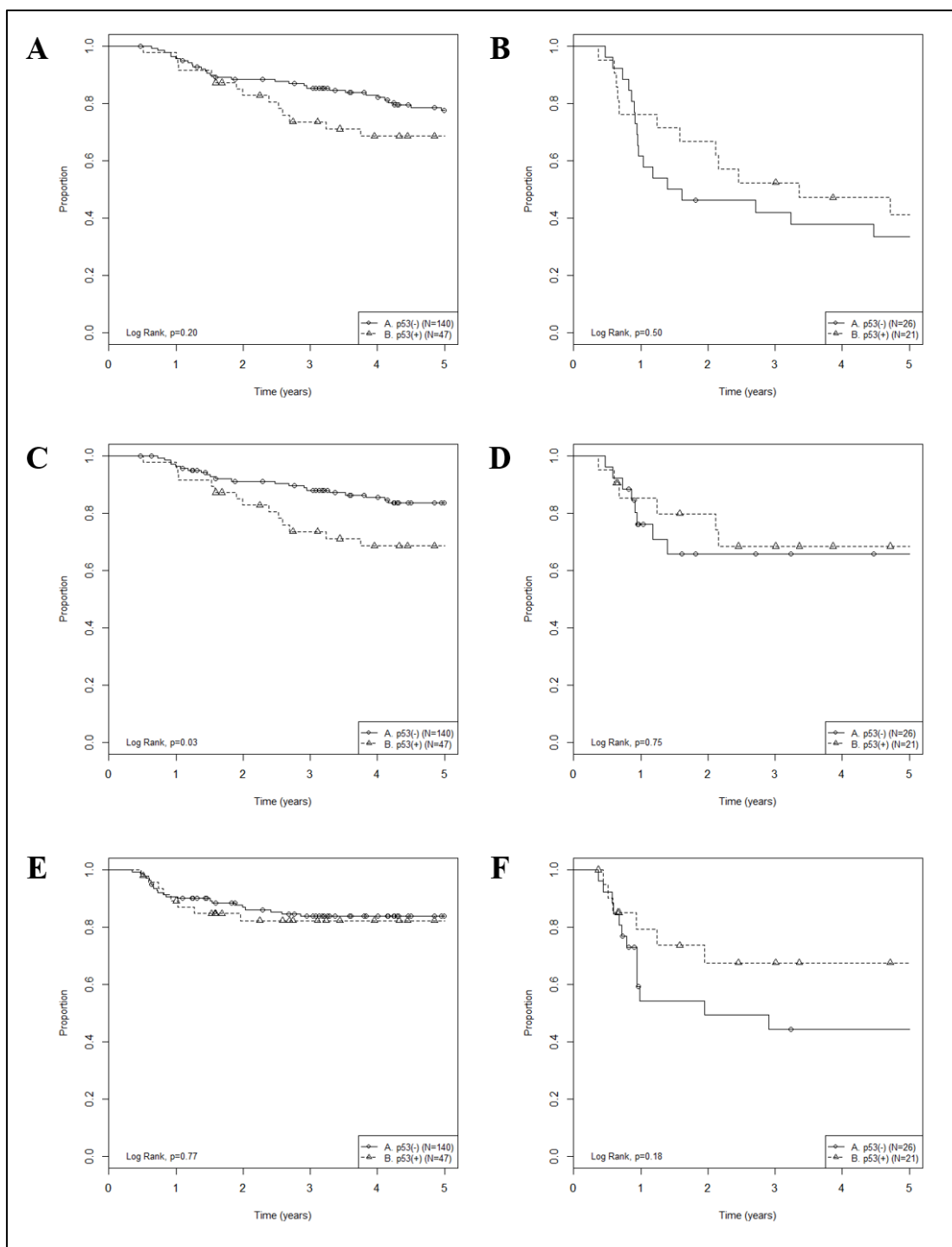Please refer to Appendices W and X for survival rates.

VII. Discussion

VII.1. The importance of this study

This was one of the few studies that evaluated multiple biomarkers from a large population-based cohort of patients. Our results support the prognostic significance of the presence of HR-HPV in OpSCC patients and this agrees with others.[57,121,122,125] Given the large sample size, we were able to compare and contrast different techniques for their advantages and disadvantages in detecting the presence of HR-HPV in OpSCC FFPE samples. Moreover, with the comprehensive data collection from BCCA, we were able to investigate important clinic-pathological factors, e.g., smoking and T-staging, that may be prognostic or to possibly guide management.

Health Canada has approved of the administration of the HPV vaccine and have included the vaccine into its publicly funded vaccination program. Although the results of vaccination are too early to be assessed, the burden of HPV-related cancers in general is expected to decline. For the individuals with HPV-related OpSCCs, we are at the best time to investigate treatment regimens and determine prognostic clinical and biological factors that may be predictive of patient outcomes.

VII.2. Representativeness of the Study Cohort to the General Cohort

One of the crucial aspects of this study was to assess the representativeness of the Study Cohort to the General Cohort such that the data can be generalized to the B.C. population of OpSCC patients. The only different inclusion criteria between the two cohorts is the availability of the tissue allowing further biomarker analysis. We found the patients in the Study cohort were significantly younger (57.3±9.4 vs. 60.1±10.4 years) and mainly from tonsil subsite (77.9% vs. 43.1%). Our data has also shown that younger patients were often never-smokers and had HPV-related OpSCC. The tonsils are susceptible for HPV viral deposition.[1] We noted that ~20% of the Study Cohort received tonsillectomies and the size of the tonsillar tissues would be adequate for experimental purposes. For the other anatomical sites, the biopsy size may be the limiting factor for the availability of tissue for analysis, hence the difference between 2 groups.

VII.3. Characteristics of HR-HPV-related OpSCC

In our Study cohort, we have observed 77.9% of HPV positive cases and this is comparable with the current literature.[57,58,121,184] Both tonsil and base of tongue share the same tissue origin and similar anatomical structure. The increased susceptibility to HPV infections may be due to the lymphoid tissue origin[221] and the crypt epithelium microanatomy of the tonsils may further increase the rate of viral deposition.[1,66] In our Study Cohort, the majority of HPV-positive cases were from the tonsillar subsite. A study assessing the HPV status of the subsites of oropharynx outside of the tonsil and the base of tongue identified a lowered frequency (17%) of HPV infection.[222] Given that the majority of samples in our Study Cohort originated from either tonsils or base of tongue, we were unable to assess the impact of HPV independently in other oropharyngeal subsites.

We have observed that HPV positive patients were more often males, compared to HPV-negative patients. The increased HPV burden among males can be explained by the lower clearance rate of the virus and lower rates of seroconversion when compared to females.[50,56,169] With changing sexual practices and decreased tobacco use, relatively younger age and non-smoking status have become characteristics of HPV-positive OpSCC patients.[223] The prognostic value of HPV status can be observed from the relatively lower proportions of HPV-positive patients that either died or developed LRR. Univariate Cox-PH and KM survival analysis further showed that HPV-positive status is associated with improved prognoses, which agrees with a myriad of studies.[57,121-125] In our Study Cohort, two patients presented with unknown primary lesions during initial TNM staging were found to be HPV-positive. The use of p16, as surrogate for HPV status, has been explored as a method for locating unknown primary tumours in patients with metastatic lymph nodes.[224]

VII.4. HR-HPV detection

With the acknowledgement that HPV status is a prognostic factor, the method of detection has not currently reached a consensus thus, we evaluated different techniques of PCR, ISH, and IHC. Given that PCR has a high sensitivity, we used the molecular platform as the benchmark to compare a subset of samples with enough tissues for DNA extraction. We found that although p16 had high sensitivity, the technique suffered from low specificity. IHC p16 detection may be a relatively easy technique and has quick turnover rates, but the staining is nevertheless an indirect detection for HPV.

Given the high sensitivity and specificity of combined DNA and RNA ISH, as compared to PCR, we therefore suggest using ISH for detecting HPV in the clinic. With an agreement rate of ~90% between DNA and RNA ISH, our data suggests either nucleic acid may be feasible. To address the ~10% discrepancy, the samples that were HPV DNA ISH positive, but RNA ISH negative, may be due to transcriptionally inactive viruses[225] or the patient was infected with an HPV genotype that was not included in the RNA ISH panel. One of the challenges for ISH is to optimize the time for tissue digestion to both avoid over and under digestion, in which the former may lead to increased background staining and the latter may lead to decreased ability for probes to hybridize.[226] Among samples that were DNA ISH negative, but RNA ISH positive, we speculate the tissues may have been under digested when performing DNA ISH. Further investigation on the perfection of robust protocol is warranted.

As the current gold standard for HPV detection is to detect HPV mRNA, RNA ISH could better indicate the transcriptional activity of the virus compared to DNA ISH. Due to its availability, we used RNA ISH targeting HPV 16 and 18 only for our study. Currently, there are other panels that contain more HPV subtypes that can be used, but the cost may also increase. For HPV-related OpSCC, the majority of cases are caused by HPV 16 (>90% of cases in our cohort and others) thus, only detecting for HPV 16 may be sufficient.[147] However, we have found the discrepancy between the HPV-HR DNA ISH and HPV 16/18 RNA ISH results as well as one sample determined to be HPV 33 using our type-specific HPV-PCR assay. Different HPV genotypes have been suggested to confer different prognoses such that HPV 16 positive OpSCC patients were found to have better OS

compared to non-HPV 16 HPV-related OpSCC patients.[227] Unless specific HPV genotypes denote different treatment guidance, then patients that are HPV 16-negative, but clinically suspected for HPV involvement, may benefit from additional HPV testing using the wide panel of HPV genotypes.

Given a few techniques available for HPV detection, we propose a new algorithm, Algorithm B (**Figure 12**). The approximate costs in Canadian dollars for p16 IHC, DNA ISH, and RNA ISH are $38, $74, and $178, as estimated from a published article and our own experience.[164] Using our Study Cohort as an example to calculate the costs between algorithm A, proposed by Westra (2014),[147] and Algorithm B, the costs would be CAD $182.50 and 196.20 per case, respectively. Additionally, in Algorithm A, 18 HPV-positive cases would be scored as HPV-negative. The consequence of misclassifying HPV status of OpSCC patients have yet to be determined, but our results support that HPV status was an important prognostic factor.
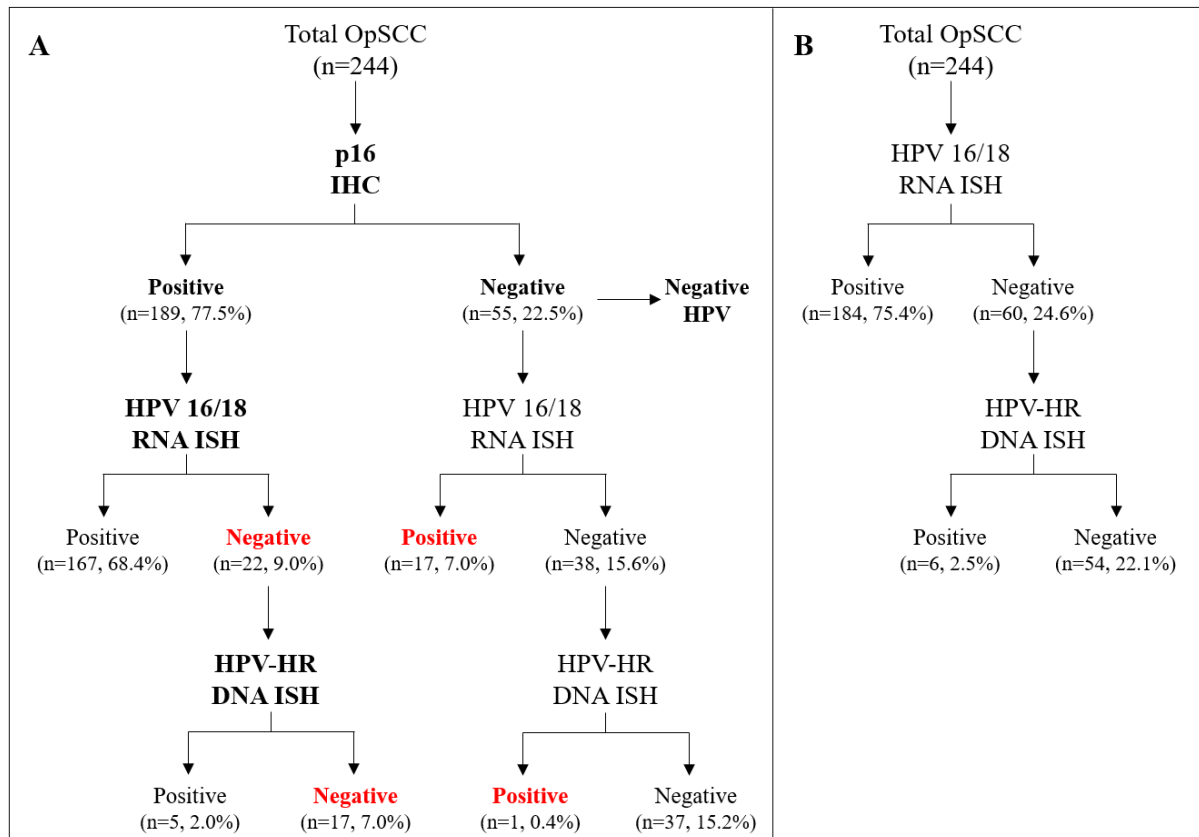
Figure 12. Comparison of HPV detection algorithms and results using the Study Cohort

Algorithm A adapted from Westra (2014)[147] uses a sequence of p16 immunohistochemistry (IHC), then *in situ* hybridization (ISH) to specifically detect HPV 16 and 18 only for those p16-positive cases, and finally ISH that detects a wide spectrum HPV genotyping (WS-HPV) for HPV 16/18 negative cases. Algorithm B, our proposed algorithm, starts testing ISH on HPV 16/18, followed by WS-HPV for those negative HPV 16/18 cases.

VII.5. Prognostic values of HPV-related biomarkers

Survival analysis did not support the prognostic values for pRB and Ki67 in our Study Cohort. We noted that pRB status did not associate with HPV status even though the HPV E7 oncoprotein is known to target pRB for degradation (**Figure 1**). We observed that approximately two-thirds of both HPV-positive and HPV-negative patients were pRB-negative which may be related to the particular antibody clone (92G2) used in this study, which specifically targeted phosphorylated pRB. When samples were scored as pRB-negative, in addition to pRB degradation due to HPV E7, under detection of unphosphorylated pRB or deletion of the gene encoding pRB are possible explanations of the negative results. Without additional investigations, such as performing genomic analysis to determine the allelic imbalance of pRB, the IHC results alone could be difficult to interpret. On the other hand, Ki67 was statistically significant for HPV status (**Table 9**) and the HPV-positive subgroup of our Study Cohort showed a higher proportion of Ki67-positive patients. When HPV is detected in OpSCC, this observation implies that the virus was the driver for carcinogenesis due to uncontrolled cellular proliferation. Given that Ki67 is a proliferation marker, Ki67 would be expected to have increased expression in concurrent with HPV detection. However, high expression levels of Ki67 have been associated with poorer prognoses[199,218] which contrasts to the association between HPV detection and improved prognoses. Alternatively, for the expression levels of Ki67 to correlate with phases in the cell cycle, the use of cut-off values for dichotomizing IHC results may not be the best approach. Survival analysis using multiple cut-off values for Ki67 expression may be a better approach, but interpretations of IHC results can have subjective bias and difficult to be implemented for clinical usage.

We found that p53 and cyclin D1 statuses were suggestive of prognostic significance. The overexpression of p53 has been associated with local treatment failure among HNSCC patients treated with RT and suggested that the biomarker may be suitable for assessing radioresistance.[228] The antibody used in this study, clone DO7, detects for both wild and mutant p53 phenotypes. HNSCC tumours that expressed HPV E6/E7 have been found to have wild-type p53.[229] Given that the half-life for the wild-type is relatively short,[200] we reason that samples interpreted as positive for both p53 and HPV (n=47) may suggest these

patients to harbour the mutant p53 phenotype. The observed poorer DSS may be related to radioresistance, but the debate of p53 phenotypes, wild vs. mutant p53, to affect radiosensitivity is still ongoing.[188,230,231] Unfortunately, determining p53 mutational status could only be achieved using techniques such as DNA sequencing.[189] If supported by future study, the confirmation of p53 mutation status may be clinically important.

The prognostic significance of cyclin D1 for all three clinical outcomes is indicative that a cut-off set at 50% is adequate. Interestingly, one study has shown that in oral cancer cell lines and oral cancer tissue samples, higher levels of cyclin D1 expression were associated with increased sensitivity to radiation,[232] which can be interpreted to confer better prognoses. Subtle molecular differences between the oral cavity and oropharynx anatomical sites may explain the radiation sensitivity for cyclin D1 expressions, but nevertheless, our data and others have shown that cyclin D1 overexpression confers poor prognoses.[209,212,213]

Expression levels of either p53 or cyclin D1 may be examined following HPV testing and be used to guide and/or monitor treatments. HPV-positive patients that also test positive for p53 may be related to a mutant p53 phenotype as discussed above. For these patients, receiving targeted therapy from agents that deplete mutant p53 while restoring the wild-type may increase their rates of survival.[233] In the current literature, one the other hand, most of the attention is placed on the HPV-positive patients resulted in slower advancement of knowledge among the HPV-negative subgroup. With increased HPV vaccination rates in Canada, the burden of HPV will be expected to decrease over time. Thus, we are at the optimal time to direct some focus into evaluating treatments for HPV-negative patients. By finding that cyclin D1-positive status was only associated with poorer OS among the HPV-negative subgroup, we hope to bring back some focus for optimizing treatments among these patients. Investigations for the effectiveness of cyclin D1 inhibitors may be conducted through clinical trials by recruiting HPV-negative/cyclin D1-positive patients.

VII.6. Prognostic values of clinical factors

Tobacco is a major contributor to carcinogenesis and its associations with increased risk for deaths and LRR were not masked by HPV status in our Study Cohort. Unfortunately, we were not able to assess for the impact of pack-years as approximately one-third of ever-smokers (n=60) did not indicate the duration and/or the quantity of their tobacco use. Pack-years can be used to stratify patients for appropriate risk groups and be assessed concurrently with HPV status for stratifying patients to receive adequate treatments.[121]

Our Study Cohort showed that HPV-positive patients can be further stratified for survival when including the assessment for tumour staging thus, accurate staging of primary tumours is crucial for treatment planning. In contrast, nodal staging, with and without consideration for HPV status, was not a prognostic factor in our cohort. One of the characteristics of HPV-positive patients is to be presented more often with enlarged neck masses and small primary tumours.[13] Thus, with increasing burden of HPV-related OpSCC, initial testing for HPV may lessen the impact of nodal staging.

Our results showed that HPV-positive patients receiving CRT had increased DSS and reduced the development for LRR. The benefits may be attributed to the treatment. Numerous studies report better outcomes from CRT as compared to RT alone.[26,29,30,33] Alternatively, the initial health of the patients may explain the observed benefits. The bimodal treatment is an aggressive approach that is only prescribed to patients after thoroughly assessing for their baseline health status.[27] Patients' health performance status can be quantified through the Eastern Cooperative Oncology Group system,[234] but the data was not available in this study. In addition, our study was not designed as a clinical trial, in which a homogenous group is randomly split to determine efficacy of treatments. On the basis of our analysis, we cannot recommend CRT over RT alone for entailing better survival among HPV-positive patients.

A recent publication reported that patients receiving induction chemotherapy with CRT at 15-20% reduction of radiation dosage, compared to standard of care, resulted in high PFS, 2-year OS, and reduced toxicity profiles.[235] Deintensification of chemotherapy is currently explored by the substitution of cisplatin with the EGFR inhibitor of cetuximab. Marur *et al.* reported 2-year PFS and OS rates of 80% and 94%, respectively, for patients

that received induction chemotherapy of cetuximab and reduced radiation dosage to 54 Gy.[236] The newer surgical procedures of Trans Oral Robotic Surgeries are also reporting improved 2-year RFS and fewer functional support, such as tracheostomy and feeding tube placement, needed for patients that received salvage surgery.[237] Notably, studies that report survival times beyond 2-years are currently minimal or non-existent. As more findings become available for the clinical feasibility of de-intensifying treatments, then survival analysis at longer follow-up times can better justify the role of treatment de-intensification.

The 8th edition of the AJCC Staging Manual will include HPV status as part of the staging process for OpSCC patients.[16] The staging will be reorganized for HPV-related OpSCC, as determined by p16-positive, and notably, only patients presented with distant metastasis will be given a Stage IV. We continue to provide support that smoking, tumour staging, and modality of treatments can impact clinical outcomes. The 8th edition of the AJCC Staging Manual continues to consider the assessment for tumour status for staging, but tobacco use was not considered to be a diagnostic factor. The explanation being that tobacco use could not be a distinguishable characteristic.[238] Time will truly determine the applicability of HPV assessments based on p16 status alone, the importance of including smoking history in the staging process, and the generalizability of the new staging system to the general population.

VII.7. Limitations of study and possible solutions for future consideration

We acknowledge that this study has several limitations. First, this was a retrospective study in which biases and missing information could potentially influence the analysis. The majority of patient charts was complete and the copies of lab and test reports were available to review. However, any incomplete information would be reliant on the dictations from clinicians. Uniformity between clinicians' assessments was also a limiting factor. For example, patients that had quit smoking were not inquired further for the number of cigarettes smoked per day and/or the number of years of smoking. The pack-years of smoking was therefore unable to be evaluated. Understandably, this limitation is exclusive for retrospective studies. On the other hand, prospective studies would have set criteria designed for accurate and consistent data collection.

Second, the number of available tissues for experimental analysis represented only ~25% of the overall OpSCC patients treated at the BCCA. Caution is warranted when generalizing our data to the larger population. The potential solution to alleviate the limitation of sample availability is the understanding of the clinicians on the importance of tissue availability for biomarker analysis and the establishment of a tissue bank for future studies.

Third, biopsy tissues were of limited sizes and this was evident by the number of samples available for DNA extraction. For research purpose, we constructed TMA blocks to alleviate the limitation of tissue size. However, TMA sections can encounter issues with missing cores or the stained cores do not contain tumour cells for the particular tissue section. The experiments could be repeated, but more time and resources would have to be spent which become impractical in the clinical setting. In a clinically practical point of view, the clinicians need to understand the importance of obtaining larger biopsy specimens for in-depth analysis.

Fourth, the majority of our Study Cohort had locally advanced stages of disease in which more aggressive forms of treatment were prescribed. Overtreating of patients resulting in higher rates of complications could have occurred and to negatively affect their clinical outcomes.

Fifth, both RT and surgery can have curative intents, but our patient population consisted only of patients that underwent radiotherapy-based treatments. With advancements in robotic surgery, the newer surgical techniques might decrease treatment sequelae. In future, comparison of the two treatment modalities may help to identify different factors responding to type of treatment and associated outcomes, including patient's quality of life.

Finally, the number of HPV-negative patients was relatively small and the prognostic significance for HPV-negative/cyclin D1-positive patients may have been skewed. A thorough analysis of a sizeable HPV-negative patients could further address the role of cyclin D1, but the current trend of increasing HPV-related OpSCC cases may limit the feasibility of the analysis.

VIII. Conclusion

The key finding for this study was that there was an increasing trend of newly diagnosed OpSCC cases between 2000 and 2008. HPV was positive in the majority of patients and HPV status remained a prognostic factor in OpSCC patients.

We conclude that HPV status is prognostic and detecting HPV DNA/RNA using ISH was more specific than using p16 IHC. With the expected increase in burden of HPV-related OpSCC, the detection method becomes crucial to triage patients for appropriate treatment regimens. Our data supported HPV-positive/p53-positive patients and HPV-negative/cyclin D1-positive patients have the worst rates of survival.

There are several future directions to explore. One direction is to gather data from patients that received surgery as the primary intent and to compare the outcomes with patients that received RT based curative treatments. Another direction is to explore comorbidities that may be associated with HPV status and to further identify factors that negatively influence survival in HPV-positive patients. With the increasing burden of HPV-related cancers, questionnaires could be conducted for the patients and clinicians to assess their knowledge and understanding of HPV. The results may be helpful to modify health policies and to minimize the stigma surrounding the nature of the virus. In the next decade, after the population had a chance to experience the effects of the nationwide HPV preventative vaccination, the burden of HPV-related OpSCC in B.C. can be revisited. Survival rates would also need to be reconsidered as newer treatment regimens may replace the current standard of care.

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Appendices

Appendix A: Inclusion criteria (ICD-O-3 Topography Codes)

C01.9 Base of tongue, NOS

C02.4 Lingual tonsil

C05.1 Soft palate, NOS

C05.2 Uvula

C09.0 Tonsillar fossa

C09.1 Tonsillar pillar

C09.8 Overlapping lesion of tonsil

C09.9 Tonsil, NOS

C10.0 Vallecula

C10.1 Anterior surface of epiglottis

C10.2 Lateral wall of oropharynx

C10.3 Posterior wall of oropharynx

C10.4 Branchial cleft (site of neoplasm)

C10.8 Overlapping lesion of oropharynx

C10.9 Oropharynx, NOS

Appendix B: Inclusion criteria (ICD-O-3 Morphological Codes)

8010/3 Carcinoma, NOS

8020/3 Carcinoma, undifferentiated type, NOS

8021/3 Carcinoma, anaplastic type, NOS

8052/3 Papillary squamous cell carcinoma

8070/3 Squamous cell carcinoma, NOS

8071/3 Squamous cell carcinoma, keratinizing, NOS

8072/3 Squamous cell carcinoma, lg. cell, non-keratinized.

8073/3 Squamous cell carcinoma, sm. cell, non-keratinized.

8074/3 Squamous cell carcinoma, spindle cell

8076/3 Squamous cell carcinoma, micro-invasive

Appendix C: Cox-PH analysis of 5-year OS for clinical and biological factors

Variables[a]	Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age				
<55	1.0		1.0	
≥55	2.3 (1.3-3.8)	**<0.01**	1.6 (0.9-2.9)	0.08
Sex				
Male	1.0			
Female	1.2 (0.7-2.0)	0.45		
Anatomical site				
Tonsil	1.0			
Base of tongue	0.9 (0.5-1.6)	0.76		
Other oropharynx[b]	2.0 (0.6-6.3)	0.25		
Smoking history				
Never-smoker	1.0		1.0	
Ever-smoker	4.6 (1.9-11.5)	**<0.01**	4.0 (1.6-10.2)	**<0.01**
Tumour stage				
T0/1	1.0			
T2	1.3 (0.7-2.5)	0.42		
T3	2.3 (1.1-4.5)	**0.02**		
T4	3.5 (1.6-7.5)	**<0.01**		
Tumour stage (reclass)				
T0/1/2	1.0		1.0	
T3/4	2.2 (1.4-3.4)	**<0.01**	2.0 (1.2-3.3)	**<0.01**
Nodal stage				
N0	1.0			
N1	1.2 (0.6-2.4)	0.69		
N2	0.9 (0.5-1.6)	0.69		
N3	1.4 (0.6-3.1)	0.49		
AJCC staging				
I/II	1.0			
III/IV	1.6 (0.7-3.7)	0.27		
Primary treatment				
RT	1.0		1.0	
CRT	0.5 (0.3-0.8)	**<0.01**	0.5 (0.3-0.8)	**<0.01**
HPV status				
Negative	1.0		1.0	
Positive	0.3 (0.2-0.4)	**<0.01**	0.6 (0.3-1.2)	0.14
p16 status				
Negative	1.0		1.0	
Positive	0.4 (0.2-0.6)	**<0.01**	1.0 (0.5-1.8)	0.90
p53 status				
Negative	1.0			
Positive	1.4 (0.9-2.3)	0.13		

Variables[a]	Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
pRB status				
Negative	1.0			
Positive	1.2 (0.8-2.0)	0.40		
Cyclin D1 status				
Negative	1.0		1.0	
Positive	2.8 (1.7-4.4)	**<0.01**	1.5 (0.9-2.7)	0.14
Ki67 status				
Negative	1.0			
Positive	0.7 (0.4-1.1)	0.14		

[a]Missing data: Anatomical Site, n=1; Tobacco use, n=4; Tumour stage, n=2; p53, n=10; pRB, n=6; Cyclin D1, n=4; Ki67, n=5

[b]Includes: Soft palate (n=2), Oropharyngeal wall (n=2), Uvula (n=1)

Abbreviations: AJCC: American Joint Committee on Cancer (7th ed.); RT: Radiotherapy; CRT: Concurrent chemoradiotherapy; HPV: Human papillomavirus; pRB: Retinoblastoma protein

Appendix D: KM survival analysis of 5-year OS for clinical factors
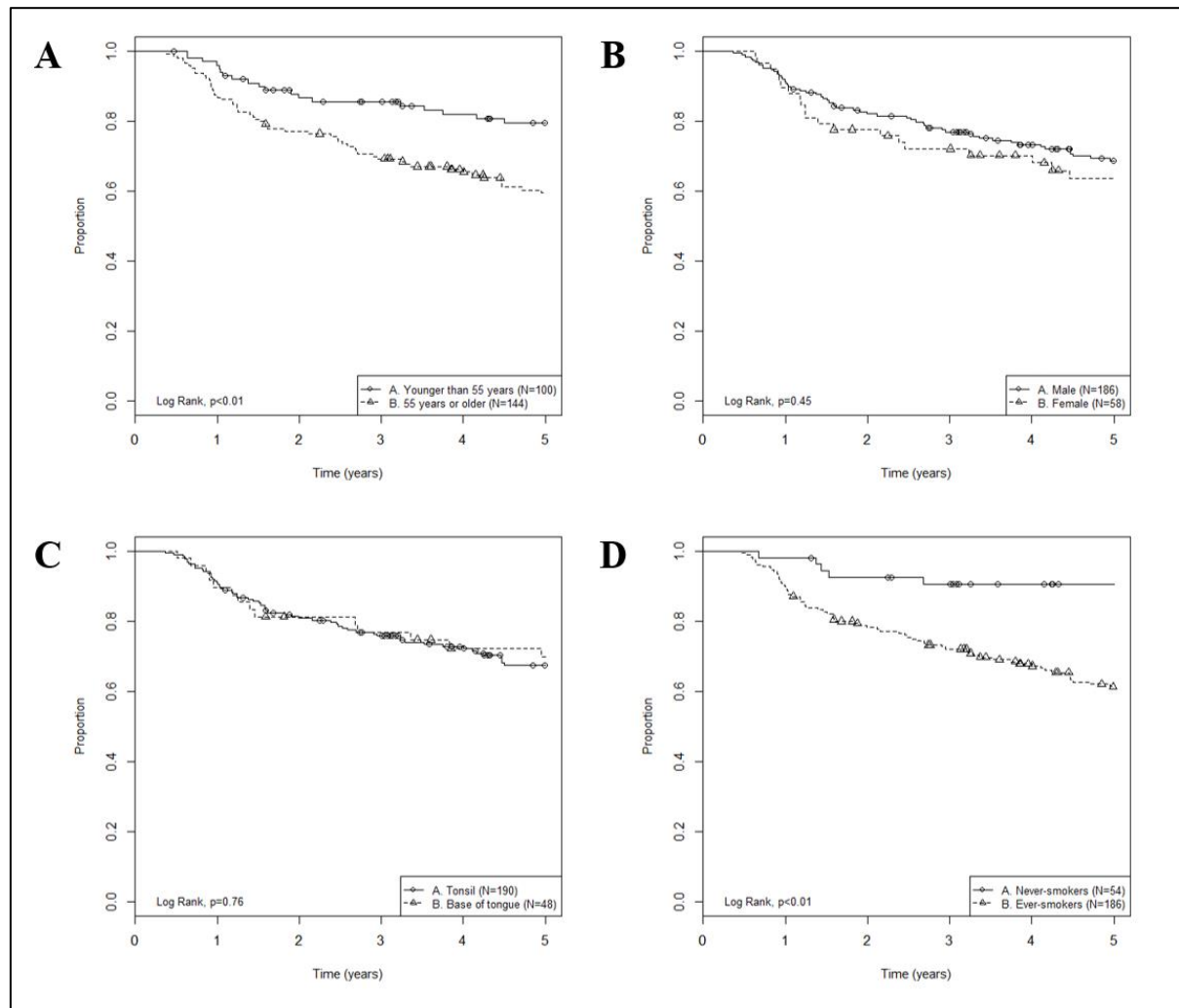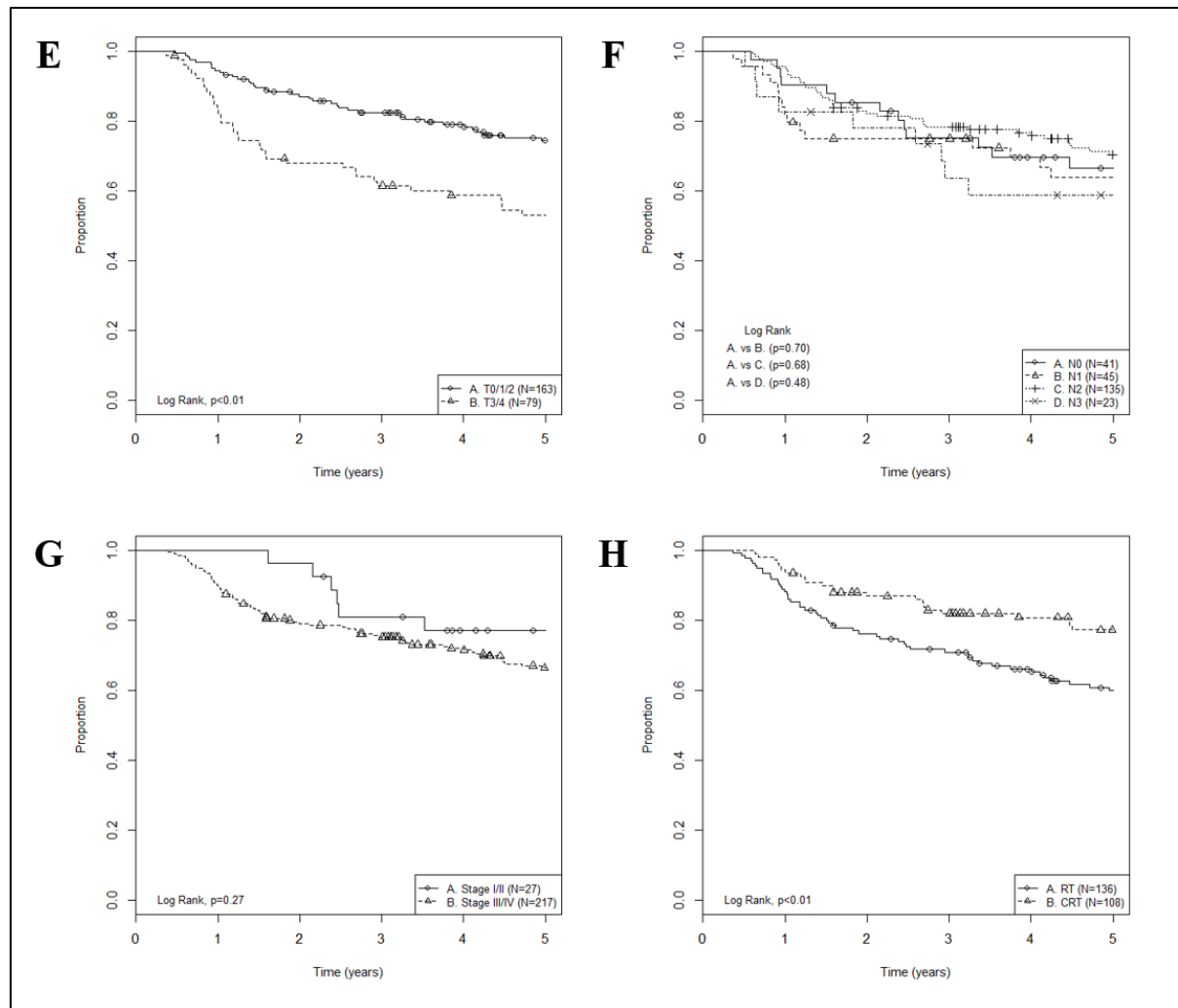
Analysis presented are factors of age (A), biological sex (B), anatomical site (C), smoking history (D), tumour staging (E), nodal staging (F), AJCC staging (G), and treatment received (H). Factors associated with 5-year OS were observed for age, smoking history, tumour staging, and treatment received. Please refer to Appendix L for survival rates.

Appendix E: KM survival analysis of 5-year OS for biological factors
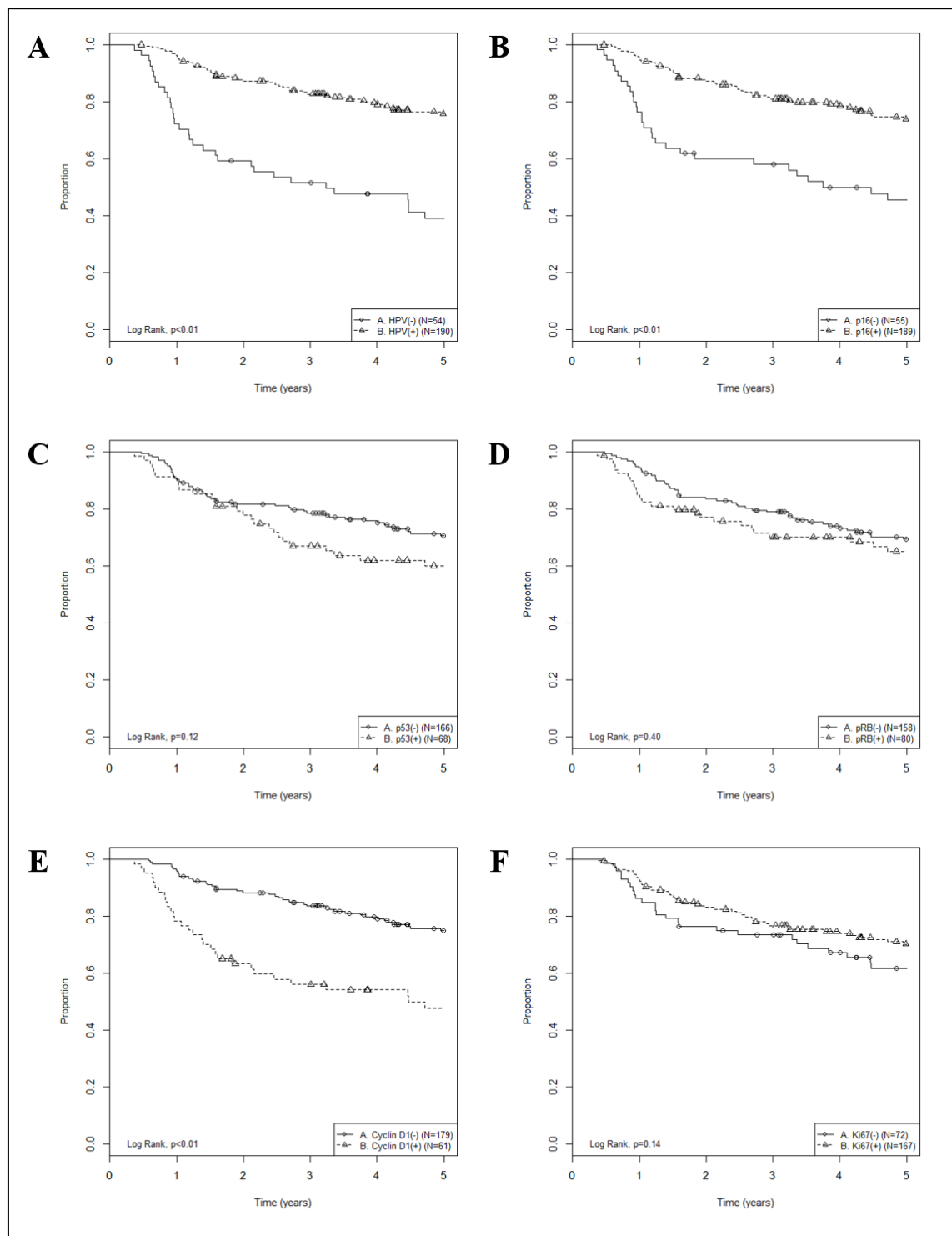
Analysis presented are statuses for HPV (A), p16 (B), p53 (C), pRB (D), cyclin D1 (E), and Ki67 (F). Factors associated with 5-year OS were observed for HPV, p16, and cyclin D1 statuses. Please refer to Appendix L for survival rates.

Appendix F: Cox-PH analysis of DSS for clinical and biological factors

Variables[a]	Univariate HR (95% CI)	p-value	Multivariate HR (95% CI)	p-value
Age				
<55	1.0		1.0	
≥55	2.1 (1.1-3.9)	**0.02**	1.7 (0.9-3.2)	0.12
Sex				
Male	1.0			
Female	0.8 (0.4-1.6)	0.50		
Anatomical site				
Tonsil	1.0			
Base of tongue	0.7 (0.3-1.5)	0.36		
Other oropharynx[b]	0.9 (0.1-6.4)	0.91		
Smoking history				
Never-smoker	1.0		1.0	
Ever-smoker	3.9 (1.4-10.9)	**<0.01**	4.0 (1.4-11.5)	**<0.01**
Tumour stage				
T0/1	1.0			
T2	1.3 (0.6-2.8)	0.56		
T3	2.4 (1.1-5.5)	**0.03**		
T4	3.4 (1.4-8.7)	**<0.01**		
Tumour stage (reclass)				
T0/1/2	1.0		1.0	
T3/4	2.3 (1.4-4.0)	**<0.01**	2.3 (1.3-4.2)	**<0.01**
Nodal stage				
N0	1.0			
N1	1.6 (0.6-4.1)	0.34		
N2	1.2 (0.5-2.7)	0.70		
N3	2.0 (0.7-5.6)	0.21		
AJCC staging				
I/II	1.0			
III/IV	1.7 (0.6-4.7)	0.31		
Primary treatment				
RT	1.0		1.0	
CRT	0.5 (0.3-0.9)	**0.02**	0.4 (0.2-0.8)	**0.01**
HPV status				
Negative	1.0		1.0	
Positive	0.4 (0.2-0.8)	**<0.01**	1.0 (0.5-2.2)	0.98
p16 status				
Negative	1.0		1.0	
Positive	0.5 (0.3-0.8)	**0.01**	1.0 (0.4-2.1)	0.92
p53 status				
Negative	1.0			
Positive	1.7 (1.0-3.1)	0.06		

Variables[a]	Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
pRB status				
Negative	1.0			
Positive	1.4 (0.8-2.4)	0.29		
Cyclin D1 status				
Negative	1.0		1.0	
Positive	2.2 (1.2-3.8)	**<0.01**	1.4 (0.7-2.9)	0.33
Ki67 status				
Negative	1.0			
Positive	0.7 (0.4-1.3)	0.31		

[a]Missing data: Anatomical site, n=1; Tobacco use, n=4; Tumour stage, n=2; p53, n=10; pRB, n=6; Cyclin D1, n=4; Ki67, n=5
[b]Includes: Soft palate (n=2), Oropharyngeal wall (n=2), Uvula (n=1)
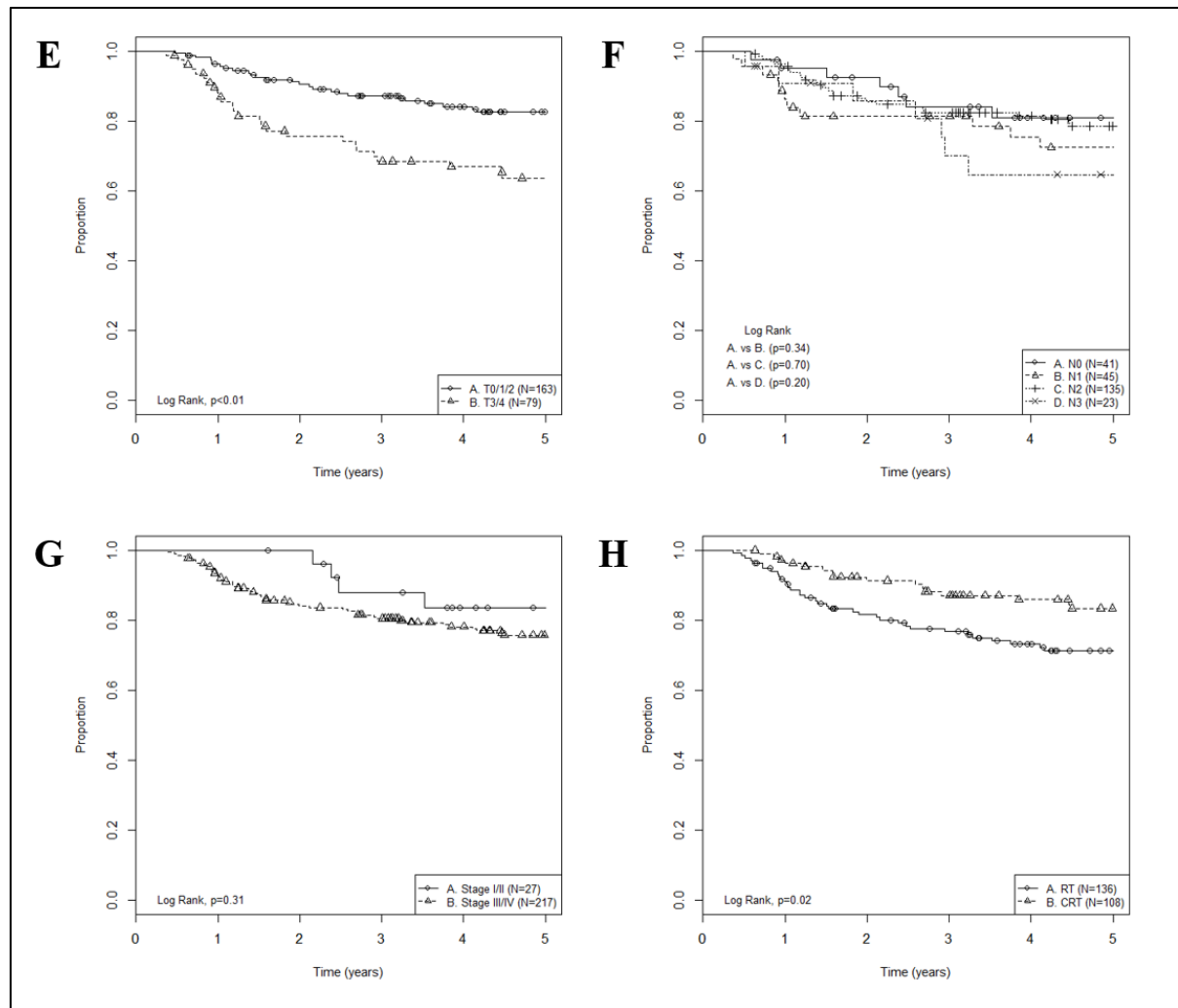Abbreviations: HPV: Human papillomavirus; AJCC: American Joint Committee on Cancer (7th ed.); RT: Radiotherapy; CRT: Concurrent chemoradiotherapy; pRB: Retinoblastoma protein

Appendix G: KM survival analysis of DSS for clinical factors
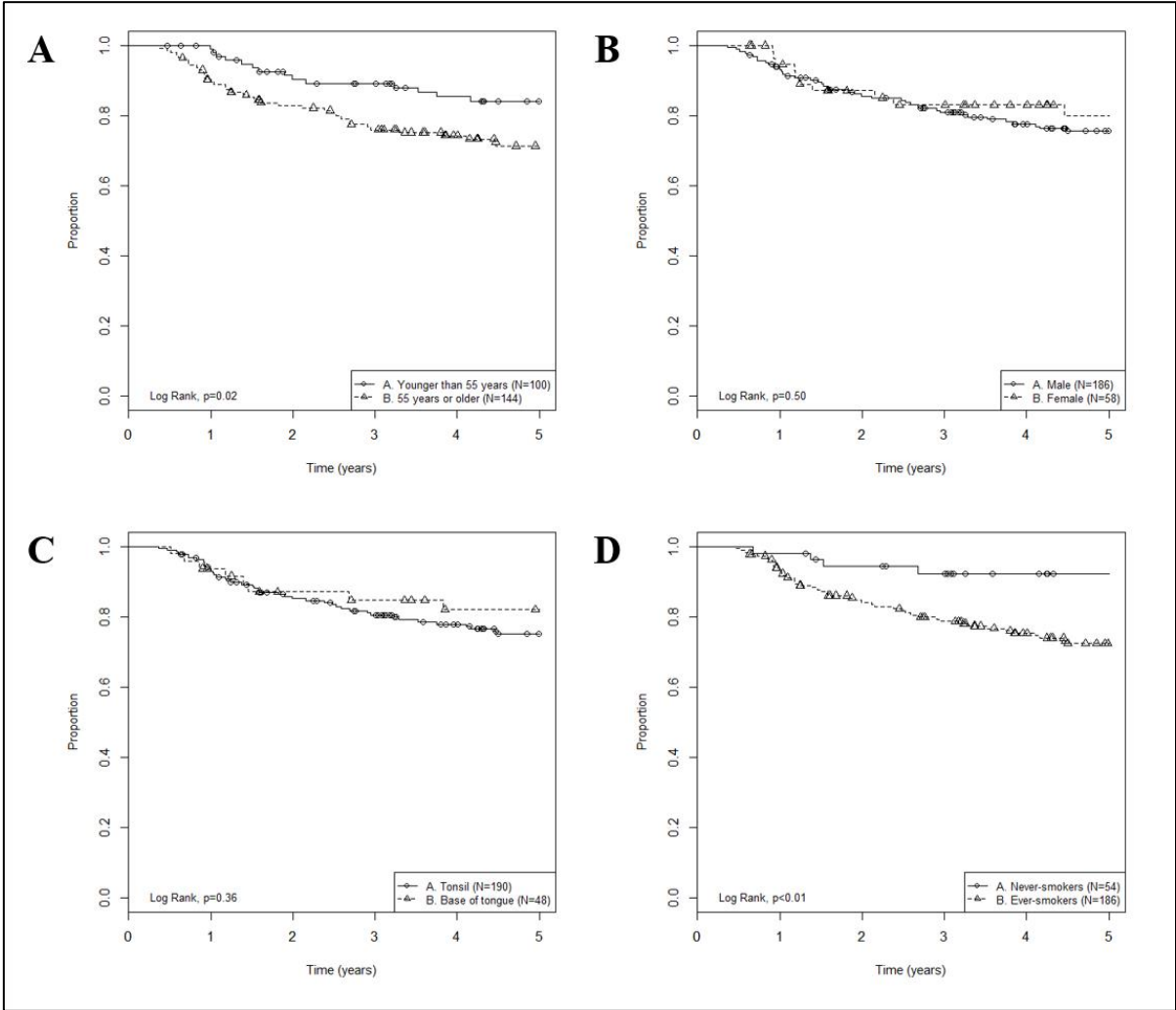
Analysis presented are factors of age (A), biological sex (B), anatomical site (C), smoking history (D), tumour staging (E), nodal staging (F), AJCC staging (G), and treatment received (H). Factors associated with DSS were observed for age, smoking history, tumour staging, and treatment received. Please refer to Appendix L for survival rates. Please refer to Appendix L for survival rates.

Appendix H: KM survival analysis of DSS for biological factors
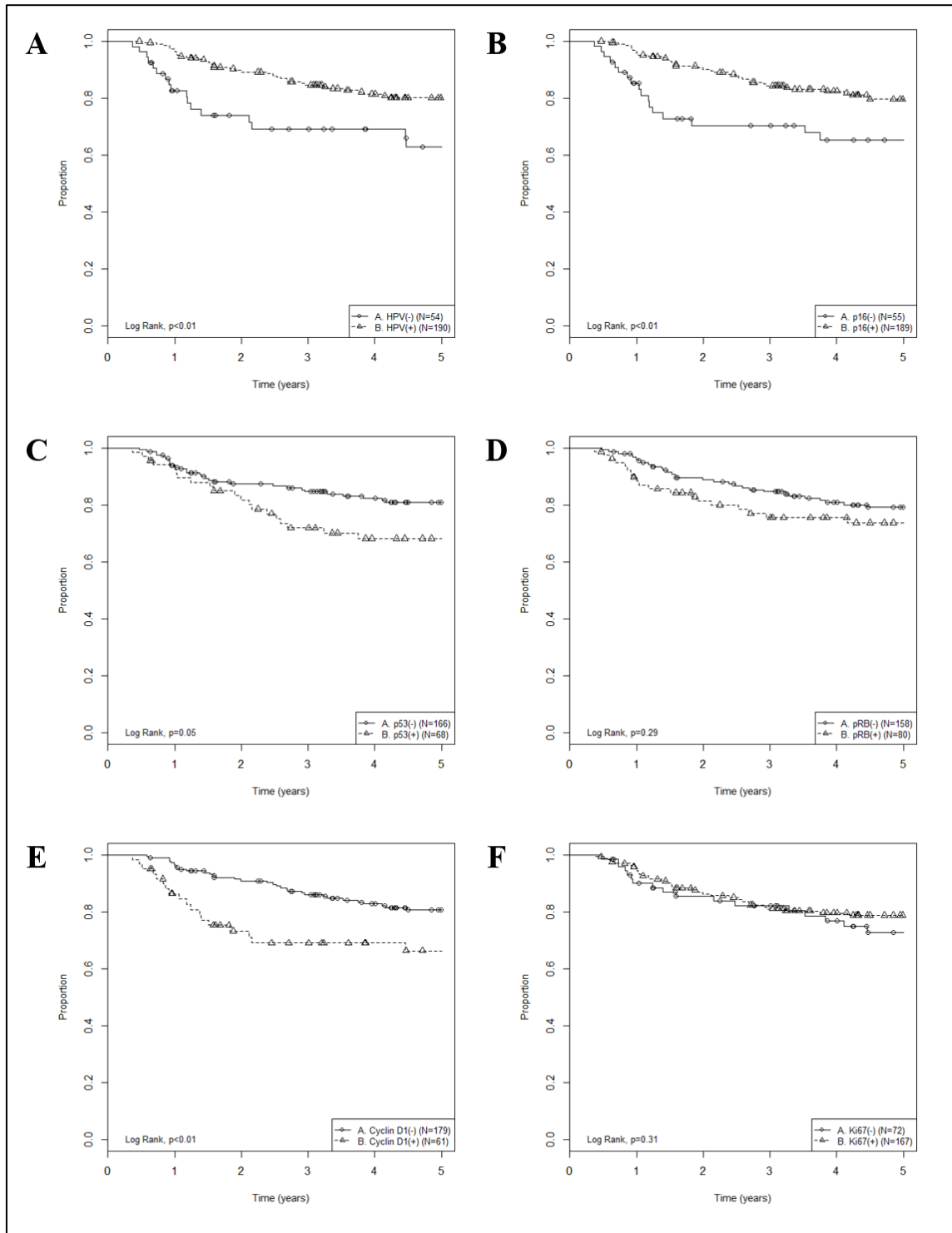
Analysis presented are statuses for the HPV (A), p16 (B), p53 (C), pRB (D), cyclin D1 (E), and Ki67 (F). Factors associated with DSS were observed for HPV, p16, and cyclin D1 statuses. Please refer to Appendix L for survival rates.

Appendix I: Cox-PH analysis of development for LRR for clinical and biological factors

Variables[a]	Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age				
<55	1.0			
≥55	1.4 (0.8-2.4)	0.28		
Sex				
Male	1.0			
Female	1.0 (0.5-1.8)	0.88		
Anatomical site				
Tonsil	1.0			
Base of tongue	0.7 (0.3-1.6)	0.42		
Other oropharynx[b]	2.0 (0.5-8.3)	0.33		
Smoking history				
Never-smoker	1.0		1.0	
Ever-smoker	5.4 (1.7-17.4)	**<0.01**	4.7 (1.4-15.5)	**0.01**
Tumour stage				
T0/1	1.0			
T2	1.6 (0.7-3.7)	0.23		
T3	2.5 (1.0-5.8)	**0.04**		
T4	3.2 (1.2-8.6)	**0.02**		
Tumour stage (reclass)				
T0/1/2	1.0		1.0	
T3/4	1.9 (1.1-3.3)	**0.02**	2.0 (1.1-3.7)	**0.02**
Nodal stage				
N0	1.0			
N1	0.8 (0.4-1.9)	0.65		
N2	0.6 (0.3-1.2)	0.18		
N3	0.8 (0.3-2.2)	0.66		
AJCC staging				
I/II	1.0			
III/IV	1.1 (0.5-2.5)	0.89		
Primary treatment				
RT	1.0		1.0	
CRT	0.4 (0.2-0.8)	**<0.01**	0.4 (0.2-0.7)	**<0.01**
HPV status				
Negative	1.0		1.0	
Positive	0.4 (0.2-0.6)	**<0.01**	0.8 (0.4-1.6)	0.51
p16 status				
Negative	1.0		1.0	
Positive	0.4 (0.2-0.7)	**<0.01**	0.9 (0.4-1.9)	0.82
p53 status				
Negative	1.0			
Positive	1.0 (0.6-1.9)	0.93		

Variables[a]	Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
pRB status				
Negative	1.0			
Positive	1.4 (0.8-2.5)	0.23		
Cyclin D1 status				
Negative	1.0		1.0	
Positive	2.7 (1.5-4.8)	**<0.01**	1.7 (0.9-3.3)	0.13
Ki67 status				
Negative	1.0			
Positive	0.6 (0.3-1.0)	0.07		

[a]Missing data: Anatomical site, n=1; Tobacco use, n=4; Tumour stage, n=2; p53, n=10; pRB, n=6; Cyclin D1, n=4; Ki67, n=5

[b]Includes: Soft palate (n=2), Oropharyngeal wall (n=2), Uvula (n=1)

Abbreviations: HPV: Human papillomavirus; AJCC: American Joint Committee on Cancer (7th ed.); RT: Radiotherapy; CRT: Concurrent chemoradiotherapy; pRB: Retinoblastoma protein

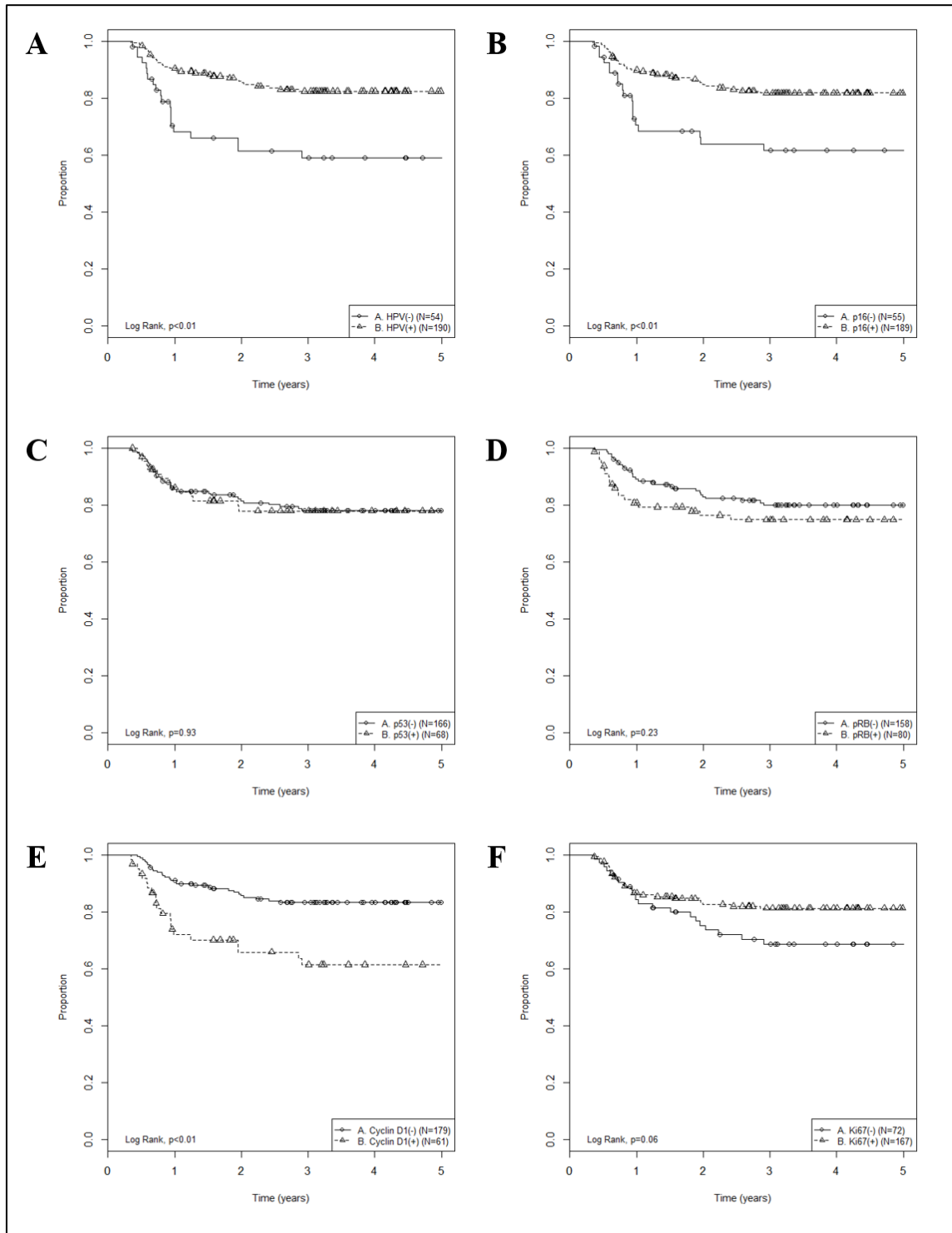**Appendix J: KM survival analysis of developing LRR for clinical factors**
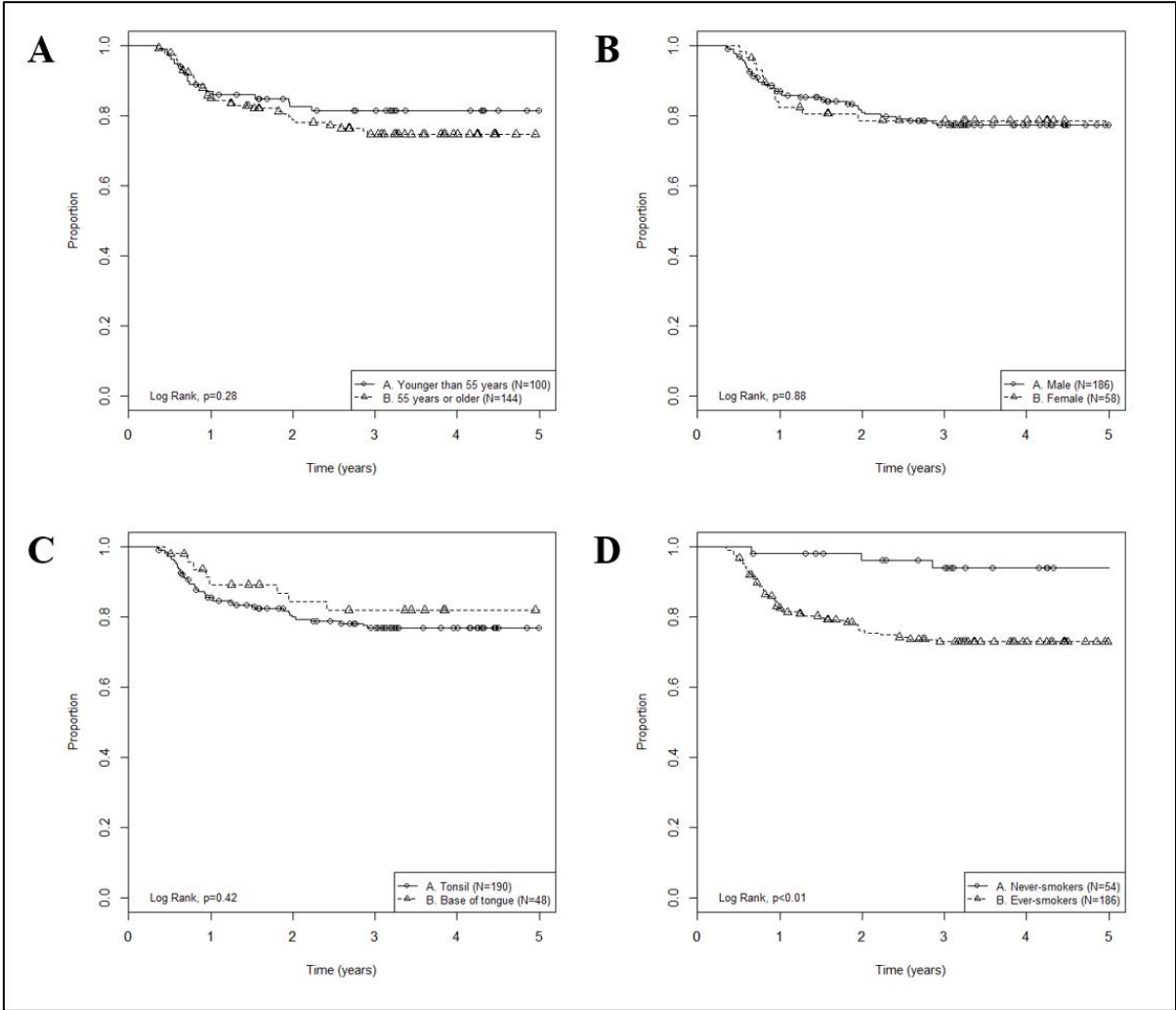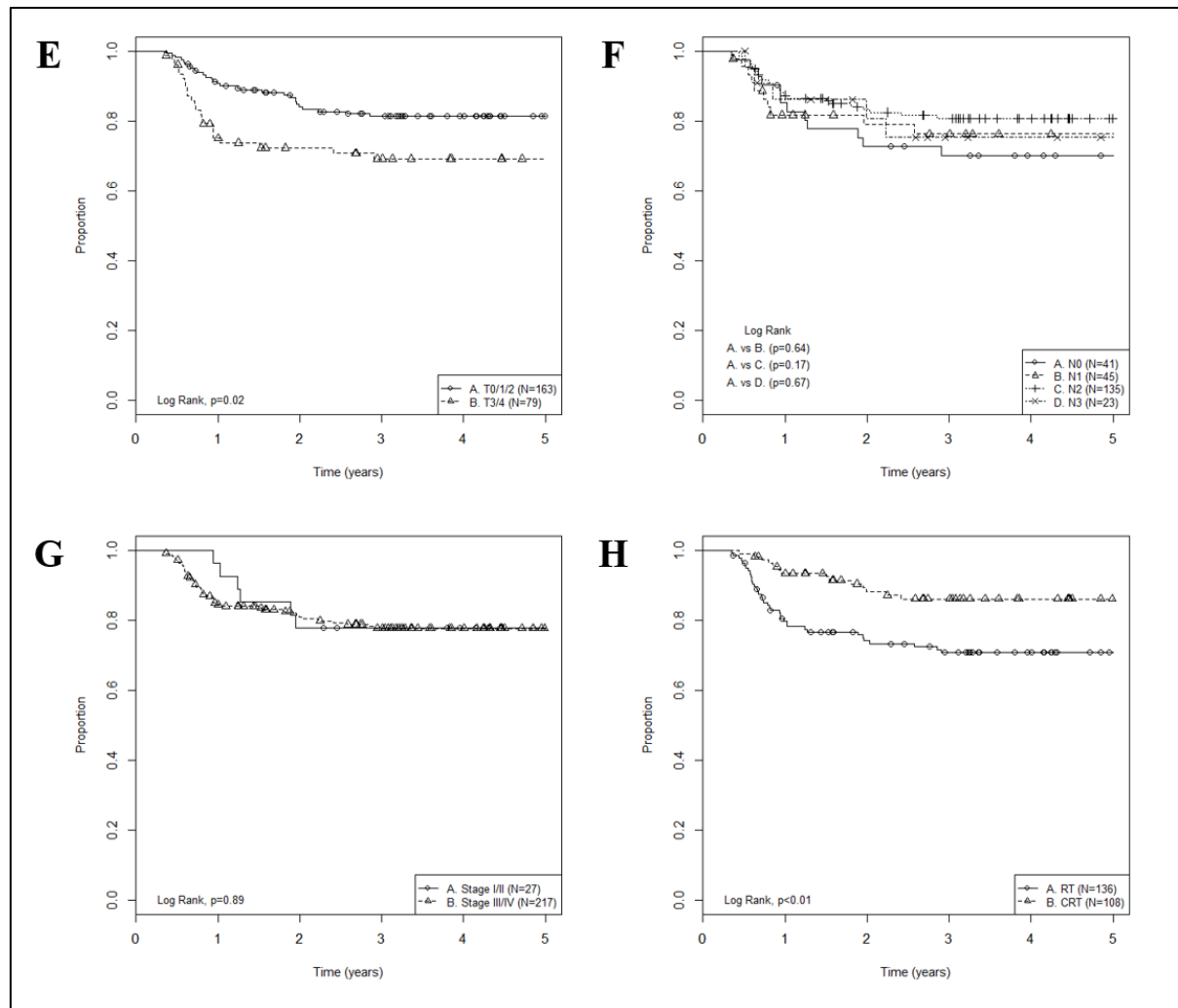
Analysis presented are factors of age (A), biological sex (B), anatomical site (C), smoking history (D), tumour staging (E), nodal staging (F), AJCC staging (G), and treatment received (H). Factors associated with development of LRR were observed for smoking history, tumour staging, and treatment received. Please refer to Appendix L for survival rates.

Appendix K: KM survival analysis for developing LRR for biological factors

Analysis presented are statuses for the HPV (A), p16 (B), p53 (C), pRB (D), cyclin D1 (E), and Ki67 (F). Factors associated with developing LRR were observed for HPV, p16, and cyclin D1 statuses. Please refer to Appendix L for survival rates.

Appendix L: KM survival analysis for rates of clinical outcomes

Variables[a]	5-year OS Rate (95% CI)	DSS Rate (95% CI)	No LRR Rate (95% CI)
Age			
<55	79.4% (71.5-88.2%)	84.1% (76.8-92.2%)	81.5% (74.2-89.6%)
≥55	59.4% (51.6-68.3%)	71.3% (63.9-79.6%)	74.7% (67.6-82.5%)
Sex			
Male	68.7% (62.1-76.0%)	75.5% (69.3-82.3%)	77.2% (71.2-83.7%)
Female	63.5% (51.8-77.9%)	80.0% (69.5-92.2%)	78.6% (68.6-90.1%)
Anatomical site			
Tonsil	67.5% (60.9-74.5%)	75.0% (68.8-81.9%)	76.8% (70.9-83.3%)
Base of tongue	69.9% (57.9-84.5%)	82.1% (71.5-94.2%)	81.9% (71.2-94.1%)
Other oropharynx[b]	N/A[c]	N/A[c]	N/A[c]
Smoking history			
Never-smoker	90.5% (82.9-98.8%)	92.3% (85.4-99.9%)	94.0% (87.6-100.0%)
Ever-smoker	61.4% (54.5-69.1%)	72.4% (65.9-79.6%)	72.9% (66.6-79.8%)
Tumour stage (reclass)			
T0/1/2	74.4% (67.7-81.7%)	82.6% (76.6-88.9%)	81.3% (75.4-87.7%)
T3/4	53.1% (43.0-65.6%)	63.7% (52.3-76.0%)	69.2% (59.4-80.6%)
Nodal stage			
N0	66.4% (52.9-83.3%)	81.0% (69.1-94.8%)	70.0% (57.1-85.8%)
N1	63.9% (50.7-80.6%)	72.4% (59.6-88.0%)	76.2% (64.3-90.4%)
N2	70.3% (62.7-78.8%)	78.5% (71.6-86.1%)	80.8% (74.3-87.9%)
N3	58.7% (41.2-83.8%)	64.6% (46.5-89.8%)	75.4% (58.6-96.9%)
AJCC staging			
I/II	77.0% (62.4-95.0%)	83.6% (70.0-99.7%)	77.8% (63.6-95.2%)
III/IV	66.4% (60.1-73.2%)	75.7% (69.9-82.0%)	77.6% (72.1-83.6%)
Primary treatment			
RT	58.9% (51.9-69.0%)	71.3% (63.7-79.7%)	70.8% (63.3-79.1%)
CRT	77.2% (69.4-86.0%)	83.4% (76.2-91.2%)	86.1% (79.6-93.2%)

Variables[a]	5-year OS Rate (95% CI)	DSS Rate (95% CI)	No LRR Rate (95% CI)
HPV status			
Negative	39.0% (27.6-55.0%)	62.8% (49.7-79.3%)	59.0% (46.6-74.8%)
Positive	75.8% (69.7-82.4%)	80.2% (74.5-86.3%)	82.4% (77.1-88.2%)
p16 status			
Negative	45.6% (33.9-61.4%)	65.2% (52.9-80.4%)	61.6% (49.3-77.0%)
Positive	73.9% (67.6-80.7%)	79.7% (73.9-86.0%)	81.8% (76.4-87.7%)
p53 status			
Negative	70.6% (63.8-78.2%)	80.8% (74.7-87.4%)	78.1% (71.9-84.8%)
Positive	60.0% (49.1-73.3%)	68.2% (57.5-80.9%)	77.9% (68.2-88.9%)
pRB status			
Negative	69.4% (62.3-77.2%)	79.1% (72.7-86.1%)	80.1% (73.9-86.7%)
Positive	65.1% (55.0-77.1%)	73.8% (64.2-84.8%)	74.8% (65.6-85.4%)
Cyclin D1 status			
Negative	74.5% (68.7-81.9%)	80.7% (74.9-87.0%)	83.3% (77.9-89.0%)
Positive	46.7% (36.1-62.9%)	66.3% (54.5-80.7%)	61.5% (49.6-76.1%)
Ki67 status			
Negative	61.8% (51.1-74.7%)	72.7% (62.3-84.9%)	68.7% (58.4-80.9%)
Positive	70.2% (63.3-77.8%)	78.8% (72.6-85.5%)	81.2% (75.4-87.6%)

[a]Missing data: Anatomical site, n=1; Tobacco use, n=4; Tumour stage, n=2; p53, n=10; pRB, n=6; Cyclin D1, n=4; Ki67, n=5

[b]Other oropharynx includes: Soft palate (n=2), Oropharyngeal wall (n=2), Uvula (n=1)

[c]Not analyzed

Abbreviations: OS: Overall survival; DSS: Disease-specific survival; LRR: Loco-regional recurrence; HPV, Human papillomavirus; AJCC, American Joint Committee on Cancer (7th ed.); RT: Radiotherapy; CRT: Concurrent chemoradiotherapy; pRB: Retinoblastoma protein

Appendix M: Univariate Cox-PH analysis of clinical outcomes in HPV-positive patients

Variables[a]	5-year OS		DSS		LRR	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age						
<55	1.0		1.0		1.0	
≥55	1.8 (0.9-3.3)	0.08	1.6 (0.8-3.1)	0.18	1.1 (0.6-2.3)	0.75
Sex						
Male	1.0		1.0		1.0	
Female	0.5 (0.2-1.3)	0.18	0.2 (0.1-1.0)	0.05	0.3 (0.1-1.1)	0.07
Anatomical site						
Tonsil	1.0		1.0		1.0	
Base of tongue	0.7 (0.3-1.6)	0.37	0.5 (0.2-1.5)	0.25	0.3 (0.1-1.2)	0.09
Other oropharynx[b]	N/A[c]		N/A[c]		N/A[c]	
Tumour stage						
T0/1	1.0		1.0		1.0	
T2	1.3 (0.6-2.8)	0.57	1.2 (0.5-3.0)	0.72	1.2 (0.5-2.9)	0.74
T3	1.5 (0.6-3.7)	0.43	1.9 (0.7-5.1)	0.20	1.8 (0.7-5.0)	0.25
T4	3.9 (1.5-10.1)	<0.01	4.4 (1.6-12.7)	**<0.01**	2.4 (0.7-8.1)	0.17
Nodal stage						
N0	1.0		1.0		1.0	
N1	1.7 (0.5-5.6)	0.39	1.3 (0.4-4.5)	0.70	1.4 (0.4-4.9)	0.56
N2	1.6 (0.6-4.5)	0.39	1.2 (0.4-3.6)	0.73	1.1 (0.4-3.1)	0.92
N3	2.5 (0.7-8.5)	0.15	2.5 (0.7-8.5)	0.15	1.3 (0.3-5.4)	0.68
AJCC staging						
I/II	1.0		1.0		1.0	
III/IV	1.7 (0.5-5.5)	0.37	1.4 (0.4-4.6)	0.57	1.2 (0.4-4.1)	0.73

[a]Missing data: Anatomical site, n=1
[b]Other oropharynx includes: Oropharyngeal wall (n=1)
[c]Not analyzed
Abbreviations: OS: Overall survival; DSS: Disease-specific survival; LRR: Loco-regional recurrence; HR: Hazard ratio; AJCC, American Joint Committee on Cancer (7th ed.)

Appendix N: Univariate Cox-PH analysis of clinical outcomes in HPV-negative patients

Variables	5-year OS HR (95% CI)	p-value	DSS HR (95% CI)	p-value	LRR HR (95% CI)	p-value
Age						
<55	1.0		1.0		1.0	
≥55	2.0 (0.7-5.6)	0.21	4.5 (0.6-33.6)	0.15	1.1 (0.4-3.4)	0.81
Sex						
Male	1.0		1.0		1.0	
Female	1.3 (0.7-2.7)	0.43	1.3 (0.5-3.3)	0.63	1.4 (0.6-3.3)	0.48
Anatomical site						
Tonsil	1.0		1.0		1.0	
Base of tongue	0.9 (0.4-1.9)	0.72	0.8 (0.2-2.4)	0.63	1.1 (0.4-3.0)	0.79
Other oropharynx[a]	N/A[b]		N/A[b]		N/A[b]	
Tumour stage						
T0/1	1.0		1.0		1.0	
T2	1.5 (0.5-4.8)	0.51	1.6 (0.3-8.2)	0.58	4.9 (0.6-39.3)	0.13
T3	2.5 (0.8-7.7)	0.10	2.8 (0.6-13.0)	0.20	4.5 (0.6-36.9)	0.16
T4	2.1 (0.5-7.7)	0.29	1.6 (0.2-11.3)	0.65	5.6 (0.6-50.3)	0.12
Nodal stage						
N0	1.0		1.0		1.0	
N1	1.5 (0.6-4.1)	0.42	3.1 (0.7-13.2)	0.12	0.7 (0.2-2.5)	0.56
N2	0.8 (0.3-1.8)	0.56	1.6 (0.4-5.8)	0.51	0.5 (0.2-1.4)	0.21
N3	N/A[b]		N/A[b]		N/A[b]	
AJCC staging						
I/II	1.0		1.0		1.0	
III/IV	1.9 (0.6-6.2)	0.29	3.1 (0.4-23.5)	0.27	1.1 (0.3-3.7)	0.89

[a]Other oropharynx includes: Soft palate (n=2), Oropharyngeal wall (n=1), Uvula (n=1)
[b]Not analyzed
Abbreviations: OS: Overall survival; DSS: Disease-specific survival; LRR: Loco-regional recurrence; HR: Hazard ratio; AJCC, American Joint Committee on Cancer (7th ed.)
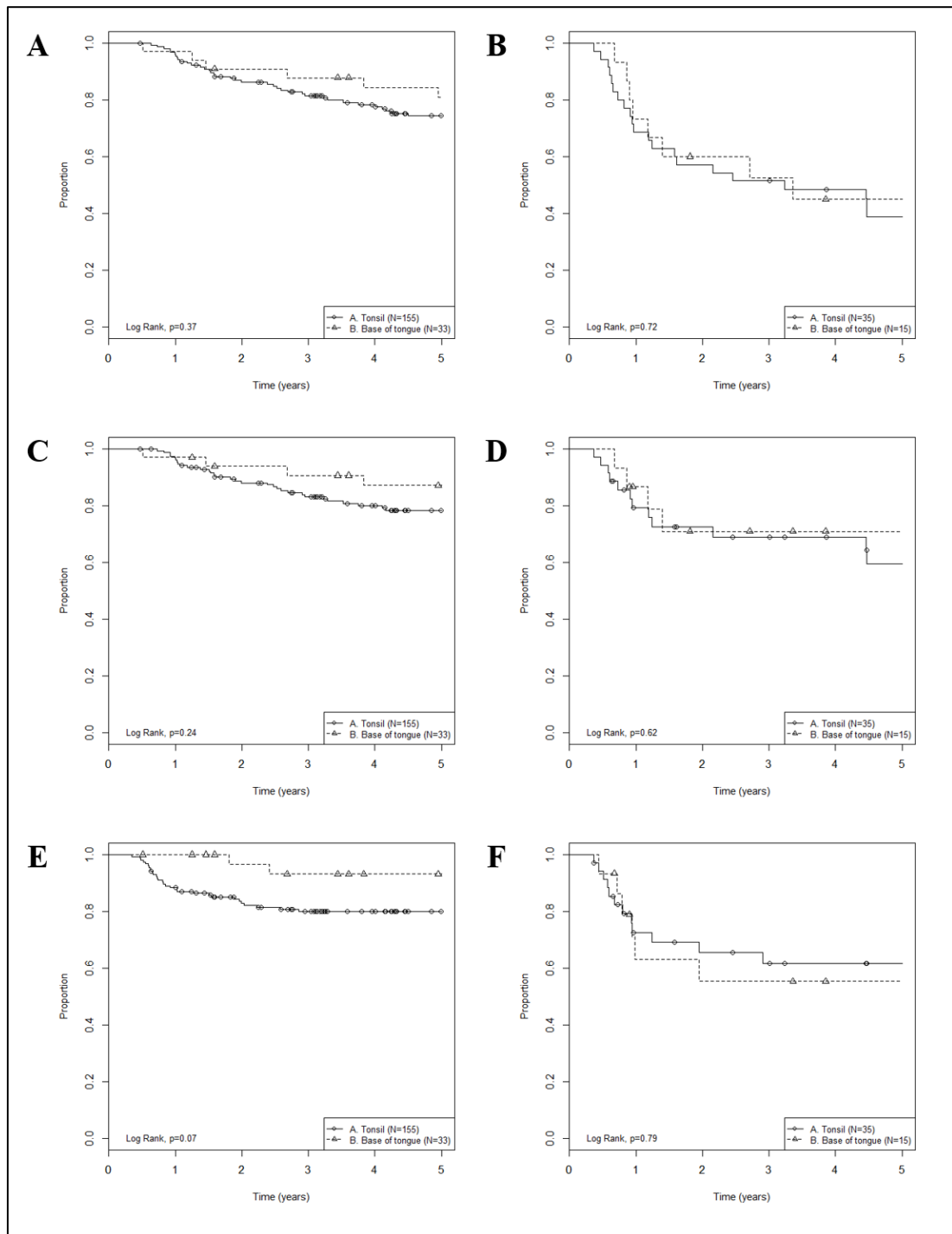
Appendix O: KM survival analysis of age
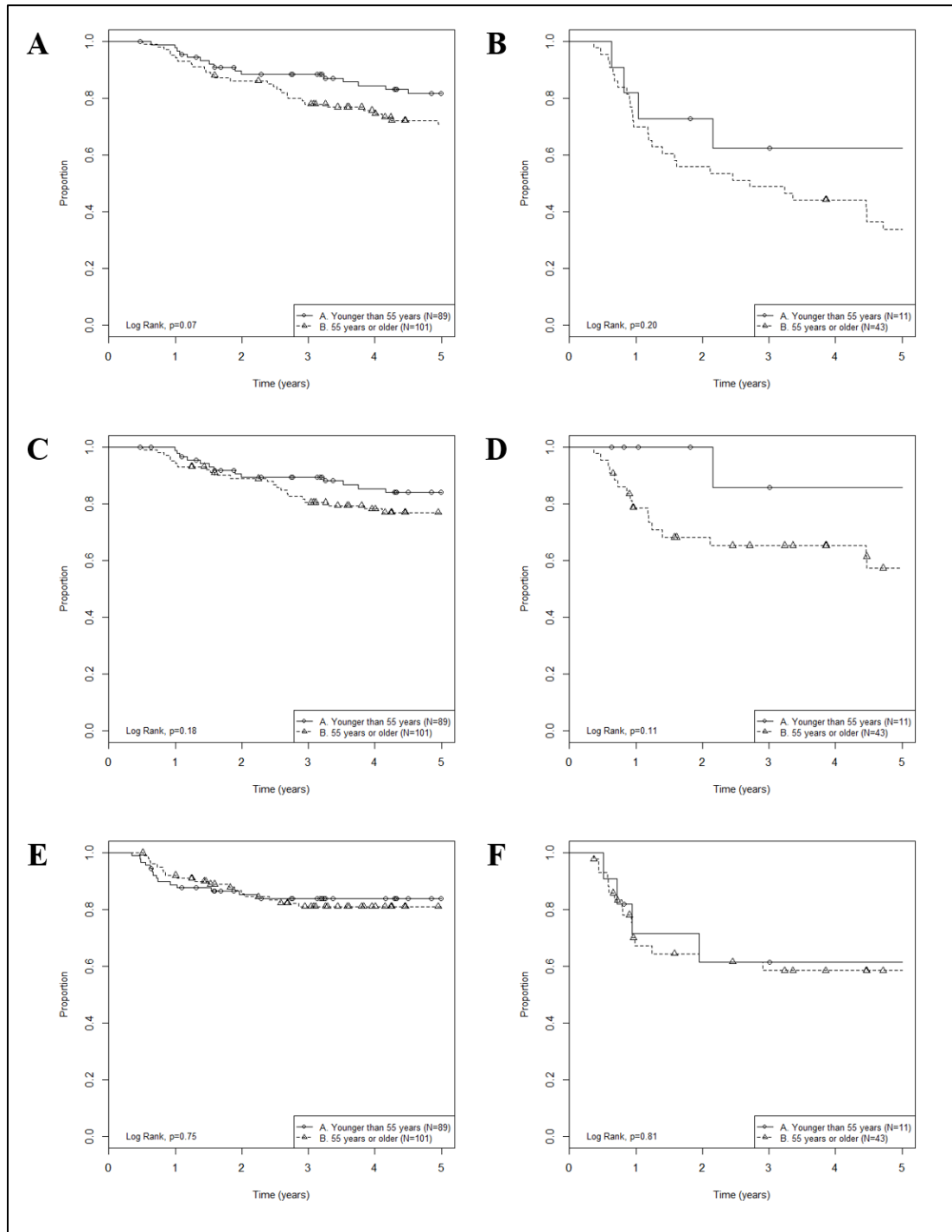
Patients were initially separated into HPV-positive (left) and HPV-negative (right) status. Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F). Please refer to Appendices W and X for survival rates.

Appendix P: KM survival analysis of sex
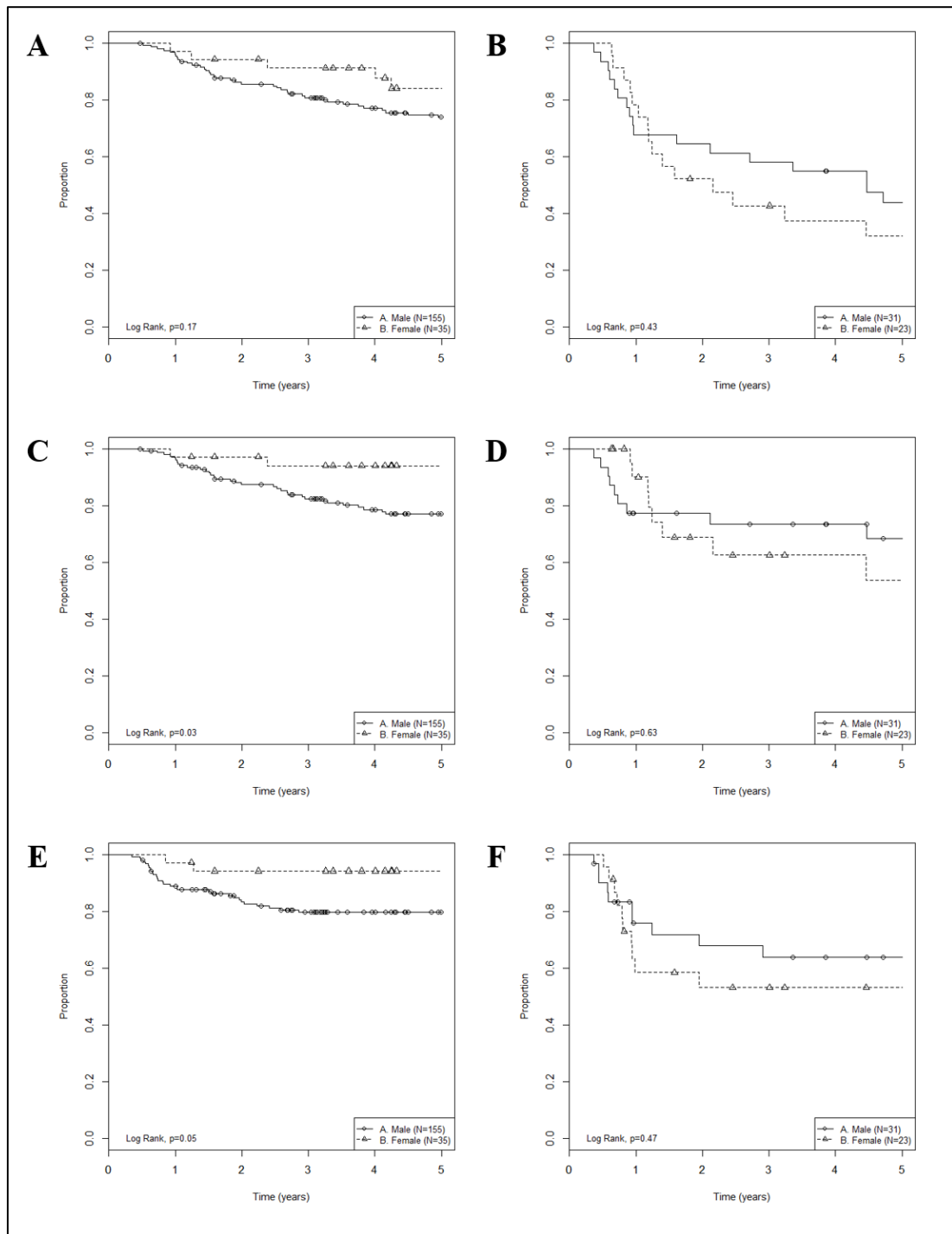
Patients were initially separated into HPV-positive (left) and HPV-negative (right) status. Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F). Please refer to Appendices W and X for survival rates.

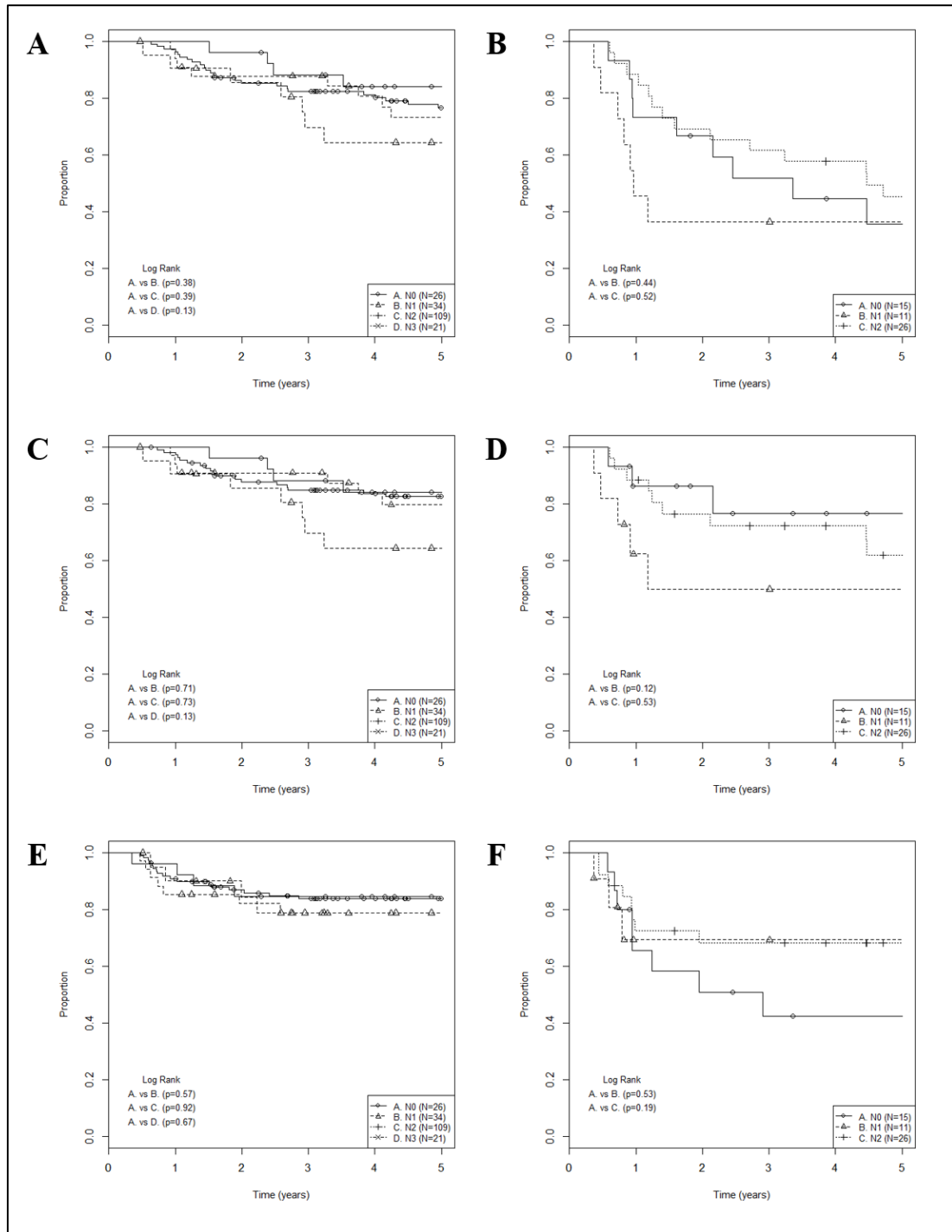**Appendix Q: KM survival analysis of anatomical site**

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status.
Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F).
Please refer to Appendices W and X for survival rates.

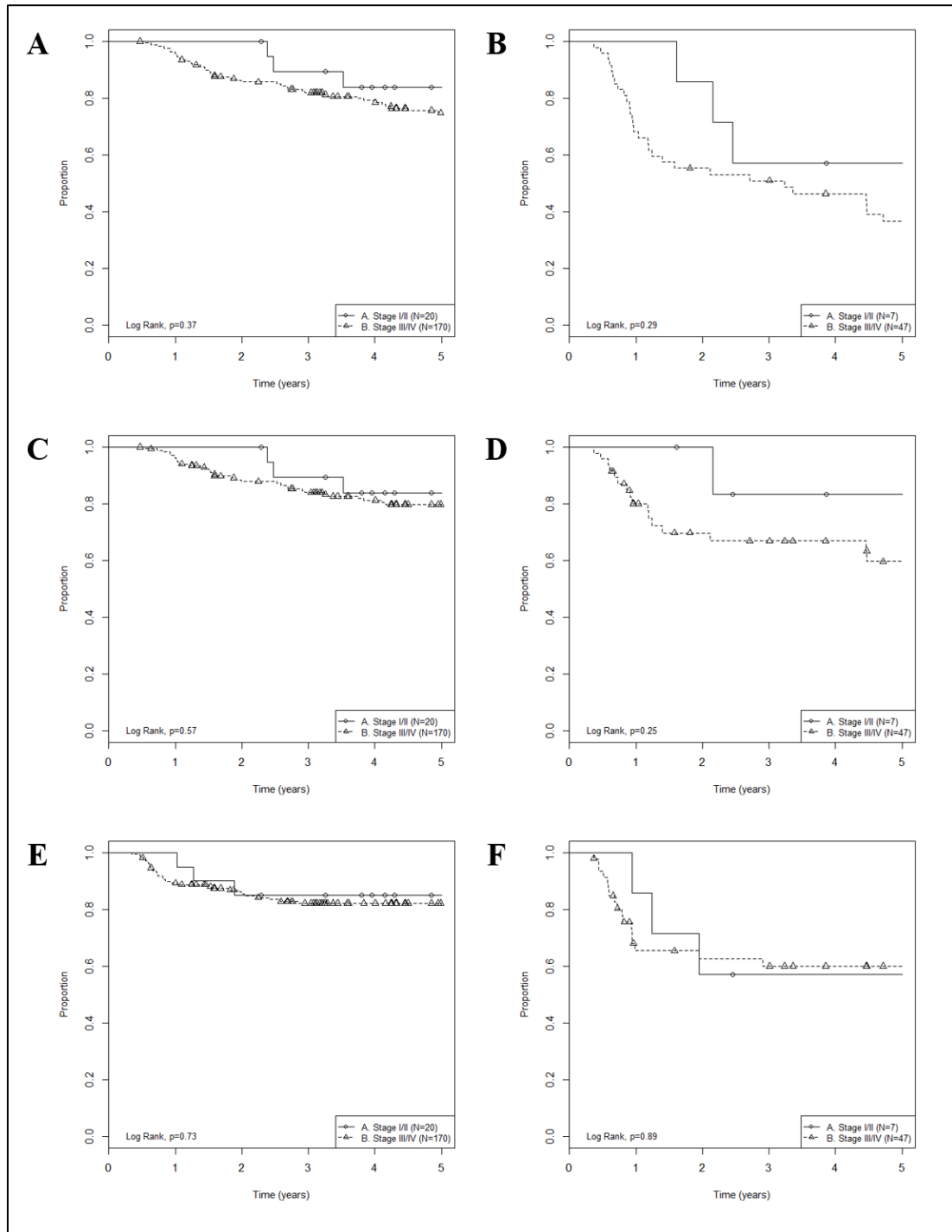**Appendix R: KM survival analysis of nodal staging**

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status.

Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F).

Please refer to Appendices W and X for survival rates.

Appendix S: KM survival analysis of AJCC staging

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status. Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F). Please refer to Appendices W and X for survival rates.

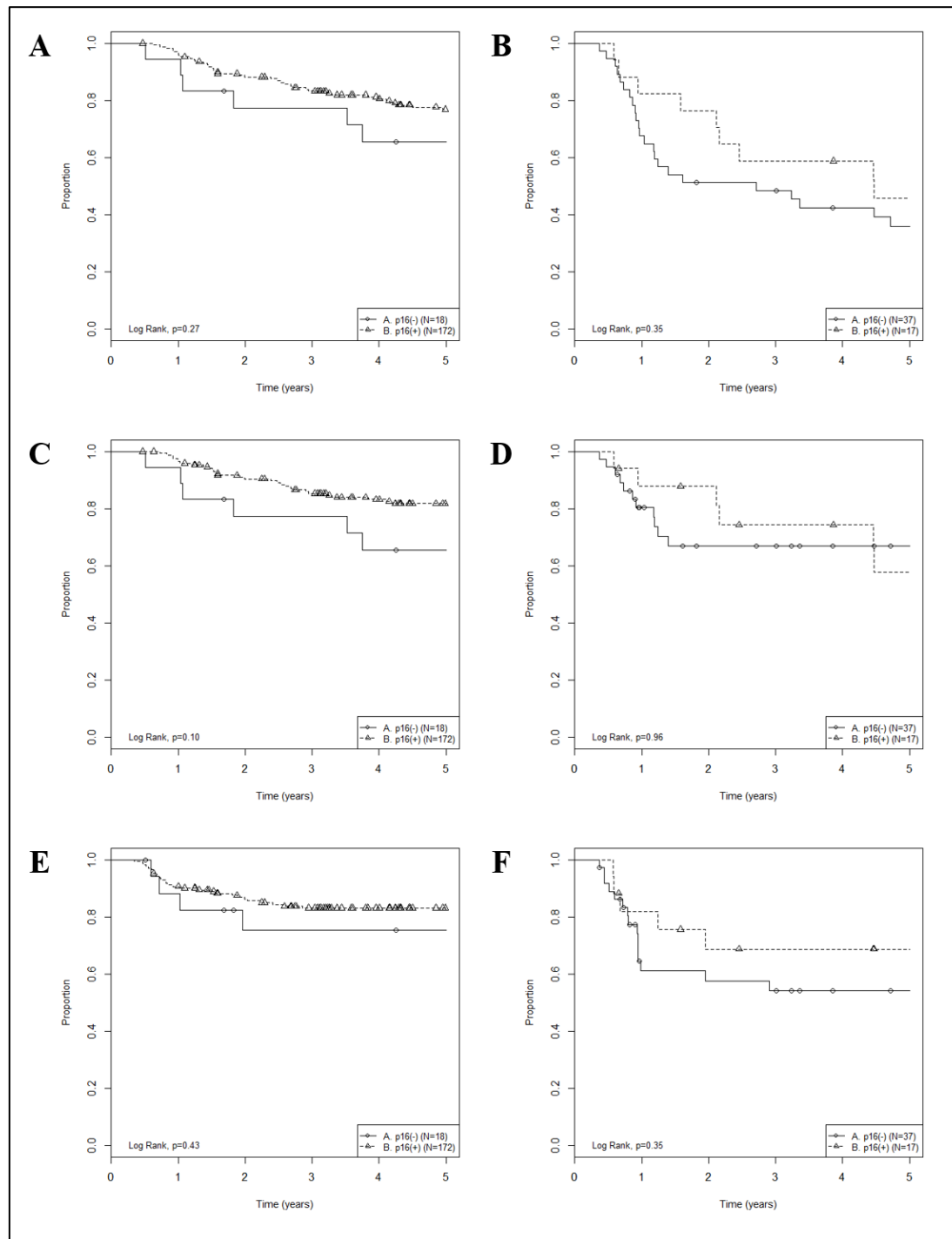**Appendix T: KM survival analysis of p16 status**

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status. Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F). Please refer to Appendices W and X for survival rates.

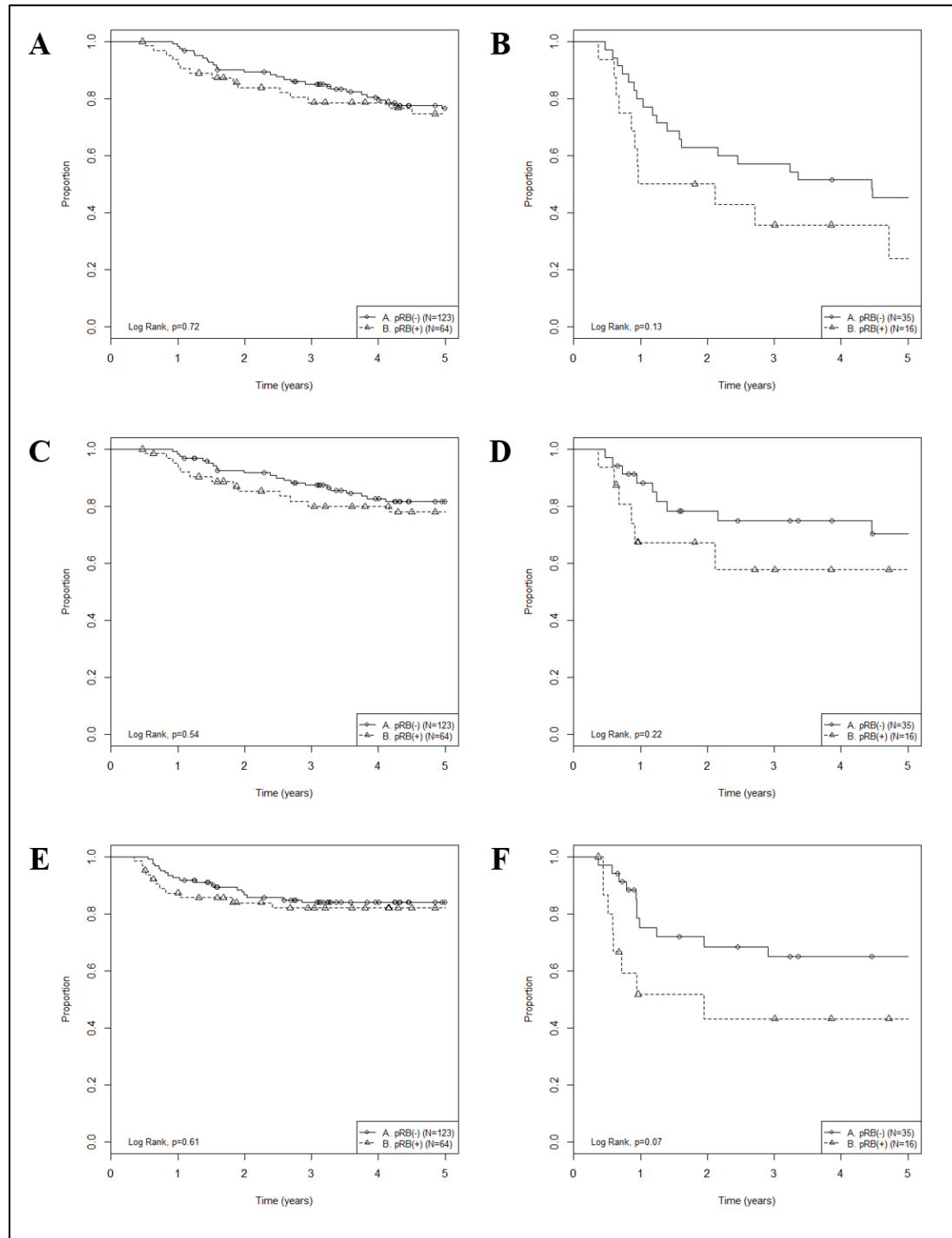## Appendix U: KM survival analysis of pRB status

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status.

Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F).

Please refer to Appendices W and X for survival rates.

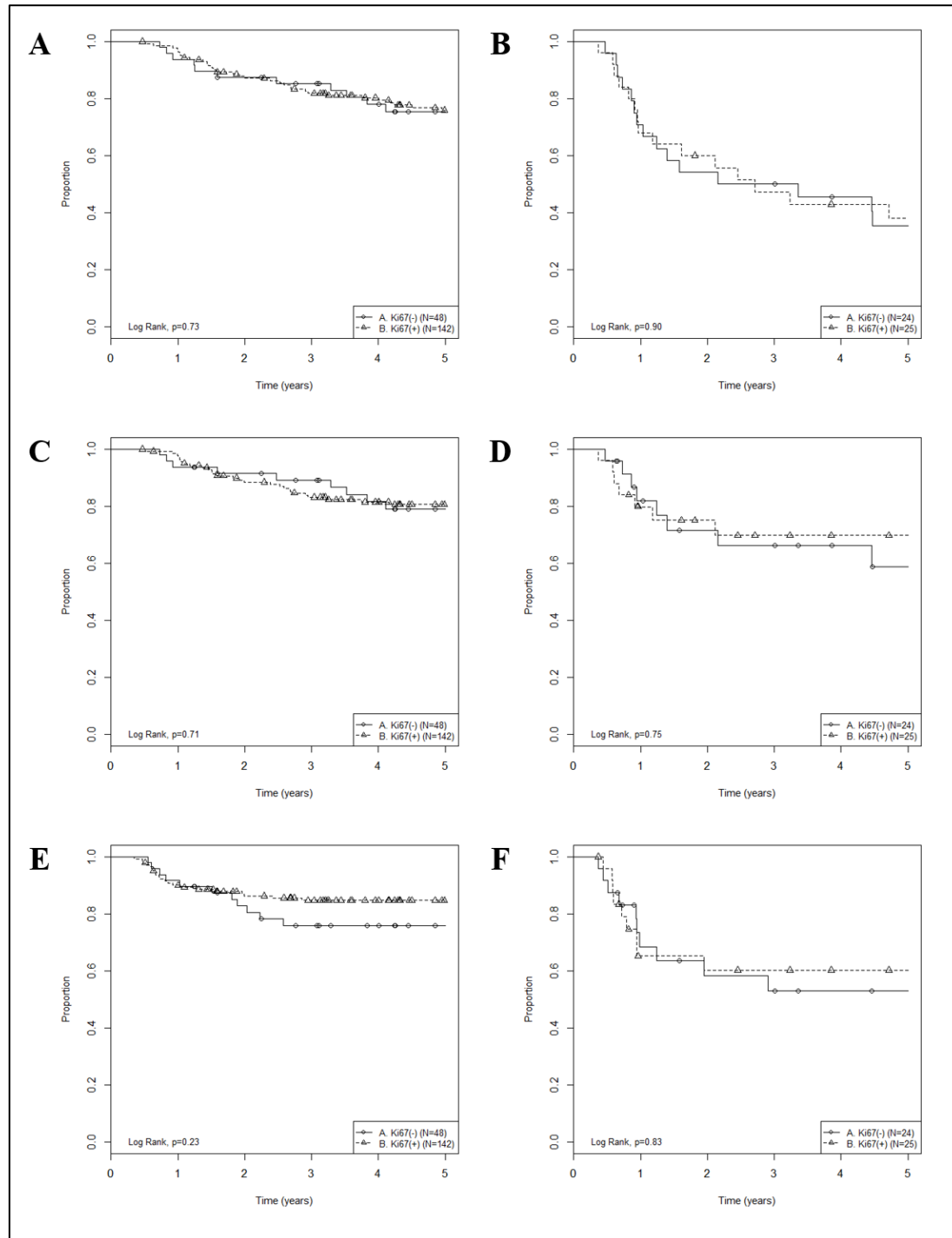## Appendix V: KM survival analysis of Ki67 status

Patients were initially separated into HPV-positive (left) and HPV-negative (right) status.
Analysis conducted for 5-year OS (A, B), DSS (C, D), and development of LRR (E, F).
Please refer to Appendices W and X for survival rates.

Appendix W: HPV-positive KM survival analysis for rates of clinical outcomes

Variables[a]	5-year OS Rate (95% CI)	DSS Rate (95% CI)	No LRR Rate (95% CI)
Age			
<55	81.6% (73.5-90.5%)	83.9% (76.3-92.4%)	83.9% (76.5-92.0%)
≥55	70.8% (62.2-80.6%)	76.9% (68.9-85.9%)	81.0% (73.5-89.4%)
Sex			
Male	73.8% (67.0-81.4%)	77.1% (70.5-84.3%)	79.7% (73.4-86.5%)
Female	84.1% (72.1-98.1%)	94.0% (86.3-100.0%)	94.2% (86.7-100.0%)
Anatomical site			
Tonsil	74.4% (67.5-81.9%)	78.4% (71.9-85.5%)	80.0% (73.8-86.7%)
Base of tongue	81.0% (68.4-96.0%)	87.1% (76.1-99.8%)	93.1% (84.3-100.0%)
Other oropharynx[b]	N/A[c]	N/A[c]	N/A[c]
Smoking history			
Never-smoker	91.7% (84.3-99.8%)	93.7% (87.0-100.0%)	93.6% (86.8-100.0%)
Ever-smoker	70.3% (62.8-78.8%)	75.6% (68.5-83.5%)	78.7% (71.9-86.0%)
Tumour stage (reclass)			
T0/1/2	78.8% (71.9-86.5%)	84.6% (78.4-91.3%)	84.6% (78.6-91.1%)
T3/4	66.6% (54.8-80.9%)	68.1% (56.4-82.3%)	76.2% (65.2-88.9%)
Nodal stage			
N0	83.9% (70.7-99.7%)	83.9% (70.7-99.7%)	84.6% (71.8-99.7%)
N1	73.3% (58.9-91.2%)	79.7% (66.3-95.8%)	78.7% (65.9-94.1%)
N2	76.7% (68.9-54.4%)	82.5% (75.5-90.2%)	83.7% (76.9-91.1%)
N3	64.3% (46.2-89.6%)	64.3% (46.2-89.6%)	78.8% (62.2-99.7%)
AJCC staging			
I/II	83.9% (68.7-100.0%)	83.9% (68.7-100.0%)	85.0% (70.7-100.0%)
III/IV	74.9% (68.4-82.0%)	79.7% (73.7-86.3%)	82.1% (76.4-88.3%)
Primary treatment			
RT	70.3% (61.6-80.3%)	74.8% (66.5-84.1%)	76.7% (68.7-85.5%)
CRT	82.1% (74.2-90.8%)	86.5% (79.4-94.3%)	89.0% (82.5-96.1%)

Variables[a]	5-year OS Rate (95% CI)	DSS Rate (95% CI)	No LRR Rate (95% CI)
p16 status			
Negative	65.5% (46.5-92.2%)	65.5% (46.5-92.2%)	75.5% (57.1-99.7%)
Positive	76.9% (70.5-83.8%)	81.8% (76.0-88.1%)	83.1% (77.5-89.0%)
p53 status			
Negative	77.6% (70.7-85.2%)	83.7% (77.5-90.4%)	83.7% (77.7-90.2%)
Positive	68.6% (56.1-83.8%)	68.6% (56.1-83.8%)	82.2% (71.7-94.2%)
pRB status			
Negative	76.5% (69.1-84.7%)	81.6% (74.7-89.1%)	84.0% (77.6-90.9%)
Positive	74.7% (64.3-86.7%)	77.9% (68.0-89.4%)	82.0% (72.9-92.3%)
Cyclin D1 status			
Negative	76.1% (69.6-83.3%)	81.3% (75.2-87.8%)	83.9% (78.3-89.9%)
Positive	76.7% (62.0-94.9%)	76.7% (62.0-94.9%)	76.5% (61.6-95.0%)
Ki67 status			
Negative	75.4% (63.7-89.3%)	78.9% (67.5-92.3%)	75.8% (64.3-89.4%)
Positive	75.9% (68.9-83.6%)	80.6% (74.1-87.6%)	84.7% (78.8-90.9%)

[a]Missing data: Anatomical site, n=1; Tobacco use, n=3; Tumour stage, n=2; p53, n=3; pRB, n=3; Cyclin D1, n=1

[b]Other oropharynx includes: Oropharyngeal wall (n=1)

[c]Not analyzed

Abbreviations: OS: Overall survival; DSS: Disease-specific survival; LRR: Loco-regional recurrence; AJCC: American Joint Committee on Cancer (7th ed.); RT: Radiotherapy; CRT: Concurrent chemoradiotherapy; pRB: Retinoblastoma protein

Appendix X: HPV-negative KM survival analysis for rates of clinical outcomes

Variables[a]	5-year OS Rate (95% CI)	DSS Rate (95% CI)	No LRR Rate (95% CI)
Age			
<55	62.3% (38.9-99.9%)	85.7% (63.3-100.0%)	61.4% (37.7-99.9%)
≥55	33.8% (22.1-51.8%)	57.4% (42.9-76.6%)	58.4% (44.6-76.6%)
Sex			
Male	43.9% (29.2-65.9%)	68.3% (52.7-88.5%)	63.9% (48.1-84.9%)
Female	32.0% (17.1-59.8%)	53.6% (33.6-85.5%)	53.1% (35.5-79.5%)
Anatomical site			
Tonsil	38.7% (25.3-59.3%)	59.4% (43.5-81.2%)	61.6% (46.5-81.7%)
Base of tongue	45.0% (25.3-80.0%)	70.9% (50.4-99.8%)	55.3% (34.0-89.9%)
Other oropharynx[b]	N/A[c]	N/A[c]	N/A[c]
Smoking history			
Never-smoker	N/A[c]	N/A[c]	N/A[c]
Ever-smoker	37.2% (25.6-54.1%)	62.8% (49.0-80.5%)	56.0% (43.1-72.8%)
Tumour stage (reclass)			
T0/1/2	51.9% (36.1-74.6%)	72.3% (56.7-92.2%)	64.1% (47.6-86.1%)
T3/4	24.3% (11.8-50.4%)	50.5% (31.3-81.6%)	54.0% (37.1-78.7%)
Nodal stage			
N0	35.6% (17.1-73.8%)	76.6% (56.2-100.0%)	42.4% (22.7-79.3%)
N1	36.4% (16.6-79.5%)	49.9% (26.2-94.9%)	69.3% (45.3-100.0%)
N2	45.3% (29.5-69.5%)	61.8% (44.7-85.5%)	68.1% (52.1-89.1%)
N3	N/A[c]	N/A[c]	N/A[c]
AJCC staging			
I/II	57.1% (30.1-100.0%)	83.3% (58.3-100.0%)	57.1% (30.1-100.0%)
III/IV	36.5% (24.8-53.9%)	59.7% (45.7-78.0%)	60.0% (46.7-77.0%)
Primary treatment			
RT	31.0% (18.8-51.0%)	61.2% (46.0-81.5%)	51.6% (36.4-73.2%)
CRT	54.4% (35.1-84.4%)	66.8% (46.1-96.9%)	71.6% (53.3-96.2%)

Variables[a]	5-year OS Rate (95% CI)	DSS Rate (95% CI)	No LRR Rate (95% CI)
p16 status			
Negative	35.9% (23.0-56.1%)	67.1% (52.7-85.4%)	54.3% (39.5-74.7%)
Positive	45.8% (26.9-77.7%)	57.8% (36.6-91.2%)	68.8% (49.3-95.9%)
p53 status			
Negative	33.6% (19.4-58.2%)	65.9% (48.9-88.7%)	44.4% (27.9-70.8%)
Positive	41.2% (24.4-69.9%)	68.4% (50.3-93.1%)	67.5% (49.0-93.0%)
pRB status			
Negative	45.4% (31.5-65.4%)	70.4% (55.7-89.1%)	64.9% (50.1-84.2%)
Positive	23.8% (8.3-68.0%)	57.7% (36.3-91.7%)	43.2% (23.4-79.9%)
Cyclin D1 status			
Negative	64.7% (45.5-91.9%)	75.5% (57.1-99.7%)	76.5% (58.7-99.5%)
Positive	24.7% (13.0-47.0%)	55.5% (38.4-80.2%)	47.7% (32.2-70.9%)
Ki67 status			
Negative	35.4% (20.1-62.1%)	58.8% (39.9-86.7%)	53.0% (35.2-79.7%)
Positive	38.1% (22.8-63.8%)	69.7% (53.1-91.7%)	60.2% (42.9-84.5%)

[a]Missing data: Tobacco use, n=1; p53, n=7; pRB, n=3; Cyclin D1, n=3; Ki67, n=5

[b]Other oropharynx includes: Soft palate (n=2), Oropharyngeal wall (n=1), Uvula (n=1)

[c]Not analyzed

Abbreviations: OS: Overall survival; DSS: Disease-specific survival; LRR: Loco-regional recurrence; AJCC, American Joint Committee on Cancer (7th ed.); RT: Radiotherapy; CRT: Concurrent chemoradiotherapy pRB: Retinoblastoma protein