Detection of High-Risk Human Papillomavirus Provides Important Diagnostic and Prognostic Information in Oropharyngeal Cancer

by

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Abstract

The prevalence of oncogenic human papillomavirus (HPV) -related oropharyngeal squamous cell carcinoma (OPSCC) is increasing worldwide. The standard for HPV diagnosis is based on p16 immunohistochemistry (IHC) on tissues, as a surrogate marker for HPV oncogenesis in clinical and trial settings with no HPV-specific testing confirmation required. HPV diagnosis is important because patients with HPV-related OPSCC have favorable prognoses compared to HPV-negative, due to unique pathologic and clinical characteristics that make the disease more responsive to certain treatment protocols. The problem with p16 overexpression as a surrogate biomarker is that it can be inaccurate in up to 25% of cases leading to misdiagnosis and mistreatment because p16 biomarker overexpression can occur via HPV-independent mechanisms. Furthermore, most methods of HPV detection in HNSCC including p16 IHC, require a fine needle aspirate (FNA) or tissue biopsy.

To address this issue, we looked at utilizing a new generation PCR technology called droplet digital PCR which is thought to be highly accurate for molecular diagnosis. We have developed a minimally invasive rapid assay using ddPCR for the early detection of 12 high-risk (hr)- HPV in salivary swab samples. We compared the hrHPV-ddPCR results to p16 IHC to measure concordance and diagnostic accuracy. We determined the distribution of hr-HPV types and survival outcomes according to p16 and HPV status in OPSCC. Our results showed that ddPCR was highly accurate compared to p16 IHC. Regarding HPV strains, we found that compared to HPV-16, other oncogenic strains were associated with lower survival and outcomes comparable to HPV- patients. Comparative survival analyses were performed for OPSCC participants according to p16, HPV,

p16/HPV combination, HPV types and presence of multiple HPV types. P16+ (vs p16-), HPV+ (vs HPV-), p16+/HPV+ (vs p16+/HPV-, p16-/HPV+ and p16-/HPV-), HPV type 16 (vs other hr-HPV strains) and single HPV+ present (vs 2-3HPV+) all have better prognosis. Further comparative survival analyses were performed between p16+ and p16-, HPV+ and HPV- and p16/HPV combination; p16+/HPV+ vs p16+/HPV-, p16-/HPV+ and p16-/HPV-, stratified according to age, sex, smoking, T-stage, N-stage and treatment. In our cohort, the prognosis of patients with discordant p16+/HPV- depends on age, particularly 50-69, former and current smokers, T1-T3 stage, N1 stage and treatment surgery+ radiotherapy (S+RT) and surgery+ chemo radiotherapy (S+CRT).

The use of hr-HPV-ddPCR testing in oropharyngeal swabs is minimally invasive, highly accurate and may improve the diagnostic accuracy of HPV-OPSCC, while providing important prognostic information. This may have potential clinical applications for early diagnosis, screening during post-treatment surveillance, cases of unknown primary tumors, cases where a tissue biopsy may be difficult or prohibitive, and in resource-limited setting. It is important to detect HPV types as some strains are more aggressive. HPV testing should be performed along with p16 IHC since discordant p16 and HPV have lower survival and HPV status may influence patient care.

Preface

This thesis is an original work by Jordana Williams. Research ethics approval was obtained from the University of Alberta Research Ethics Board - Biomedical Panel, "Droplet Digital Polymerase Chain Reaction for Detection of Human Papillomavirus in Patients with Cervical and Oropharyngeal Cancer", Study ID: Pro00062302, 2016-Apr-29 and Health Research Ethics Board of Alberta - Cancer Committee, "Droplet Digital Polymerase Chain Reaction (ddPCR) for Early Detection of Human Papillomavirus (HPV) - Related Oropharyngeal and Cervical Cancer: A Prospective Cohort Study", Study ID: HREBA.CC-18-0216, 2019-Nov-22. The research conducted for this thesis is part of a University of Alberta collaboration, led by Vincent Biron, MD, PhD, FRCSC, with Morris Kostiuk, PhD being the research scientist, and Hadi Seikaly, MD, MAL, FRCSC, Jeff Harris, MD, MHA, FRCSC, and Daniel O'Connell, MD, MSc, FRCSC contributing to this research. Chapter 1 introduction and literature review, chapter 3 results, chapter 4 discussion and chapter 5 conclusion and future directions are my original work. I performed some of the technical aspects involved in chapter 2 methods with Dr. Morris Kostiuk's guidance. Dr. Morris Kostiuk was involved in conceptualizing the study, lab and data analysis. Dr. Vincent Biron was involved in conceptualizing the study, patient recruitment, patient specimen collection, lab and data analysis.

Chapter 1 introduction and literature review of this thesis have been published as Williams J, Kostiuk M, Biron VL. "Molecular Detection Methods in HPV-related Cancers." *Front Oncol.* (2022) DOI: 10.3389/fonc.2022.864820. J. Williams was responsible for designing, writing, editing and revising the manuscript. M. Kostiuk contributed to the editing of the first draft. V. Biron was responsible for the supervision and

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Parts of the methods, results, discussion and conclusion are reproduced from the following study and are being prepared for publication.

1. A Novel High-risk HPV Detection Panel for Increased Diagnostic Accuracy in Oropharyngeal Squamous Cell Carcinoma

2. Detection of High-Risk Human Papillomavirus Provides Important Prognostic Information in Oropharyngeal Cancer

Dedications

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List of Abbreviations

1HPV+	one HPV type
2-3HPV+	two to three HPV types
Ab	antibodies
AC	adenocarcinoma
ACC	adenoid cystic carcinoma
ACOG	American College of Obstetricians and Gynecologists
Ag	antigens
AJCC8	American Joint Commission on Cancer 8th edition
AP	alkaline phosphatase
ASC	atypical squamous cells
ASCCP	American Society for Colposcopy and Cervical Pathology
ASCO	American Society of Clinical Oncology
ATHENA	Addressing the Need for Advanced HPV Diagnostics
ATP	adenine triphosphate
CAC	cervical adenocarcinoma
CAP	College of American Pathologists
CC	cervical cancer
CDK	cyclin-dependent kinase

CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary DNA
CI	Confidence interval
CIN	cervical intraepithelial neoplasia
CNV	copy number variations
cRNA	complementary RNA
CRT	Chemo radiotherapy
CSCC	cervical squamous cell carcinoma
ctDNA	circulating tumoral DNA
CTP	cytosine triphosphate
ddPCR	droplet digital polymerase chain reaction
DFS	Disease-Free Survival
DKA	Dual Kinetic Assay
DSS	Disease-Specific Survival
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
E6AP	E6-associated protein
FFPE	formalin-fixed, paraffin-embedded
FNA	fine needle aspirate

GLOBOCAN	Global Cancer Observatory
GTP	guanine triphosphate
HC	Hybrid Capture
HDR	Homology directed repair
HMSC	HPV-related multiphenotypic sinonasal carcinoma
HNCUP	Head and neck cancer of unknown primary
HNSCC	Head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	Hazard ratio
hr-HPV	high-risk HPV
HSIL	high-grade squamous intraepithelial lesion
IARC	International Agency for Research on Cancer
IC	internal control
IHC	immunohistochemistry
ISH	in-situ hybridization
LBC	liquid-based cytology
lr-HPV	low-risk HPV
LSIL	low-grade squamous intraepithelial lesion
mAbs	monoclonal antibodies

mRNA	messenger RNA
NGS	next generation sequencing
NHEJ	non-homologous end joining
NLR	negative likelihood ratio
NPV	negative predictive value
OCSCC	oral cavity squamous cell carcinoma
OPSCC	oropharyngeal squamous cell carcinoma
OS	Overall Survival
PCR	polymerase chain reaction
PLR	positive likelihood ratio
PPV	positive predictive value
Rb	retinoblastoma protein
S/CO	signal to cutoff
RLU	relative light units
RNA	ribonucleic acid
RT	radiotherapy
RT-PCR	real-time PCR
RT-qPCR	real-time quantitative PCR
SCC	squamous cell carcinoma

S + CRT	Surgery + Chemoradiation Therapy
SNSCC	sinonasal squamous cell carcinoma
S + RT	Surgery + Radiation Therapy
STI	sexually transmitted infection
TMA	transcription-mediated amplification
TTP	thymine triphosphate
USPSTF	US Preventive Services Task Force
VIA	visual inspection of cervix with acetic acid
VL	viral load

Chapter 1: Introduction and Literature Review

Sections 1.1-1.8 of this chapter have been previously published as:

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At the end of Section 1.5: Cervical Cancer Screening and Diagnostic tools for HPV Detection, next-generation sequencing and MassARRAY were added in pages 27-29.

At the end of Section 1.6: OPSCC Screening and Diagnostic tools for HPV Detection, studies using next-generation sequencing and MassARRAY were added in pages 34-36.

1.1 Introduction

Human Papillomavirus (HPV) is the most common sexually transmitted infection (STI) in the world (1) and classified as a carcinogenic infectious agent by the International Agency for Research on Cancer (IARC) (2). Both sexually active men and women will be infected at least once without developing any symptoms or cancerous diseases in their lifetime (1). However, only some HPV strains are oncogenic. These have been shown to cause most cervical cancers, some head and neck cancers, particularly in the oropharynx (3,4) and to a lesser extent in the sinonasal region (5). HPV testing is important clinically, for the accuracy of diagnosis, patient-centered treatment and prognostication (3,6–11).

Cervical cancer screening and diagnosis is minimally invasive. It combines liquid-based cytology stained Papanicolaou stain (Pap smear) and HPV testing using deoxyribonucleic acid (DNA)/ ribonucleic acid (RNA) PCR-based methods (12,13). The association between cervical squamous cell carcinoma (CSCC) and HPV is well established as HPV is known to cause most cervical cancers (1–3,12,13). In developed countries, cervical cancer has been effectively controlled by cytological screening, which involves physician-administered cervical samples and directed cervical exams which are interpreted by a trained cytopathologist. However, in low- and middle-income countries where the burden of cervical cancer is the highest (1,2), such established screening programs are not available or feasible. Some of the barriers that affect the success of screening programs include the availability of physicians, trained personnel that can interpret sample results, access to equipment and technology and social and cultural issues (14). To overcome these drawbacks, recent studies have investigated the use of self-sampling swabs for HPV detection to replace pap smears and cervical exams as first-line screening. Their results showed that self-sampling has greater sensitivity compared to traditional cytology and similar sensitivity to clinician-collected specimens (14–16). The studies suggested that self-sampled HPV testing can be cost-effective and can be used as a primary screening strategy or in addition to existing screening programs. By self-sampling, the cost of testing can be lowered, the level of screening attendance will be increased, and can attract long-term under-screened women or never screened women to participate (17). However, HPV assays that have been developed have limited sensitivity, specificity and replicability in resource-limited settings (12,13,18).

For head and neck cancers, p16INK4a (p16) immunohistochemistry (IHC) is a widely used surrogate marker for oncogenic HPV (19,20). Since HPV-related SCC in the head and neck region is predominantly seen in the oropharyngeal, p16 IHC testing is considered an acceptable clinical standard for the diagnosis of oropharyngeal SCC. Although sinonasal SCC is thought to be associated with HPV in many cases, p16 or direct HPV testing is not routinely done for these cancers (21,22). Most methods of HPV detection in head and neck SCC including p16 IHC, require a fine needle aspirate (FNA) or tissue biopsy (19,20). This can often be limiting because special equipment is needed to acquire FNA samples and tissue biopsies are often invasive and resource intensive as they are obtained under general anesthesia.

Droplet digital polymerase chain reaction (ddPCR) is a promising technology for the minimally invasive detection of oncogenic HPV. It allows for the quantification of the absolute amount of target nucleic acid present with high precision and reproducibility (23). DdPCR involves partitioning a single nucleic acid sample into up to 20,000 uniform, nanoliter-sized water-in-oil droplets, amplifying them by PCR, analyzing each droplet individually and reporting the results digitally (23,24). This method quantifies the absolute amount of target nucleic acid present with high accuracy and reproducibility that is several orders of magnitude higher than traditional PCR (24). DdPCR is a highly sensitive method for the identification of oncogenic HPV as it is able to quantify gene expression with extremely low copy numbers (25–27). This method can be applied in the early detection of oncogenic HPV in swabs from the oropharynx, sinonasal and cervix.

1.2 Epidemiology

HPV infection is recognized as one of the major causes of viral-related cancers in both men and women. It is classified into two categories: low-risk HPVs (LR-HPVs) which are responsible for skin warts on hands, feet and around genitals and anus, and high-risk HPVs (hr-HPVs) associated with anogenital (cervical, anal, vaginal, vulvar and penile) and head and neck cancers (oropharyngeal and sinonasal) (1). There are more than 200 genotypes of HPV but only a few are considered carcinogenic. There are as many as 15 hr-HPV types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) and globally, HPV 16 is the most frequent oncogenic type (1–4). It is estimated that 4.5% of all cancers worldwide (630,000 new cancer cases per year) are attributable to HPV infection: 8.6% in women and 0.8% in men. **Table 1.1** summarizes the epidemiology of HPV-related Cervical (CSCC and CAC), OPSCC and Sinonasal Carcinoma.

Table 1. 1: Epidemiology Summary of HPV-associated Cervical (CSCC and CAC), OPSCC and Sinonasal Carcinoma

	CSCC and CAC	**OPSCC**	**Sinonasal Carcinoma**
Incidence	Decreasing	Increasing	Decreasing
Prevalence	Higher in developing countries	Higher in developed countries	Higher in developed countries
Sex	100% female	> 70% male	Male and female about similar rates
Age	Under 50	Under 60	50s
Etiology	Almost all are caused by HPV	Tobacco and alcohol remain important causes, along with HPV	Environmental toxins such as tobacco and wood dust etc, along with HPV

HPV genotype	50% HPV16, 20% HPV18	> 90% HPV16, HPV18	82% HPV 16, 12% HPV 31/33, 6% HPV 18

Cervical cancer (CC), which includes the two major histology types; squamous cell carcinoma (SCC) and adenocarcinoma (AC), is the fourth most common cancer among women worldwide (3,15,28,29) affecting women under 50 years of age (4) with approximately 570,000 new cases in 2018 (13.1/100,000 women) (1,27). Almost all cervical SCCs (CSCC) and some cervical ACs (CAC) are HPV-related, and AC is rare compared with SCC (29,30). Globally, HPV 16 and 18 together account for 71% of cervical cancer and this percentage rises to 90% for HPV 6/11/16/18/31/33/45/52/58 (4) HPV 16 is the more dominant type in CSCC while HPV18 is more prevalent in CAC (29). In 2018 CC was responsible for 3.3% of deaths due to cancers by causing more than 300,000 deaths, with more than 85% of the deaths occurring in low- to middle-income countries (1). About 98% of CC deaths are attributed to hr-HPVs (1). It is estimated that the highest CC attributable to hr-HPV is in Africa (31.5/100,000 women/year) specifically in Sub-Saharan Africa (75.3/100,000 women/year) and lowest in Asia (10.2/100,000 women/year) (1). Hr-HPVs are more prevalent in developing countries mostly due to shortage and/or lack of healthcare access, higher prevalence of immunocompromised patients, a paucity of screening programs, and low vaccination rates (1).

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide(7,31,32) with 710,000 cases per year (7). HNSCC represents a large and diverse group of malignancies, which have been historically attributed to tobacco and alcohol consumption (3,4). Although the incidence of HNSCC is declining in some parts of the world largely due to a decrease in tobacco use, developed countries (eg. United

States, Canada, Australia, Sweden) have experienced an increase in the incidence of oropharyngeal cancer over the past two decades due to HPV infection, especially in men under 60 years of age (7). HNSCC accounts for about 6% of HPV-attributable cancer (38,000 cases globally), most of which are in Northern America and Europe (3,4). HPV 16 and 18 are responsible for 85% of HPV-related cancers of the head and neck (4,7). Most HPV-related HNSCC arise in the oropharynx (>90%) but has also been detected in other sites including the oral cavity, larynx, nasopharynx and sinuses (3,4).

The sinonasal tract is the second anatomic subsite of the head and neck for HPV-related carcinomas (33,34). However, sinonasal malignancies are rare, accounting for approximately 0.2% of all cancers and 3 to 5 % of head and neck cancers (5,35). The mean age of patients with sinonasal malignancies is about 62 years, and more prevalent in Caucasian men (5). The overall incidence is estimated to be 5 to 9 per million for males and 2 to 5 per million for females based on WHO statistics taken from the Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) dataset for 9 countries. Environmental toxins such as tobacco and industrial agents such as wood dust, thorium dioxide, lacquer paints, isopropyl oils, formaldehyde, solder and welding materials are risk factors for developing sinonasal malignancies (5,35). The incidence of sinonasal cancer has been declining in most countries due to decreasing tobacco use and efforts to reduce occupational exposures (5,35). However, there is increasing acknowledgment that a subset of malignancies is HPV-related but how it's transmitted is unknown (34). HPV type 16 (82%) is the most prevalent, followed by type 31/33 (12%) and type 18 (6%) (33). The most common sinonasal histologic type is SCC (SNSCC) which accounts for about 60-75% and it is estimated that 20% to 62% of SNSCC is HPV positive (36).

1.3 HPV Carcinogenesis

An understanding of transformation processes initiated by HPV infection has relied on the study of premalignant uterine cervical cells and has led to a recognized model of HPV carcinogenesis. The model parallels the normal HPV life cycle with initial infection, establishment and maintenance, but with persistent infection of basal or stem cells, carcinogenesis can be initiated (37). Persistent infection, with HPV causing genomic instability, is considered a necessary but not sufficient event for the development of cancer (38). There are a variety of molecular mechanisms involved in HPV-associated carcinogenesis that includes the overexpression of HPV oncoproteins E6 and E7 altering multiple signaling pathways and inducing genomic instability. Cancer-associated phenotypes are caused by HPV DNA integration in the host genome, immune evasion, changes in global DNA methylation (39–41) and the buildup of genetic and epigenetic modifications or mutations in genes whose encoded proteins act in diverse signaling pathways (42).

The HPV oncoproteins E5, E6 and E7 play a role in infiltrating many signaling pathways to create favorable conditions for cellular transformation. The E5 protein has been demonstrated to play an important role during the productive viral life cycle of HPV (43). E6 and E7's role in the initiation and progression of HPV-related cancers has been extensively demonstrated, and together they have been shown to be necessary but not sufficient for HPV-driven cellular transformation (44). E6 targets p53 by forming a complex with the E3 ubiquitin-protein ligase E6-associated protein (E6AP) for proteasomal degradation and can also bind p53 and block transcription of tumor-suppressive genes (39,41,45). The degradation of p53 aids in productive viral replication and allows for the

accumulation of genetic mutations which can lead to transformation, dysplasia and cancer (45). Both LR and HR E6 oncoproteins can bind to p53 but LR E6 cannot induce degradation (40,45). HR HPV E7 binds a cell cycle regulator, retinoblastoma protein (Rb) and other retinoblastoma pocket proteins, p105, p107 and p130 for degradation which results in the release and activation of transcription factor E2F (45). This promotes the expression of S-phase genes resulting in increased cell proliferation and viral gene transcription (45). E7 further induces cell proliferation by promoting G1-S phase entry of the cell cycle through the inhibition of cyclin-dependent kinase (CDK) inhibitors p21 and p27 leading to increased activity of CDK2 (41,45). The degradation of Rb and increased E2F activity results in a feedback loop causing an increased expression of the biomarker p16INK4a (p16) which controls the crucial G1–S phase transition (46). LR HPV E7 proteins are still able to target Rb but with a lower affinity compared to HR HPV E7 proteins, possibly contributing to their difference in progression to cancer (45).

1.4 HPV Attributes, Screening, Diagnosis, Treatment and Prevention

Almost all cervical cancers are caused by persistent infections with oncogenic strains of HPV, leading to the development of premalignant lesions and eventually invasive cancer (40). Since hr-HPV is well established as the main cause of almost all cervical cancers, it has been effectively controlled by screening and diagnosis. Primary screening involves Pap smears that detect morphologic changes in the cervical epithelium (such as abnormal cells, precancerous and cancerous lesions) caused by early HPV infections (30).

It is followed by HPV DNA testing if the Pap smears result showed malignancy or co-screening together with HPV DNA testing on the same cytology sample which gives greater sensitivity and specificity (30). HPV-related cervical cancer histology includes cervical squamous cell carcinoma (CSCC) (70%) cervical adenocarcinoma (CAC) (25%) or mixed histology tumors (30). Non-HPV-related cervical cancer is rare representing < 1% of newly diagnosed cases, with histologies including cervical neuroendocrine, small cell, and large cell carcinomas (30). In the comparison of the two major histologies, SCC develops from the ectocervix's squamous epithelia and AC develops from the endocervix's glandular epithelia (29). Studies suggest that the incidence of AC appears to be increasing in some countries while SCC incidence is decreasing (29,47). The rise is seen among young women, partly due to cohort effect and partly due to cytology screening, which is less effective for detection of AC compared to SCC (29). Although there is growing evidence that ACs have different epidemiology, prognostic variables, patterns of dissemination, and treatment failure following therapy compared to SCCs, both are staged and treated similarly (47). Silva classification, which stratifies invasion in three patterns, is used to determine HPV-related CAC (47,48). Even though p16 expression is considered to be a surrogate marker for HPV association, p16 IHC testing is not absolutely necessary for the classification, and HPV analysis is not necessary for the diagnosis (48). HPV-related CSCC causes pre-cancerous lesions but there is no known precancerous lesion in the very rare non-HPV-related CSCC (48). According to WHO guidelines, HPV DNA testing is used to detect HPV-related CSCC but p16 IHC is also recommended since morphology alone cannot distinguish the two types (48). Cervical cancer is a continuous single disease process advancing gradually from mild cervical intraepithelial neoplasia (CIN1) to more

severe degrees of neoplasia and micro-invasive lesions (CIN2 or CIN3), and finally to invasive disease (30). The primary treatment for early-stage cervical cancer is surgery, and later stage with chemotherapy and/or radiation (37).

HPV- related OPSCC is clinically distinct, affecting younger patients with fewer comorbidities, responding favorably to treatment and portending survival outcomes compared to HPV negative OPSCC, affecting older patients with a significant history of tobacco use and alcohol consumption (49,50). HPV 16 induces over 90% of HPV-related OPSCC followed by HPV 18 and 45 presented at less than 2% each (44). Most HPV-related OPSCC present with small primary tumors, but often cystic, multilevel nodal disease (9,51). The histology is predominantly non-keratinizing SCC with basaloid morphology (9,51). OPSCC is usually tested for hr-HPV by surrogate marker p16 IHC and discretionally, additional molecular HPV-DNA testing may also be performed (9,22). For early-stage OPSCC with minimal or no nodal disease the treatment is generally either primary surgery and/or definitive radiotherapy (RT) (30). Patients with more advanced disease or the presence of extensive nodal metastases are generally treated with combined modalities including surgery, radiation and/or chemoradiation (30).

While the incidence of sinonasal carcinoma is low, their histology is among the most diverse of all head and neck sites with several uncommon and distinct subtypes, several SCC variants, interesting etiologic lesions and HPV- related tumors (52). HPV-related sinonasal carcinoma histologic types are SCC and variants (non- or partially-keratinizing, papillary, adenosquamous and basaloid), small cell carcinoma, undifferentiated and carcinoma with adenoid cystic-like features (33), which is now known as HPV-related multiphenotypic sinonasal carcinoma (HMSC) (53–56). HMSC is rare and

histologically characterized by multiple patterns of differentiation including squamoid, ductal and myoepithelial, similar to adenoid cystic carcinoma (ACC) (51). There is increasing histologic and epidemiologic evidence suggesting that a subset of SNSCC may be caused by HPV and detection may be a biomarker for improved survival similar to HPV-positive OPSCC but definitive conclusions are hampered by small sample sizes and inconsistent HPV detection methods (57). The available literature has shown conflicting results with some studies showing that HPV-related SNSCC is associated with better outcomes, while others have reported that HPV status is not a significant prognostic factor (36). HPV testing in these cancers is not widely performed by pathologists. The primary treatment modality is surgery with or without adjuvant RT with some evidence suggesting that adjuvant RT may prolong the disease-free interval among patients who develop local recurrence (53,58). **Table 1.2** summarizes a few attributes of HPV- related Cervical cancers (CSCC and CAC), OPSCC and Sinonasal Carcinoma.

Table 1. 2: Summary of attributes of HPV- related Cervical (CSCC and CAC), OPSCC and Sinonasal Carcinoma

	CSCC	**OPSCC**	**Sinonasal Carcinoma**
Histopathology	Keratinizing SCC, large cell nonkeratinizing, small cell nonkeratinizing	Non keratinizing SCC with basaloid morphology	non- or partially-keratinizing, papillary, adenosquamous, basaloid, small cell, ductal myoepithelial
Molecular diagnosis	HPV-DNA testing, p16 IHC	p16 IHC (and HPV-DNA)	Not recommended
Early-stage primary treatment	Surgery	Surgery and or RT	Surgery and or RT
Treatment sensitivity to chemotherapy and radiation	Moderate	High	High

Because almost all cervical cancers and rising proportions of OPSCCs are attributable to HPV infections, universal access to vaccination against HPV could effectively reduce the incidence of these and other HPV-associated cancers (49). When the occurrence and transmission of anogenital HPV is decreased with vaccinations, the incidence and sexual transmission of oral HPV should also decrease, lowering the incidence of HPV-positive OPSCC (30). Universal HPV vaccination has been introduced into the national immunization program in most developed countries. In Canada, HPV2, HPV4 and HPV9 are available for both sexes from the age of 9 or Grade 6 and are administered as a two-dose series as part of the national immunization program (59). Overall, HPV vaccination has been effective in the prevention of persistent HPV16 and HPV18 infections (39). However, immunization programs are not established in developing countries and the uptake of HPV vaccine is low, and hence HPV-related diseases continue to rise. Potentially, HPV vaccination can prevent over 90% of cervical and other HPV-related malignancies globally and will be the most effective way to protect young people from HPV-related diseases (30). However, screening and HPV testing will continue to play a key role as prophylactic vaccines are most effective prior to HPV exposure and eradication of HPV through vaccinations is still decades away (30,60).

1.5 Cervical Cancer Screening and Diagnostic Tools for HPV Detection

Cervical cancer screening and diagnosis is combined liquid-based cytology stained Papanicolaou stain (also known as Pap smear) and HPV testing using DNA/RNA PCR-

based methods (12,13,61). Papanicolaou carried out the first prospective studies of the vaginal cycle by working with guineapigs and in 1943, jointly with Traut, he outlined detailed studies of cycle-dependent epithelial changes in the vaginal epithelium of the human female (62). Epithelial cells are collected from the external surface of the cervix and lower part of the cervical canal using a cervical sampling brush or spatula, processed into a thin layer on a glass microscope slide, stained with Papanicolaou stain, and evaluated by a cytopathologist using a microscope (62). The cytopathologist evaluates the sample by comparing the histologic structure to the normal squamous epithelium from the vagina and ectocervix (62). Höffken et al (62) summarized the histology and cytology of a normal squamous epithelium from the vagina and ectocervix as shown in **Table 1.3.**

Table 1. 3: Histology and cytology of normal squamous epithelium from the vagina and ectocervix

Histology	**Cytology**	**Cytometry** **C=cell diameter** **N=nuclear diameter**	**Proliferation grade**
Basal cell layer (stratum basale)	Basal cells, basophilic with dense cytoplasm, nucleus round or oval	C 12-20 µm N 8-10 µm	Not seen in normal smears
Parabasal cell layer (stratum spinosum profundum)	Parabasal cells, basophilic with dense cytoplasm, nucleus round or oval	C 15-25 µm N 8-10 µm	1
Intermediate cell layer (stratum spinosum su perficiale)	Small intermediate cells, polygonal, basophilic, pale-staining cytoplasm, nucleus vesicular, with fine granules	C 20-40 µm N 7-9 µm	2
Superficial cell	a) Large intermediate cells,	C 40-60 µm	3

layer (stratum superficiale)	polygonal, basophilic, eosinophilic, nucleus still vesicular	N 6- 8 µm	
	b) Surface cells, polygonal, eosinophilic, basophilic, nucleus pyknotic	C 40-60 µm N 6 µm	4

The Bethesda System which was adopted in 1988 and revised in 1991 to replace the cervical intraepithelial neoplasia (CIN) method, is the current Pap smear reporting system. Burd et al (13) summarized the cytology and histology terminology for HPV-associated squamous lesions of the cervix as shown in **Table 1.4**. The histologic diagnoses are reported as normal, atypia, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma (SCC) (12,13,63,64).

Table 1. 4: Cytology and histology for HPV-associated squamous lesions of the cervix

Bethesda System	**CIN System**	**Interpretation**
No epithelial abnormalities or benign cellular changes	Normal	Normal
Atypical squamous cells (ASC): ASC-US (undetermined significance), ASC-H (cannot exclude HSIL		Atypia, Squamous cells with abnormalities greater than those attributed to reactive changes but not meeting the criteria for a squamous intraepithelial lesion
Low-grade squamous intraepithelial lesion (LSIL)	CIN 1	Koilocytosis, mild dysplasia, mild abnormalities caused by HPV infection
High-grade squamous intraepithelial lesion (HSIL). (Perform p16 IHC to upgrade or downgrade; if negative, classify as LSIL and if positive, classify as HSIL)	CIN 2-3	Moderate dysplasia, severe dysplasia, carcinoma in situ, suspicious; more severe abnormalities that have a higher likelihood of progressing to cancer if left untreated

Squamous cell carcinoma	Invasive squamous cell carcinoma, invasive glandular cell (adeno)carcinoma	Invasive squamous cell carcinoma (cervical cancer) Atypia, glandular epithelial cells

Cytology screening is one of the most successful public health prevention activities worldwide. It has led to significant reductions in cervical cancer incidence and mortality, but it has significant limitations such as low sensitivity and poor reproducibility (60). HPV testing was found to be more advantageous than cytology largely due to direct early detection further upstream in cervical carcinogenesis (60). Some of the benefits include: 1) higher sensitivity and reproducibility but somewhat lower specificity, 2) ability to be automated, centralized, and be quality checked for large specimen throughput, 3) more cost-effective than cytology, if deployed for high volume testing and 4) the ability to use self-sampling, which has the potential to increase screening to remote areas or to women who are not directly reached by primary healthcare in urban areas (60,65). In 2008, the 3-year prospective study, ATHENA (Addressing the Need for Advanced HPV Diagnostics) was initiated in the US and it is the first and largest screening study to evaluate the performance of HPV primary screening (66). The results indicated that co-testing, cytology and HPV, provided minimal increased protection against the development of CIN2 or worse compared to HPV primary screening. This led the FDA to approve in 2014, HPV primary screening tests for women ages 25-65. Women tested for hr-HPV 16 and /or 18 are referred for Colposcopy and those positive with the other hr-HPVs should be triaged with cytology and, if the latter is positive (ASC-US or worse), colposcopy is recommended. The important development was that the majority of women who tested

HPV negative are to be screened no sooner than 3 years later (60,61,66,67). **Table 1.5** shows the cervical cancer screening recommendations from American College of Obstetricians and Gynecologists (ACOG), American Society for Colposcopy and Cervical Pathology (ASCCP) and US Preventive Services Task Force (USPSTF).

Table 1. 5: Cervical cancer screening recommendations from ACOG, ASCCP, USPSTF

Testing	**ACOG**	**ASCCP**	**USPSTF**
Pap only	Every 3 years	Every 3 years	Every 3 years
Pap-HPV co-test	Every 5 years, age 30–65	Every 5 years, age 30–65	Every 5 years, age 30–65
High-risk HPV only	Every 3 years, age > 25	Every 3 years, age > 25	Every 5 years, age 30–65

Primary HPV testing followed by cytology was accepted in Canada and Europe because of its safety relative to co-testing and reduction of required tests nearly in half with a consequent reduction in the cost for screening programs (60). Combining primary HPV screening with cytology triage provides increased confidence in the absence of cervical abnormalities and allows for longer intervals between screening rounds compared to standard cytology (60,66).

Immunohistochemistry (IHC) for p16INK4a (p16) is commonly utilized as a surrogate marker for the presence of hr-HPV E7 in tumor tissues and has become the clinical standard for HPV testing **(9,22,68)**. Most routine laboratories testing surgical pathologies usually have accessible IHC with pathologists that can easily perform the methods and adequately interpret the staining reactions **(69)**. The IHC assay is widely used

in the diagnosis of abnormal cells, to identify its origin based on the binding of antibodies (Ab) to specific antigens (Ag) in tissue sections **(70)**. It is visualized by a histochemical chromogen reaction or by fluorochromes visible by using conventional microscopy or fluorescence microscopy **(70)**. IHC is generally performed on 4–6 μm thick formalin-fixed, paraffin-embedded (FFPE) tissue slices or on frozen fresh tissue with thickness 8–90 μm **(70)**. IHC assays detect distinct tissue components by capturing target antigens with specific antibodies tagged with proper labels binding to the tissues, and the reaction is visualized using fluorochromes (substances that absorbs or emits light) or chromogens (substances that produce distinct color) **(70)**. While most pathologists use strong nuclear and cytoplasmic expression for a positive result, a few interpret only cytoplasmic staining as positive **(68)**. The *College of American Pathologists (CAP)* and the *8th edition of the American Joint Commission on Cancer (AJCC8)* recommend that for a result to be considered positive, a threshold of at least 70–75% of tumor cells must show moderate to strong nuclear and cytoplasmic staining of the neoplastic cells. The threshold of at least 70% of positive tumor cells might be too high because it was found that there is a presence of nuclear and cytoplasmic staining in 50–70% of tumor cells associated with hr-HPV in a subset of patients **(71)**. IHC for the detection of p16 expression is a highly sensitive surrogate marker for transcriptionally active HR HPV infection in CSCC (in the triage of women with positive screening results and to identify pre-cancer biopsies) **(72)**.

In situ hybridization (ISH) is a method used to detect nucleotide sequences based on the complementary binding of a nucleotide probe (complementary DNA (cDNA), complementary RNA (cRNA) or synthetic oligonucleotide) to a specific target sequence of RNA or DNA in cells, tissue sections or an entire tissue (73). The hybrids that form

between the labeled probe and the specific target sequences can be visualized and detected by various methods (73). Tissue samples are prepared by the treatment with proteases to facilitate access of the target nucleic acid to increase hybridization efficiency and reduce nonspecific background staining (73). The probes used have radioisotope labels or non-isotope labels (biotin, fluorescein, digoxigenin, alkaline phosphatase, or bromodeoxyuridine are used) (73). The radioisotope labeling is considered as the most sensitive, but others believe that nonisotopic methods are just as sensitive (73). The radioisotope labeling hybridization sites are observed by autoradiography with X-ray film or liquid emulsion and nonisotopic labeling hybridization sites are observed by histochemistry or immunohistochemistry (73). HPV detection procedure in ISH occurs within the nuclei of infected cells which makes it the only molecular method that reliably detects and identifies the location of specific nucleic acid sequences in tissues, which is evaluated microscopically (74). The presence of HPV in tissue samples being tested is indicated by the development of appropriate precipitate within the nuclei of the epithelial cells and the condition of the virus can be classified as integrated or episomal by the presence of punctuating signals and diffuse signals, respectively (74). ISH is highly specific (100%) but not sensitive (83%) for HPV infection compared p16 immunohistochemical staining (73,74).

Polymerase chain reaction (PCR) is a widely used technique that allows a specific stretch of DNA to be copied exponentially in a short amount of time (75–77). There are five primary components of PCR and it is summarized in **Table 1.6.** They are: 1. template DNA, is the double-stranded DNA segment to be copied; 2. deoxynucleotide triphosphates (dNTPs), are the building blocks of DNA (adenine triphosphate (ATP), thymine

triphosphate (TTP), guanine triphosphate (GTP) and cytosine triphosphate (CTP)); 3. polymerase enzyme, Taq DNA polymerase joins the nucleotides together; 4. oligonucleotide primers, DNA sequence complementary to the target DNA; 5. buffer solution of favorable ionic strength and pH (75).

Table 1. 6: Summary of PCR components and description

Component	Description
Template DNA	Double-stranded DNA segment to be copied
dNTPs	The building blocks of DNA. The 4 nucleotides are ATP, TTP, GTP, CTP
Polymerase enzyme	Taq DNA polymerase enzyme joins the nucleotides together creating a mirror image of the template
Oligonucleotide primers	DNA sequence complementary to the target DNA where DNA polymerase binds and initiates DNA synthesis
Buffer solution	A solution to contain the DNA sample of favorable ionic strength and pH

PCR uses Taq DNA polymerase derived from the thermophilic bacterium *Thermus aquaticus* for its heat stability as it allows the enzyme to withstand the heating needed to denature DNA and maintain activity at relatively high temperatures which improves primer specificity **(75)**. There are three core steps involved in PCR as summarized in **Table 1.7**. Step 1: Denaturation is heating the PCR tube components at high temperatures (94-96°C) weakens the DNA and breaks the two complementary strands apart. Step 2: Annealing is cooling the PCR tube components (55°C) which allows the DNA primers to bind themselves to the complementary sites on the template strands. Step 3: Extension is

heating the PCR tube components (72°C) which permits the DNA polymerase to copy the template strands by adding nucleotides onto the ends of the primers and producing two molecules of double-stranded DNA **(75)**. The procedure is normally repeated through a number of cycles thereby, increasing the amount of the target DNA exponentially **(75)**.

Table 1. 7: Summary of the steps and events in PCR

Steps	Event
1. Denaturation	A very small PCR tube is heated to 94-96 °C which denatures the DNA and splits the two complementary strands apart.
2. Annealing	The tube is cooled which allows the DNA primers to bind themselves to the complementary sites on the template strands
3. Extension	DNA polymerase copies the template strands by adding nucleotides onto the ends of the primers and producing two molecules of double-stranded DNA

PCR is an integral component of many protocols and is perhaps the key technique of molecular biology (75). PCR has broad applications including medical diagnostics and as such, it is used to detect HPV. PCR is a selective technique capable to reproduce and increase the amount of target HPV sequences present in biological specimens exponentially, following repeated cycles of amplification (77). PCR based assays have wide-ranging specificity and sensitivity determined by a few factors such as; the size of the PCR product, the spectrum of HPV DNA amplified and ability to detect multiple types, the primer sets chosen, reaction conditions and performance of the polymerase enzymes in the reaction (77). Most primer sets are designed to target the L1 gene or the E6 and E7 oncogenes (78). PCR primers directed at the E6 or E7 regions have been described as

preferable because the L1/E1 regions are often lost during the integration of viral DNA into host genomic DNA and targeting the L1 or E1 region may miss advanced disease (77).

The most current HPV detection methods that are commercially available are type-specific target amplification DNA PCR and signal amplification DNA ISH, which are approved for cervical samples (77,78). HPV DNA PCR is a target amplification technique that effectively amplifies small amounts of DNA sequences in a biological specimen containing diverse cell types, using primers that can be specific for a single HPV type or target sequence shared by multiple types (78). HPV DNA PCR can also be used as a non-quantitative technique but information about the abundance of a particular DNA species is not provided (78). DNA ISH is a signal amplification technique that utilizes labeled DNA probes (that can be type specific to one HPV type or multiple HPV types or mixed in a single reaction to cover a range of HPV types) that binds to a specific target sequence of DNA forming hybrids which is visualized using microscopy (73,78). The performance of DNA PCR and DNA ISH is comparable but direct comparison suggests that DNA ISH may be more practical as a diagnostic tool due to its ability to reliably differentiate relevant HPV infection from passenger virus or contaminant (78). Furthermore, DNA ISH adaptation to FFPE tissues make it compatible with standard tissue processing procedure, using nonfluorescent chromogens allows hybridized DNA to be visualized using conventional light microscopy and the introduction of various signal amplification steps has increased sensitivity (78).

Hybrid Capture 2 (HC2) HPV DNA test was developed by Digene Corporation (Gaithersburg, MD) and is now marketed by Qiagen (Germantown, MD), was FDA approved in 1999, and replaced the original Hybrid Capture (HC1) tube-format assay,

which was approved in 1995. It was the only test available until 2009. The HC2 is a signal amplification nucleic acid hybridization assay in microtiter format for cervical specimens collected using a specialized HC2 DNA device or HC cervical sampler (cervical broom) (13). The specimen release and denature target DNA after treatment, and a mixture of multigene RNA probes specific for 13 high-risk HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58,59, and 68 is added (13). If hr-HPV DNA is present, it combines with the probes and the resultant DNA-RNA hybrids are captured onto wells of a microtiter plate that are coated with monoclonal antibodies to DNA-RNA hybrids (13). The addition of a second monoclonal antibody conjugated to the alkaline phosphatase binds to the captured hybrids in multiples resulting in dephosphorylation of a chemiluminescent substrate which produces light (13). When the alkaline phosphatase acts on several copies of the substrate, the target/signal level is increased, and light emitted is quantified in relative light units (RLU) on a luminometer (13). HR probe may cross-react with LR HPV that is not in the probe mixture which will adversely affect the sensitivity (77,79). The HC2 test has a cutoff of 1 RLU and an RLU greater than or equal to 1 indicates the presence of HR HPV DNA and an RLU less than 1 indicates either absence of HR HPV DNA or HR HPV DNA levels below the limit of detection of the test (13). The test has a sensitivity of 0.2 to 1 pg/ml which is equivalent to 1,000 to 5,000 genome copies of HPV but does not distinguish the specific HPV genotype present (13,79). HC2 test does not contain an internal control and hence, it is impossible to evaluate the quality of the specimen or the presence of potentially interfering materials (13,79).

The Cervista HR HPV test (Third Wave Technologies, Madison,WI, now Hologic/Gen-Probe, San Diego, CA) was FDA approved in 2009. It utilizes proprietary

Invader Chemistry to generate signal amplification of a fluorescent probe to detect HPV DNAs from 14 HR types, including the same 13 types detected by the HC2 test plus HPV66 (13,79). The analytical sensitivity of the Cervista HPV HR test varies depending on HPV type, with limits of detection of 1,250 to 2,500 copies per reaction for HPV16, 18, 31, 45, 52, and 56, 2,500 to 5,000 copies per reaction for HPV33, 39, 51, 58, 59, 66, and 68, and 5,000 to 7,500 copies per reaction for HPV35 (13,79). Similar to HC2, it does not identify the individual HPV type (13,79). Cervista uses a lower sample requirement of 2 ml (vs 4ml) and has lower cross-reactivity with some LR HPV types compared to HC2 (13,79). Its analytical sensitivity is comparable to HC2 but uses the human histone 2 gene as an internal control to ensure the efficacy of the specimen and eliminate false-negative results (80).

The APTIMA HPV assay (Hologic Gen-Probe Inc., San Diego, CA) was FDA approved in late 2012. The assay qualitatively detects E6/E7 messenger RNA (mRNA) transcripts of 14 high-risk HPV types and using a noninfectious RNA transcript as extrinsic process control (13). The assay performs pooled HR HPV detection that does not distinguish between the 14 targeted HR types like HC2 and Cervista HR HPV assays. The 3 main steps in the assay, which occur in the same tube involve target capture, target amplification using transcription-mediated amplification (TMA), and detection (79). The assay uses 1 mL of liquid-based cytology, and a lesser amount is inadequate for testing (79). The cells are lysed so that mRNA can be released and allowed to hybridize to capture oligonucleotides attached to magnetic microparticles (81). The target mRNA is isolated using capture oligomers linked to magnetic microparticles containing sequence-specific regions which attach to specific sites of the target mRNA (79,81). A temperature reduction

of the reaction to room temperature causes the complex of the capture oligomer and target to be extracted from the solution and to be hybridized with the magnetic particles (81). The captured HPV mRNA bound to the microparticles are retrieved from the reaction tube using magnets and the supernatant is aspirated then washed to remove potential inhibitors (81). The captured HR HPV mRNA is amplified by (TMA), detected by hybridization protection assay (HPA) using chemiluminescent labels (13,79). A luminometer is used to measure the resultant signal in RLUs and results are interpreted based on the analyte signal to cutoff (S/CO) value (79). Internal control (IC) is added to each reaction and the signal in each reaction is distinguished from the HPV signal by the differential kinetics of light emission from probes with different labels (79). Target RNA amplification is detected using probes with slow emission of light (glowers) and IC amplification is detected using probes with rapid emission of light (flashers) (79). The Dual Kinetic Assay (DKA) is a method used to differentiate between the signals from the flasher and glower labels (79). The analyte S/CO is calculated from the analyte RLU of the test sample and the analyte cutoff for the run (79). If the S/CO ratio is <0.50, a negative result is generated, and if the S/CO ratio is ≥ 0.50, a positive result is generated (79). The system is automated with high output and the full process from sample preparation to result detection can be automated on the TIGRIS system (Hologic) (13,79).

The Cobas 4800 HPV test (Roche, Pleasanton, CA) was approved by the FDA in 2011 but has been available in the European market since 2009. It is a target amplification assay that detects the same 14 HR HPV types as the Cervista and APTIMA tests but also distinguishes hr-HPV types 16 and 18 (13,79,80). It simultaneously detects the L1 gene of HPV16 and HPV18 as individual reactions and the other 12 hr-HPV as a pooled result by

using multiplex real-time PCR (RT-PCR) and nucleic acid hybridization with four different fluorescent reporter probes (13,79). There are four fluorescent-labeled cleavage primer probes used for detection of amplification of the HPV DNA that target the L1 region; one specific for HPV 16, one specific for HPV 18, one for non-16/18 genotypes, and one for β-globin (79). The test is automated and the system consists of two separate instruments: the Cobas z 480 instruments for automated nucleic acid extraction and the Cobas x 480 analyzers for PCR amplification and detection reactions in a single tube (13). The system is designed to process up to 280 cervical specimens collected in PreservCyt solution in one day (13). False negatives can occur though since the L1 gene is lost upon integration into the human genome in a considerable proportion of cancers (13,79). The overall intra-laboratory agreement is 98.3% and genotyping agreement is 98.2% (79). Inter-laboratory reproducibility studies showed 94.6% overall agreement and 93.7% genotyping agreement (79).

The OncoE6^{TM} Cervical Test (Arbor Vita Corporation, Fremont, CA) is a qualitative lateral flow assay (strip test) that detects the elevated level of E6 oncoprotein expressed from HPV infected cells associated with the most common oncogenic HPV types 16 and 18 (82,83). The presence of elevated E6 oncoprotein levels suggests that there is an existing malignant cell or an increased risk of future malignancy (82,83). The assay uses cell lysates samples from cervical swab specimens or from specimens collected in PreservCyt® solution (82). The lysate is incubated with highly specific mouse monoclonal antibodies (mAbs) to E6 oncoprotein from hr-HPV types 16 and 18 bound with alkaline phosphatase (AP) (82). The test strip made out of nitrocellulose with two capture lines consisting of the immobilized mAbs to E6 16/18 is placed in the lysate/mAb-AP mix (82).

By capillary action, the lysate/mAb-AP mix pass through the test strip, and a complex (capture mAb-E6-detector mAb) may form if E6 16 and/or 18 is present and becomes visible as purple lines at the respective locations (either 16 or 18) when the enzyme substrate is added (82). If the test is valid and a purple test line at any intensity is seen, the result is positive and no line indicates a negative result (82). The assay was validated in several clinical studies. Valdez et al (84) conducted cervical cancer screening study in rural China and their results showed that OncoE6^{TM} Test had a 70.3% sensitivity and 98.9% specificity for CIN3 detection compared to HPV DNA testing (careHPV) and visual inspection of cervix with acetic acid (VIA). Torres et al (85) performed a cervical cancer screening in remote areas in Brazil and their results showed that OncoE6^{TM} has overall 50% sensitivity and 99% specificity for CIN3+ and specificity is a high priority in remote geographic settings due to the difficulties of follow up. Krings et al (83) demonstrated that OncoE6^{TM} has a high sensitivity in the detection of HPV 16 or 18 in 3 different types of self-sampled specimens and their results showed 90% sensitivity with the Delphi Screener lavage and the cytobrush sample in PreservCyt media and 95% sensitivity for the swab sample. They suggested that using OncoE6^{TM} testing and self -sampled specimens will allow highly effective cervical cancer screening in remote areas, thereby increasing the effectiveness of preventive strategies (83).

The comparison of diagnostic tools of CSCC is summarized in **Table 1.8**. In testing women with abnormal cytology, HPV testing is more sensitive (97.4% vs 56.4%), more reproducible (Cohen's kappa coefficient k = 0.60 - 0.93 vs k = 0.46) but less specific (94.3% and 97.3%) compared to cytology for the detection of cervical pathology (13,79). For the detection of CIN2+ in women with abnormal cytology, p16 IHC sensitivity

compared to cytology is 85.7% vs 54.7% and for specificity 88% and 61%, respectively (79). All of the FDA-approved assays for HPV detection use either target or signal amplification techniques and are approved for use with liquid-based cytology. For the sensitivity comparison of HC2, APTIMA and Cobas 4800 (96.3%, 95.3% and 95.2% respectively), HC2 is most sensitive and for specificity (19.5%, 28.8% and 24.0%), APTIMA is most specific (79). The HC2, Cervista, and Cobas 4800 tests target HPV DNA while APTIMA tests target E6/E7 mRNA and have improved specificity compared to the other assays. They all have similar sensitivity for the detection of cervical dysplasia (79).

Table 1. 8: Summary of performances of the tests for Cervical Cancer

Test	**Sensitivity %**	**Specificity %**	**Reproducibility**
Cytology	53.3	92	
p16 IHC	85.7 (88*)	54.7 (61*)	
Cytology	53.3	92	k = 0.46
OncoE6^{TM}	50-70	99	
HPV testing	73.0	56.9	k = 0.60 -0.93
HPV testing methods			
HC2	96.3	19.5	
APTIMA	95.3	28.8	
Cobas 4800	95.2	24.0	

*If performing p16 on only HPV-positive women

Next-generation sequencing technology is used for DNA and RNA sequencing, detect variants or mutations, and sequence thousands of genes or entire genomes in a short time (86). NGS technologies were first released between 2004 and 2006, revolutionizing

biomedical research and leading to a significant increase in sequencing data output (87). There are numerous NGS platforms using various sequencing technologies (87) from different companies being used today and Barba et al (88) reviewed the history, development and applications. The workflow involves several key steps: nucleic acid extraction, then library preparation which involves addition of sequencing adapters that interacts with the NGS platform followed by PCR amplification, next is sequencing in which the library is loaded onto the sequencer which then reads the sequences and distinct sequences have specific matrices, the sequence information is analyzed by the bioinformatics software and data is then interpreted (86). NGS was used to detect HPV in cervical cytology samples and their results were similar to Cobas 4800 but also detected HPV types that were in the NGS panel and therefore it has great potential to improve HPV screening programs (89). NGS was capable of detecting and genotyping HPV in cervical cancer from 82 FFPE and 10 plasma samples and NGS analyses showed full agreement with ddPCR analyses of HPV+ and HPV- plasma samples (90).

MassARRAY System (Agena Bioscience) developed by Sequenom, Inc. San Diego, United States, which was introduced in October 2011 and sold to Agena Bioscience in June 2014, is based on matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) mass spectrometry for the precise detection of DNA molecules (91). Samples are started with purified DNA or RNA (10-40ng needed) placed in 96- well plate and primers are added then amplified, followed by the addition of shrimp alkaline phosphatase to dephosphorylate unincorporated dNTPs, and lastly the addition of assay extension primers and termination mixes for single base extension reaction to ensure that the extension products are differentiated by their molecular mass. The plate is placed in the

MassARRAY system and sample processing is fully automated and two 96 well plates can be analyzed at the same time. In the automated system, CleanRESIN is added to minimize the effects of salt in the reaction then nanoliters of the analytes are dispersed onto the SpectroCHIP Array which is then transferred to the MassARRAY analyzer for processing (91). In a vacuum chamber, samples are exposed to laser radiation to cause molecular ionization and desorption (91). Variations in the mass of the positively charged DNA ions cause them to migrate through the vacuum tube at varying speeds, which ultimately results in varying arrival times for the ions (91). The MassARRAY System measures the mass and shows a mass spectrum that distinguishes the many genetic targets based on the arrival time of each individual ionized DNA analyte (91). The MassARRAY System was used in cervical HPV detection for 19 HPV strains (14 hr-HPV and 5 lr-HPV) using FFPE and they found that MassARRAY is highly sensitive, reliable and cost-effective when applied in institutions with a high case load (92). Another cervical HPV study analyzed collected screening samples from women participating in the Danish organized cervical cancer screening program and compared MassARRAY to PCR-based enzyme assay and they found that they have comparable sensitivity for ≥CIN2 (94.3% vs 92.6%) and ≥CIN3 (95.5% vs 94.0%) but MassARRAY had lower specificity (79.2% vs 89.2%) (93). Since MassARRAY is designed for high sensitivity and operates without established clinical cutoffs, the high sensitivity translates into substantially lower specificity (93). This emphasis on high sensitivity, however, conflicts with cervical cancer screening guidelines, which call for balancing sensitivity and specificity to minimize overdiagnosis (93).

1.6 OPSCC Screening and Diagnostic Tools for HPV Detection

The current recommendation for HPV testing for OPSCC from the *College of American Pathologists (CAP)* and A*merican Society of Clinical Oncology (ASCO)* guidelines is p16 IHC, and additional molecular HPV-DNA testing may also be performed at the physician's discretion. However, HPV testing is not recommended for other HNSCC (9,21,22). There is evidence that p16 IHC shows strong diffuse cytoplasmic and nuclear staining in >70% of the tumor cells in SNSCC, though a lower rate than that for OPSCC (94), can be used as a surrogate marker (21,33,94). But since SNSCC is not studied as much as OPSCC due to its rarity, the favorable effect of HPV diagnosis is inconclusive and therefore p16 IHC testing is not a routine practice (52,95). Future research studies are essential to better understand the role of HR HPVs in sinonasal carcinoma. p16 IHC is currently used as a highly sensitive surrogate marker for detecting transcriptionally active HPV in OPSCC (both primary and metastatic sites) (21). Other HPV testing methods are also utilized such as viral DNA detection by PCR or ISH as well as combined detection of p16INK4a IHC and HPV DNA-PCR are frequently applied (68). The E6 oncoprotein testing has also been used to detect HPV in HNSCC. Menegaldo et al (96) detected HPV16/18 E6 oncoproteins in 34 OPSCC and (Cancer of Unknown Primary) CUP using OncoE6^{TM} and their results showed 94% and 88% sensitivity when applied to the primary tumor and neck nodes respectively and 100% specificity in both primary and neck lesions. Chernesky (97) evaluated HPV E6 oncoproteins and nucleic acids in FNA and oral samples from patients with OPSCC using commercial assays. Their results showed that for FNA samples, the overall agreements of p16 antigen staining of tumor were 81.4% (k 0.53) for OncoE6™, 94.9% (k=0.83) for Aptima HPV E6/E7 mRNA and 91.1% (k 0.73) for and

cobas HPV DNA (97). There were lower agreements with tumor markers for saliva and oral swab samples; 23.7–24.0% (k 0.02) for OncoE6™, 55.9–68.0% (k 0.24–0.37)) for Aptima HPV E6/E7 mRNA and 78.9–86.9% (k 0.49–0.58) for cobas HPV DNA (97). Agustin et al (71) summarized the benefits and drawbacks of HPV detection techniques for OPSCC as shown in **Table 1.9** with the addition of OncoE6™ testing. p16 IHC sensitivity in OPSCC is around 80–90% and specificity vary from 80-90% (71). p16 IHC is a cost-effective method, and its diagnostic performance is considered high enough to diagnose HR HPV infection in OPSCC (71). DNA PCR techniques are known to be stable and reproducible and have a sensitivity of 98% and specificity of 84% (68,71). RT PCR detection of HPV mRNA E6/E7 has a sensitivity of 97% and specificity of 100% and is considered by some authors to be the gold standard to diagnose HPV related OPSCC but requires fresh/frozen specimens and is technically demanding therefore not useful for routine screening (71). HPV DNA ISH allows for direct visualization of the virus within the tumor cells and minimizes the risk for a false-positive test result that may derive from tissue contamination with viral DNA (68) HPV DNA ISH has a sensitivity of 85% and specificity of 88% (68).

New HPV biomarkers have been studied in the management of HPV-related OPSCC. Antibodies against E6 protein have been associated with a 132-fold increased risk in developing OPSCC and develop more than 10 years before HPV-related OPSCC diagnosis (71). Research showed that these E6 antibodies are detectable in less than 1% of healthy individuals but other studies have shown that most HPV-positive OPSCC patients (>90%) present an HPV16 E6 antibody response in blood at the time of their HPV16-OPSCC diagnosis (71). Some researchers suggest that E6 serology could be considered for

HPV OPSCC monitoring especially tracking residual disease or recurrence, but more validation and research is needed before consideration for clinical routine application (71).

The detection of HPV circulating tumoral DNA (ctDNA) from plasma by using ultra-sensitive droplet digital PCR (ddPCR) has garnered growing clinical interest in HNSCC and CSCC. HPV ctDNA detection in the plasma of HPV-related OPSCC patients using ddPCR is highly sensitive and specific in identifying HPV16 and HPV33 subtypes in a similar distribution as reported in major genomic profiling studies (98). Their results suggested that HPV16 and HPV33 ctDNA ddPCR could be used in early detection screening trials and in disease response monitoring. The HPV ctDNA in CSCC detection using ddPCR may predict relapse and their results suggest that monitoring HPV ctDNA could help evaluate treatment options for patients with residual HPV ctDNA after treatment (99). ddPCR and RT-PCR performances were compared in the detection of HPV ctDNA in cervical neoplasia at different stages of the disease and ddPCR offers sensitive detection and absolute quantification of low target DNA compared to RT-PCR (100).

The quantitative method of ddPCR is characterized by its high sensitivity, its accuracy and its reproducibility inter-laboratories and intra-laboratories (31,71). The ultrasensitive ddPCR can be operated at a very low cost compared to other innovative technologies (71). These properties of ddPCR can be applied to detect samples in swabs with very low amounts of DNA.

Table 1. 9: Summary of HPV detection techniques used in OPSCC

Detection method	Advantages	Disadvantages	Sensitivity %	Specificity %
p16 IHC	High sensitivity Inexpensive FFPE tissues manageable	Moderate specificity	80-90	80-90
DNA PCR	HPV genotype information High sensitivity FFPE tissues manageable Easy and inexpensive	No information about viral transcription High risk of contamination (intrinsic and extrinsic)	98	84
E6/E7 mRNA RT-PCR	High sensitivity and specificity Detects active viral infection Gold standard for research	Time-consuming Non-FFPE tissues manageable (fresh or frozen tissue only) RNA fragility RNA degradation over time, expensive	97	100
E6/E7 mRNA ISH	High specificity and sensitivity In situ detection of a transcriptionally active HPV infection FFPE tissues manageable	RNA degradation over time Expensive	87-100	88-100
HPV DNA ISH	In situ detection of HPV DNA High specificity FFPE tissues manageable	Low sensitivity	85	88
OncoE6^{TM}	High specificity, easy to use	Low sensitivity Need to be validated with a larger cohort	88-94	100
Serology for antibodies against E6 protein	Present in more than 90% of patient with OPSCC related to HPV16 Easy to set up	Lack of clinical data and retrospect		

HPV circulating tumoral DNA by ddPCR	Early detection of recurrences in posttreatment monitoring High sensitivity and specificity. Low cost	Need to be validated

NGS system was used to detect HPV in FFPE from 44 HN tumors and they found that NGS had 100% specificity compared to PCR or p16 IHC and 50% sensitivity compared to PCR and 75% sensitivity compared to p16 IHC (101). Mattox et al. (102) directly compared next-generation sequencing (NGS), ddPCR, and RT-qPCR in detecting HPV16 DNA in plasma and oral rinse from 66 patients diagnosed with HPV16-positive OPSCC. Their results showed that NGS and ddPCR both had comparable sensitivity (68.3% vs 69.8% compared to RT-qPCR (20.6%) in plasma samples ($p<0.001$) like other comparison studies (103–106). For HPV detection in oral rinses, NGS was the most accurate with a sensitivity of 75.0%, which was significantly higher than ddPCR (8.3%, $p<0.001$) and RT-qPCR (2.1%, $p<0.001$). But Hanna et al.(107) analyzed plasma and saliva from 21 HPV+ OPSCC using ddPCR and they have similar sensitivity in plasma samples but higher sensitivity in saliva compared to Mattox oral rinse (86.7% vs 75%). Several studies compared NGS and ddPCR technologies in clinical molecular diagnostics and they both have their strengths and limitations. ddPCR is more cost-effective for rapid genotyping (108,109), serial monitoring (108), shorter turnaround time (109,110), has higher precision at low concentration targets (109,111), absolute quantification (109,110) and more straightforward data interpretation (109,110) compared to NGS. NGS provides higher discovery power, enabling the detection of known and unknown mutations (108,110) but ddPCR is more appropriate for screening or diagnosis for cancers that have

well-defined and consistent genetic markers such as in the identification of HPV types, providing absolute quantification rather than ratios or relative amounts at a lower cost.

A study used both RT-qPCR and MassARRAY for HPV detection and they found that non-invasive liquid biopsy-based (ie saliva) can be very helpful in the identification and treatment of HPV-HNSCC (112). HPV detection methods in oropharyngeal, nasopharyngeal and oral cavity cancers were compared using PCR-MassARRAY (PCR-MA), p16 IHC and ISH and results showed that PCR-MassARRAY had the highest sensitivity and specificity (99.5% and 100% vs 94.2% and 85.5% vs 82.9% s and 81.0%, respectively) (19). They found that PCR-MA had high throughput, can quickly identify HPV types and has a minimal DNA (5 ng) needed, making it useful for testing small tissue samples (19). Additionally, they discovered that PCR-MA in conjunction with p16 IHC was the most effective method for HPV testing in FFPE head and neck tumor tissue specimens, providing an accurate assessment of HPV presence, type, and activity (19). Several studies compared ddPCR and MassARRAY in plasma samples. Weber et al (113) performed molecular profiling from cell-free DNA (cfDNA) comparing ddPCR, MassARRAY and NGS and they found that MassARRAY had highest precision and ddPCR had the lowest. Leest et al (114) detected mutations in circulating tumor-specific DNA (ctDNA) in metastatic non-small cell lung cancer patients using both MassARRAY and ddPCR and they found that they had 90% concordance. Mehrotra et al (115) evaluated MassARRAY and ddPCR for cfDNA genotyping both compared against NGS. According to their findings for mutation identification, dPCR and MassARRAY had 83% and 77% concordance with NGS with 100% and 79% sensitivity, respectively (115). With the use of allele-specific primers or probes, ddPCR can perform absolute quantification of a mutant

gene copy number in the background of a wild-type sequence while also detecting low-level mutations utilizing small amount of nucleic acid samples (115). Boysen et al (116) analyzed ctDNA from patients with colorectal cancer comparing ddPCR and MassARRAY and they had 87% concordance but ddPCR was more sensitive with detection limit of 0.1%. Given that the predicted detection limit of MassARRAY is approximately 5%, it may be used as a screening tool to find mutations, while ddPCR identifies a specific mutation that is known in advance (116). Although MassARRAY detects novel variants or mutations, ddPCR is highly precise at low-concentration targets and more appropriate for screening or diagnosis for cancers that have well-defined and consistent genetic markers such as in the identification of HPV types. Our aims are limited to screening or identification of known variants so ddPCR is a more appropriate tool.

1.7 Droplet Digital Polymerase Chain Reaction (ddPCR) for HPV Detection

ddPCR quantifies the absolute amount of target nucleic acid molecules encapsulated in discrete, volumetrically defined water-in-oil droplet partitions (117). It was first commercially available in 2011 (118) but the concept of ddPCR was first raised by Sykes in 1992 in which DNA molecules are quantified using Poisson distribution and diluting templates to single-molecule level (119). Samples are prepared in a similar manner as PCR reactions that use TaqMan hydrolysis probes or DNA binding dyes (Eva Green®) but in smaller volume-precise reactions or partitions which are then run individually, and positive reactions are checked and calculated among each partition using Poisson distribution

(119,120). The system involves 3 main parts as follows (also summarized in **Table 1.10**): 1. droplet generation, in which samples are placed in a droplet generator to partition each sample into 20,000 uniform, nanoliter-sized droplets enabling precise target amplification; 2. amplification, in which samples are placed in a thermal cycler to amplify each droplet following the PCR principle involving denaturation, annealing and extension; and 3. droplet reading, in which the droplet reader reads spaced out individual droplets fluorescence in two channels. (117)

Table 1. 10: Summary of the steps and events in ddPCR

Steps	**Events**
Droplet generation	Samples are placed in a droplet generator using specially developed reagents and microfluidics to partition each sample into 20,000 uniform, nanoliter-sized droplets enabling precise target quantification. The target and background DNA are distributed randomly into the droplets. **Figure 2.1** shows the partitioning of discrete droplets and the distribution of target and background DNA (117)
Droplet amplification	Droplets are transferred in a thermal cycler to amplify each droplet. The amplification of target molecules follows similar principles of RT-PCR which involves denaturation, annealing and extension (117)
Droplet reading	Droplets are streamed in a single file in the reader which calculates the target DNA concentration by counting the fluorescent positive and negative droplets in two channels. Positive droplets containing at least one copy of the target DNA molecule demonstrate increased fluorescence compared to negative droplets. **Figure 2.2** shows the separation of individual droplets and readings measured in two channels (117)

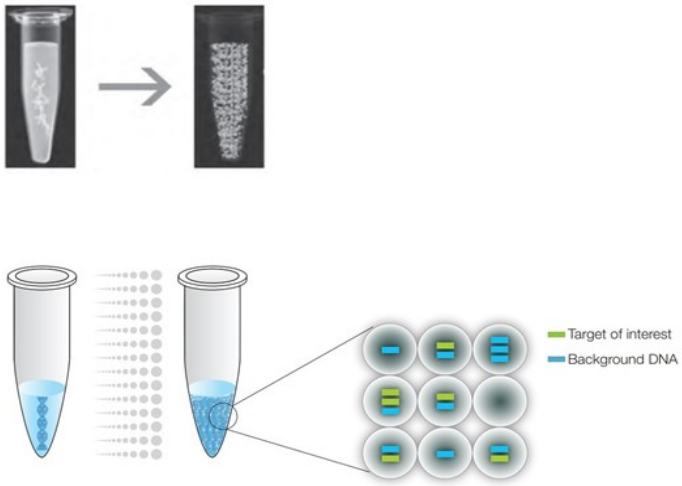


Figure 1. 1: The target and background DNA are randomly dispersed into the droplets of a ddPCR sample, which is partitioned into 20, 000 uniform, nanoliter-sized droplets **(117)**.

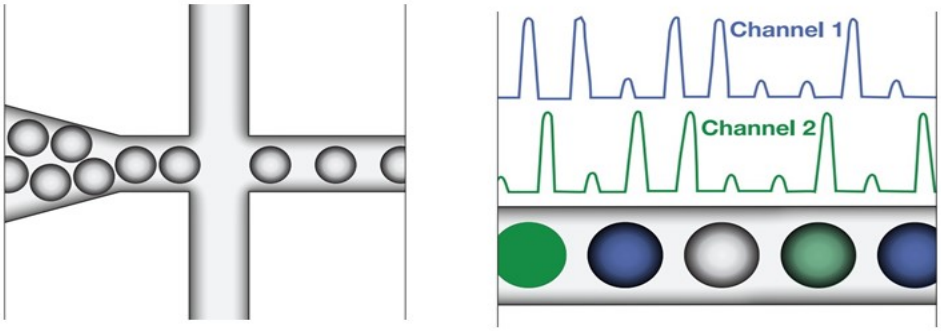


Figure 1. 2: Droplet reader reads spaced out individual droplets fluorescence in two channels in positive droplets with at least one copy of the target DNA molecule demonstrates increased fluorescence compared to negative droplets **(117)**.

ddPCR has a broad range of applications as summarized in **Table 1.11**, in both research and clinical diagnostic applications such as: 1. absolute quantification for target

DNA measurements, viral load analysis and microbial quantification, 2. genomic alterations such as gene copy number variations (CNV), 3. detection of rare sequences, 4. gene expression and microRNA analysis, 5. next-generation sequencing (NGS), 6. single-cell analysis and 7. genome edit detection (117).

Table 1. 11: Summary of the applications and capabilities of ddPCR

Applications	**ddPCR capabilities**
1. Absolute quantification	ddPCR's immense droplet partitioning provides quantification of DNA copies without standard curves giving more precise and reproducible data making it ideal for target DNA measurements, viral load analysis and microbial quantification (117).
2. Genomic alterations such as gene copy number variation (CNV)	CNVs are deletions and amplifications of genome segments involved in phenotypic variability, complex behavioral traits, and disease. ddPCR's droplet partitioning provides a large number of replicates that precisely measure copy numbers (117).
3. Detection of rare sequences	ddPCR increases sensitivity by partitioning the target mutant DNA away from highly homologous wildtype DNA (117).
4. Gene expression and microRNA analysis	ddPCR provides stand-alone absolute quantification with sensitivity and precision of expression levels, especially low-abundance microRNAs (117).
5. Next-generation sequencing (NGS)	Absolute quantification and accuracy of NGS sample preparations and validate sequencing results or CNVs (117).
6. Single-cell analysis	ddPCR enables quantification of low copy number (117).
7. Genome edit detection	dPCR provides fast, accurate, and cost-effective evaluation of HDR (Homology directed repair) and NHEJ (Non-homologous end joining) generated by CRISPR-Cas9 or other genome editing tools (117).

1.7.1 ddPCR HPV Detection in CSCC and OPSCC

The high sensitivity, specificity and absolute quantification for target DNA measurement by ddPCR are particularly of interest for HPV detection. Several studies have used ddPCR to detect HPV DNA and viral load (VL) in CSCC. HPV VL is an important determinant of virus persistence in HPV-related diseases and therefore VL quantification is a useful tool in preventive strategies as well as a biomarker for monitoring treatment response and prognosis estimation in HPV-related diseases (120,121). ddPCR was used to detect HPV in CSCC by using FFPE tissues, cervical liquid cytology samples and cell lines. Malin et al (120) detected HPV VL in FFPE tissues and cervical liquid cytology and their results showed that ddPCR was highly sensitive in detecting HPV and VL at the lowest dilution level, there was no difference in VL between tumors with multiple and single HPV infections and women's age, and HPV genotype and genera were associated with VL. Larsson et al (121) compared ddPCR with RT-qPCR in quantifying HPV VL in FFPE tissues and liquid-based cytology (LBC). Their results showed that DNAs extracted from FFPE tissue samples yielded lower amplification signals compared to LBC samples and ddPCR were found to quantify copy numbers that are 1 to 31 times higher than RT-qPCR numbers (121). Rotondo et al (27) used ddPCR to quantify HPV DNA in CIN specimens and human cell lines and their results showed the reliability of ddPCR in the simultaneous detection and quantification of different HPV types in one experimental run and low template copy-number conditions. ddPCR exhibited high sensitivity, accuracy and specificity in quantifying HPV DNA sequences and the method was repeatable and reproducible (27).

HPV detection using ddPCR has been demonstrated in OPSCC FFPE tissues, tissue biopsy, FNA biopsy, and swabs. Schiavetto et al (122) detected HPV DNA in OPSCC

FFPE tissues and showed comparable results to the clinical standard technique p16 IHC. Antonsson et al (123) detected HPV 16 VL in OPSCC FFPE tissues and showed large variations among HPV 16 positive OPSCC ranging from 1 copy per cell to over 900 per cell compared to CSCC where high VL is associated with an increased risk of CIN progression. Biron et al (26) detected HPV 16 in OPSCC tissues, FNA and swabs and they showed that adequate amounts of RNA were extracted using commercially available kits and the sensitivity and specificity of HPV E6 and E7 ddPCR for the detection of p16 positivity was 91.3% and 100%, respectively compared against p16 IHC. Isaac et al (25) detected HPV 16 in OPSCC swabs showed 92% sensitivity and 98% specificity against fresh tissue p16 IHC, which is the clinical reference standard. The excellent sensitivity and specificity of HPV detection using ddPCR in swabs without the need for invasive tissue biopsy have several potential applications for both diagnosis and disease surveillance. Furthermore, ddPCR method is reported to be accurate, repeatable, reproducible (27,118,124) and cost-effective (25,26,98).

1.7.2 ddPCR for Cervical HPV Self-sampling

Several studies have demonstrated the effectiveness of self-sampling vaginal swabs as a screening tool for CSCC in the minorities and lower socioeconomic groups, remote or hard-to-reach areas, and low-resource settings. The HPV self-sampling was effective in detecting HPV and as sensitive as clinician cytology samples to detect CIN2 or higher (15,16,125–127). Wright et al (18) study found that HPV testing of the self-sampled vaginal swab is less specific but as sensitive as cytology for detecting high-grade cervical disease in women age 35 years and older while Sancho-Garnier et al (127) study found that the sensitivity and specificity of hr-HPV testing using self-sampled vaginal swabs is very

similar to that of clinician-collected cervical specimens. Gustavsson et al (128) showed that self-sampling and repeated HPV tests detected more than twice as many women with CIN2+ compared to Pap smear cytology. Irregular or absenteeism to cervical screening is a major barrier to eliminating cervical cancer and there are many reasons for low participation such as cultural reluctance (14,16,128), limited access to health care or geographical isolation (129), lack of health insurance, low health literacy, language barriers and lack of awareness (16). HPV self-sampling is a great tool to increase cervical screening and several studies reported high uptake in participation (14,16,125,129–132). Moses et al (131) reported that there was a high uptake of self-sampling hr-HPV testing and it was highly acceptable in the community for cervical cancer screening which exceeded 99%, whereas the standard of care, VIA reached only 48.4% in a low-resource setting. Women have positive experiences and highly accepted HPV self-sampling screening strategy (14,15,130). Furthermore, in a randomized trial performed by Haguenoer et al (132), they showed that HPV self-sampling is a cost-effective method to increase participation in a cervical cancer screening program. With the substantial amount of studies performed on HPV testing of self-sampled specimens with positive outcomes, it has been proposed to be considered as a screening tool (14,15,125,129,131,132). Self-sampling at home followed by hr-HPV testing has been proposed to increase screening recruitment among underserved groups for convenience and avoid the need for a gynecological clinical exam in women with negative tests (127). Most of the HPV self-sampling was tested using commercially available HC2 (18,127), Cobas (15) and other PCR-based methods particularly RT-PCR (125,126,128,129,131,132) and PCR based testing is preferred to HC2 as it is more sensitive (132). Because the viral load in the vagina

is lower than the cervix, a test with high analytic sensitivity appears to be required for self-sampling to ensure equivalent accuracy between clinician and self-sampled specimens (132). It has been demonstrated that ddPCR exhibits high sensitivity, accuracy, specificity, repeatability and reproducibility compared to RT-PCR in quantifying HPV DNA (31,71,100) and therefore it can be used to test the self-sampled swabs. Since ddPCR method is reported to be accurate, repeatable, reproducible (27,118,124) and cost-effective (23,25,26,98) it is an ideal method for routine diagnostic testing.

1.8 Literature Review Conclusion

The routine practice for cervical cancer diagnosis is minimally invasive and utilizes liquid-based cytology followed by HPV testing using commercially available p16 IHC, DNA/RNA ISH, or DNA/RNA PCR. For OPSCC, the main HPV detection method available is for FNA, fresh, frozen or FFPE tissues using p16 IHC and/or DNA/RNA PCR. For other HPV-related HNSCC, however, HPV testing is not a standard procedure. The sinonasal tract is the second anatomic subsite of the head and neck for HPV-related carcinomas and favorable HPV prognosis is unresolved, therefore more research studies are essential to better understand the role of HR HPVs in sinonasal carcinoma.

Self-sampling HPV testing could be used in the future to replace Pap smears and cervical exams as first-line screening for cervical cancer. However, to ensure similar or better accuracy compared to clinician collected samples, a test with high analytical sensitivity and specificity is required. For HPV-related HNSCC, swabs will be sufficient for diagnosis without the need for highly invasive tissue biopsy. p16 IHC is the most widely used method due to its availability in labs but results can be highly variable as the criteria for interpretation is not standardized. Commercially available HPV testing methods

approved for cervical samples including HC2, Cervista, Aptima and Cobas 4800 all have comparable sensitivity and specificity. In comparison to cytology and p16 IHC, they have higher sensitivity but lower specificity.

The new generation of HPV assay such as ddPCR is highly sensitive and can be performed on non-invasive samples such as those obtained using swabs. ddPCR has the potential clinical applicability in early HPV detection for screening, diagnosis and disease surveillance. It can amplify a target sequence from minimal RNA samples and provides significantly higher precision and sensitivity for specific DNA/RNA compared to traditional PCR.

1.9 Study Rationale

The standard for the diagnosis of HPV-related OPSCC is based on biomarker p16-IHC on tissues alone without HPV testing required, both in clinical and trial settings. The problem with p16 overexpression as surrogate marker is that it can be inaccurate in up to 25% of cases (133) leading to misdiagnosis and mistreatment. HPV-testing may improve the accuracy of HPV-related OPSCC diagnosis. Additionally, p16-IHC as well as several other methods of HPV detection require a tissue biopsy. Early HPV diagnosis without a tissue biopsy has received considerable interest in the literature. This may have significant clinical applications for OPSCC surveillance, cases of unknown primary tumors, cases where a tissue biopsy may be difficult or prohibitive, such as patients with significant comorbidities, and in resource-limited settings. A new generation PCR technology, ddPCR has been reported to be highly accurate for molecular diagnosis and has the potential clinical applicability in early HPV detection. We employed this to detect HPV in

oropharyngeal swabs. Furthermore, a multinational study with a large OPSCC cohort have shown that HPV discordant patients have worse prognosis and therefore it is important to determine both p16 and HPV.

1.10 Research Hypothesis and Objectives

We hypothesized that ddPCR can provide clinically important diagnostic and prognostic information in the management of HPV-related OPSC. Our primary objective is to investigate the diagnostic and prognostic value of ddPCR to detect hr-HPV in salivary swabs from patients with OPSCC. We measured three main outcomes: the accuracy of HPV-ddPCR measured against clinical standard p16-IHC, the distribution of hr-HPV strains in OPSCC and the survival outcomes according to p16 and HPV.

Chapter 2: Materials and Methods

2.1 Setting

This was a single-center prospective cohort validation study at the University of Alberta tertiary care Otolaryngology-Head and Neck Surgery referral center in Edmonton, AB, Canada. Ethics board approval was obtained from Health Research Ethics Board – Biomedical Panel (Pro00062302) and Health Research Ethics Board of Alberta – Cancer Committee (HREBA.CC-18-0216).

2.2 Participants

Participants eligible for this study were recruited from January 2015 to June 2022 under the University of Alberta Health Research Ethics protocols. Three distinct adult participant groups were identified and recruited: 1) patients with a clinical diagnosis of OPSCC, 2) patients with benign tonsils and squamous papillomas (negative control) and 3) patients with non-OPSCC (HNCUP, OCSCC, sinonasal carcinoma and non-SCC). Patients who were unable to undergo an oropharyngeal swab, patients with unavailable p16 IHC, had previous HNSCC treatment and improperly processed samples were excluded from the study.

For participants enrolled in this study, chart reviews were performed to obtain the following data points: age, sex, date of diagnosis, date of death or last known alive, treatment types, tobacco smoking history, clinical and pathologic TNM staging (AJCC 8th edition), tumor histopathology, tumor subsite, and p16 IHC positivity. Smokers were defined as patients with a >10 pack-year tobacco smoking history. Former smokers were

defined as those with any pack-year smoking history and quit 12 months prior to the diagnosis.

2.3 Oral/oropharyngeal Swabs

Pre-treatment salivary swabs were obtained at the University of Alberta Head and Neck clinic or in the operating room prior to tumor resection. Swabs were collected and processed using an established protocol previously reported (25).

2.4 Tissue Pathology and p16 IHC

As per standard clinical practice, each patient with an oral/oropharyngeal tumour underwent panendoscopy with biopsy of the tumour, and the pathology was reported by a head and neck pathologist at the University of Alberta to confirm SCC diagnosis. In accordance with prevailing guidelines, p16 IHC was performed using a monoclonal antibody to p16 on 4 μm sections cut from formalin-fixed, paraffin-embedded tissue blocks (25). Control tonsil specimens were sent for pathologic evaluation at the time of tonsillectomy and the findings were interpreted by an anatomic pathologist for confirmation of non-malignant tonsil tissue. Since p16 is insignificant without carcinoma, p16 IHC was not performed on benign tissues and these patients were used as negative controls instead (25).

2.5 RNA Extraction, Quantification, and cDNA Synthesis

RNA from swab samples was extracted using the RNeasy Plus Mini Kit (Qiagen Cat #74134, Germantown, MD, USA) following the manufacturer's protocol. The 15 ml

conical tubes containing patient swabs in 5 ml of RNAlater solution were vortexed for 20 seconds. Following vortexing, the RNAlater solution, containing cells, was transferred to new 15 ml conical tubes and the tubes were centrifuged at 3000 x g for 12 minutes to pellet the cells. The supernatant was aspirated to within 100 ul, followed by the addition of 650 ul of Buffer RLT plus containing 40 mM DTT to solubilize the cell pellets then vortexed for 10 seconds. The solution containing the solubilized cells was loaded onto a QIA shredder (Qiagen Cat #79656) to homogenize the cells and then centrifuged for 30 seconds at 8,000 x g. The flow-through was loaded onto a gDNA Eliminator Column which eliminates genomic DNA contamination, effectively followed by centrifugation for 30 seconds at 8,000 x g. An equal volume of 70 % ethanol was added to the subsequent flow-through to help precipitate RNA molecules out of the aqueous solution, mixed well by pipetting, and the mixture was transferred to a RNeasy Mini spin column and centrifuged for 15 seconds at 8,000 x g. Following RNA binding, the Mini column was washed as per the manufacturer's instructions and the RNA was eluted with 54 ul RNAse free H_2O.

(**Note**: RNA extraction during COVID-19 Pandemic from 15-Apr-2020 to 5-Nov-2021). Swab samples were collected in 15 ml conical tubes containing 800 ul Buffer RLT with 40 mM DTT to minimize exposure to the virus by the deactivation of the viral particles that are exposed to the lysis buffer. The tubes were swirled gently and then transferred 750 ul of buffer/sample into QIAshredder column. The rest of the RNA extraction followed the above procedure. RNA extractions were performed within 24 hours of collection).

RNA concentration was quantified using the Qubit RNA HS assay kit (Invitrogen Cat # Q32855) on a Qubit 3.0 fluorometer as per the manufacturer's instructions. RNA (up to 200 ng) in a 20 ul reaction was used to synthesize cDNA using the iScript^{TM} Reverse Transcription Supermix

for RT-qPCR (BIO-RAD) and the C1000 Touch™ Thermal Cycler (catalog #185-1197 BIO-RAD) as per the manufacturer's protocols. Following the reaction, the cDNA was diluted in nuclease-free water up to a maximum concentration of 2 ng/ul, and either stored at -20° C or used directly for ddPCR.

2.6 Hr-HPV Identification Using Evagreen

ddPCR was carried out using the QX200™ ddPCR™ EvaGreen Supermix, (catalog # 186-4035 BIO-RAD) the QX200™ Droplet Generator (catalog #186-4002 BIO-RAD), the QX200 Droplet Reader (catalog #186-4003 BIO-RAD) the C1000 Touch™ Thermal Cycler (catalog #185-1197 BIO-RAD) and the PX1™ PCR Plate Sealer (catalog #181-4000 BIO-RAD) as per manufacturer's instructions.

2.6.1 For the Multi-primer Reactions

Reactions were set up following the manufacturer's protocols using 12 ul/reaction of 2x QX200™ ddPCR™ EvaGreen Supermix, 2.4 ul cDNA and 9.6 ul primer mixture in RNAse free H_2O. Final primer concentrations were 50 nM of each primer. For the initial screening of each sample, 2 multi-primer sets of hr-HPV primers were developed, with each set containing primer pairs for 6 types of hr-HPV to cover the 12 hr-HPV types. An additional 3 multi-primer sets of hr-HPV primers were developed and contained primer pairs for 4 types of hr-HPV per set. Final primer concentrations were 50 nM for each primer. Samples were first assayed with the multi-primer sets using EvaGreen ddPCR™.

2.6.2 For the Individual Primer Reactions

Reactions were set up following the manufacturer's protocols using 12 ul/reaction of 2x QX200™ ddPCR™ EvaGreen Supermix, 7.2 ul cDNA and 4.8 ul primer mixture in H_2O. Final primer concentrations were 50 nM of each primer. Reactions for both the multi-primer and individual primer reactions were set up as outlined above in 96 well plates, mixed using a Mixmate Vortex Shaker (Eppendorf) and 20 ul of the reaction mixture was transferred to DG8™ Cartridge for QX200/QX100 Droplet Generator (catalog #186-4008 BIO-RAD) followed by 70 µl of Droplet Generation Oil for EvaGreen (catalog #186-4006 BIO-RAD) into the oil wells, according to the QX200 Droplet Generator Instruction Manual (#10031907 BIO-RAD). Following droplet generation, 40 ul of the reaction was transferred to wells of a 96 well plate and the reactions were carried out in the thermocycler using the following parameters: For Step 1) 95º C for 5 minutes, Step 2) 95º C for 30 seconds and 58º C for 1 minute (Step 2 repeat 39 times for a total of 40), Step 3) 4º C for 5 minutes Step 4) 90º C for 5 minutes and Step 5) 4º C infinite hold. All steps had a ramp rate of 2º C/second. The steps are summarized in **Table 2.1.**

Table 2. 1: Summary of the thermocycler program for individual primer reactions

Cycling Step	Temp, °C	Time	Ramp Rate	Number of Cycles
1. Enzyme activation	95	5 min	2°C/ sec	1
2. Denaturation	95	30 sec		40
Annealing/extension	58	1 min		40
3. Cooling	4	5 min		1
4. Enzyme deactivation	90	5 min		1
4. Hold	4	Infinite		1

Following thermocycling, the reactions were read in the QX200 Droplet Reader, and the amplified amplicons were identified using the QuantaSoft™ Software (BIO-RAD). Samples showing clear positive results, and samples deemed possibly to be positive by amplitude shift of droplet fluorescence in the multi-primer assays, were further assayed using individual primer pairs corresponding to the primers present in the positive multi-primer assay. A three times greater amount of cDNA was used in the individual primer pair assays. Primer concentrations remained at 50 nM for each primer.

E6 Primers used for EvaGreen ddPCR are shown in **Table 2.2.** Primers for HPV types 18, 31, 33, 39, 45, 51, 56 and 59 were previously reported (134). Primers for HPV type 35, 52 and 58 were designed for this study and validated by ddPCR™ using the following cDNAs: Type 35 cDNA, DQ057314.1 Human papillomavirus type 35 isolate SA1505 E6 protein (E6) gene; Type 52 cDNA, KY077832.1 Human papillomavirus type 52 isolate KOR_M10-5237 E6 (E6) gene; Type 58 cDNA, KC190291.1 Human papillomavirus type 58 isolate USA_990989 E6 (E6) gene. Double stranded DNA was diluted to approximately 1100 molecules per ul and 2.4 ul were used for each ddPCR™ reaction.

Table 2. 2: Primer sequences used in ddPCR

HPV type	**EvaGreen ddPCR Primer sequences**		**Probe based ddPCR[a]**
HPV 16	5'-TCAGGACCCACAGGAGCG	Forward	CAGAAAGTTACCACAGTTATGCACAG
			AGC- FAM
	5'-CCTCACGTCGCAGTAACTGTTG	Reverse	
HPV 18	5'-AGAGGCCAGTGCCATTCGT	Forward	TCCTGTCGTGCTCGGTTGCAGC- HEX
	5'-GTTTCTCTGCGTCGTTGGAGT	Reverse	
HPV 31	5'ATTCCACAACATAGGAGGAAGGTG	Forward	ACAGGACGTTGCATAGTATGTTGGA-

			FAM
	5’-CACTTGGGTTTCAGTACGAGGTCT	Reverse	
HPV 33	5’-ATATTTCGGGTCGTTGGGCA	Forward	ACCTCCAACACGCCGCACAGC- HEX
	5’-ACGTCACAGTGCAGTTTCTCTACGT	Reverse	
HPV 35	5’-TCAGGACCCAGCTGAAAGACC	Forward	CTTTCTTCTACCTCGTTGCACAAAT
			CATGC - FAM
	5’-ACTCCGCTGTAATTCTTGTTTGC	Reverse	
HPV 39	5’GCAGGAAGCTATACAGGACAGTGTC	Forward	CCGTTTTGTGGTCCAGCACCG- HEX
	5’-CTTGGGTTTCTCTTCGTGTTAGTCT	Reverse	
HPV 45	5’-GGACAGTACCGAGGGCAGTGTAA	Forward	CATGTTGTGACCAGGCACGGCA- FAM
	5’-TCCCTACGTCTGCGAAGTCTTTC	Reverse	
HPV 51	5’-AAAGCAAAAATTGGTGGACGA	Forward	CATGAAATAGCGGGACGTTGGACG-
			HEX
	5’-TGCCAGCAATTAGCGCATT	Reverse	
HPV 52	5’-ACGAATTGTGTGAGGTGCTGG	Forward	TTTGCACTGCACACACTGCAGCC-
			FAM
	5’-ACTTGTATACCTCTCTTCGTTGTAGC	Reverse	
HPV 56	5’-TGCATTGTGACAGAAAAAGACGAT	Forward	CCCGGTCCAACCATGTGCTATTAGAT
			GA- HEX
	5’-CTCCAGCACCCCAAACATG	Reverse	
HPV 58	5’-TGTGCATGAAATCGAATTGAAATGC	Forward	CTCAGATCGCTGCAAAGTCTTTTTGC-
			FAM
	5’ACACTTTACATACTGCAAATGGATTTCC	Reverse	
HPV 59	5’TGTATGGAGAAACATTAGAGGCTGAA	Forward	AGACACCGTTACATGAGCTGCTGATA
			CGC- HEX
	5’TGGACATAGAGGTTTTAGGCATCTATAA	Reverse	

[a,] Probe based ddPCR uses forward and reverse primers shown for EvaGreen ddPCR in addition to probes shown, labelled with HEX or FAM fluorophore.

2.7 Hr-HPV Identification Using Probe-based ddPCR

ddPCR was carried out using the ddPCR™ Supermix for Probes (No dUTP) (catalog # 186-3024 BIO-RAD), the QX200™ Droplet Generator (catalog #186-4002 BIO-RAD), the QX200 Droplet Reader (catalog #186-4003 BIO-RAD) the C1000 Touch™ Thermal Cycler (catalog #185-1197 BIO-RAD) and the PX1™ PCR Plate Sealer (catalog #181-4000 BIO-RAD) as per manufacturer's instructions.

Reactions were set up following the manufacturer's protocols using 12 ul/reaction of 2x ddPCR Supermix for Probes (No dUTP), 1.2 ul/reaction of 20x target primers/probe for one specific HPV type E6 transcript, 1.2 ul/reaction 20x target primers/probe for a second specific HPV type E6 transcript, 2.4 ul cDNA (at up to 2 ng/ul where concentration was high enough) and 7.4 ul nuclease-free water in a 96 well plate. Primer/probe sequences and multiplex reaction combinations are shown below. Reactions were mixed 3 times for 30 seconds at 1000 RPM using a Mixmate Vortex Shaker (Eppendorf) and 20 ul of the reaction mixture was transferred to DG8TM Cartridge for QX200/QX100 Droplet Generator (catalog #186-4008 BIO-RAD) followed by 70 µl of Droplet Generation Oil for Probes (catalog #186-3005 BIO-RAD) into the oil wells, according to the QX200 Droplet Generator Instruction Manual (#10031907 BIO-RAD). Following droplet generation, 40 ul of the reaction was transferred to wells of a 96 well plate and PCR reactions were carried out in the thermocycler using the following parameters: Step 1: 95° C for 10 minutes, Step 2: 94° C for 30 seconds and 60° C for 1 minute (Step 2 repeat 39 times for a total of 40), Step 3: 98° C for 10 minutes and Step 4: 4° C infinite hold. All steps had a ramp rate of 2° C/second. The thermocycler program is summarized in **Table 2.3.** Following

thermocycling, the reactions were read in the QX200 Droplet Reader, and the RNA targets were quantified using the QuantaSoft™ Software (BIO-RAD).

Table 2. 3: Summary of the thermocycler program for probe-based ddPCR

Cycling Step	Temp, °C	Time	Ramp Rate	Number of Cycles
1. Enzyme activation	95	10 min		1
2. Denaturation	94	30 sec		40
Annealing/extension	60	1 min	2°C/ sec	40
3 Enzyme deactivation	98	10 min		1
4. Hold	4	Infinite		1

E6 hr-HPV primers and probes are shown in **Table 2.2**. Primers and probes for HPV types 18, 31, 33, 39, 45, 51, 56, and 59 were previously reported (134). Probes for Types 31, 33 and 39 were slightly modified for melting temp GC content and /or removal of Guanadine from 5'end and validated using the cDNAs shown below. Primers for HPV type 16 were previously reported (26).

Primers and probes for HPV type 35, 52 and 58 were designed for this study and validated by ddPCR™ using the following cDNAs: Type 35 cDNA, DQ057314.1 Human papillomavirus type 35 isolate SA1505 E6 protein (E6) gene; Type 52 cDNA, KY077832.1 Human papillomavirus type 52 isolate KOR_M10-5237 E6 (E6) gene; Type 58 cDNA, KC190291.1 Human papillomavirus type 58 isolate USA_990989 E6 (E6) gene.Double stranded DNA was diluted to approximately 1100 molecules per ul and 2.4 ul were used for each ddPCR™ reaction.

2.7.1 Primers/probes Combinations for Multiplexing

Type 16 FAM was paired with type 18 HEX, type 31 FAM was paired with type 33 HEX, type 35 FAM was paired with type 39 HEX, type 45 FAM was paired with type 51 HEX, type 52 FAM was paired with type 56 HEX and type 58 FAM was paired with type 59 HEX.

2.7.2 E6 cDNA Sequences Used for Primer/probe Validations

Type 18, KC662569.1 Human papillomavirus type 18 isolate B8890 E6 (E6) gene, partial cds; Type 31, KC662562.1 Human papillomavirus type 31 isolate B848 E6 (E6) gene, complete cds; Type 33, KC662567.1 Human papillomavirus type 33 isolate B8454 E6 (E6) gene, complete cds; Type 35, DQ057314.1 Human papillomavirus type 35 isolate SA1505 E6 protein (E6) gene, complete cds; Type 39, KC470246.1:107-583 Human papillomavirus type 39 isolate Rw15, complete genome; Type 45, KC662573.1 Human papillomavirus type 45 isolate B3587 E6 (E6) gene, complete cds; Type 51, KF436887.1:97-552 Human papillomavirus type 51 isolate BF315, complete genome (E6); Type 52,KY077832.1 Human papillomavirus type 52 isolate KOR_M10-5237 E6 (E6) gene, complete cds; Type 56, DQ007173.1 Human papillomavirus type 56 isolate BR4114 E6 gene, partial cds; Type 58, KC190291.1 Human papillomavirus type 58 isolate USA_990989 E6 (E6) gene, complete cds; Type 59, KC470266.1:55-537 Human papillomavirus type 59 isolate Qv33361, complete genome. Double-stranded DNA was diluted to approximately 1100 molecules per ul and 2.4 ul were used for each ddPCR^{TM} reaction.

Positive controls using EEF2 HEX were paired with MKI67 (proliferation marker) FAM and with CDKN2A (p16 biomarker) FAM. Probes were prepared as follows. Reactions were set up following the manufacturer's protocols using 12 ul/reaction of 2x ddPCR^{TM} Supermix for Probes (No dUTP), 1.2 ul/reaction of 20x target primers/probe (FAM or HEX, BIO-RAD), 1.2 ul/reaction 20x reference primers/probe (FAM or HEX, BIO-RAD), 2.4 ul cDNA (at 0.5 ng/ul) and 7.4 ul nuclease-free water.

The catalog numbers for the positive controls: Primers/probe for CDKN2A FAM (Unique Assay ID: dHsaCPE5045104 (BIO-RAD), Primers/probe for EEF2 HEX (Unique Assay ID: dHsaCPE5050049 (BIO-RAD) and Primers/probe MKI67 FAM (Unique Assay ID: dHsaCPE5050322 (BIO-RAD).

2.8 Statistics

R version 4.2.3 and R Studio (version: 2023.09.1+494) were used for statistical calculations. Descriptive statistics were conducted to determine the proportion of the different groups of patients included in the study. Cohen's kappa was used to compare agreement between HPV-ddPCR and p16 IHC status for OPSCC, non-OPSCC and benign groups. Sensitivity, specificity, PPV, NPV, PLR, NLR and accuracy with 95 % confidence intervals (CI) was calculated as previously reported (25,26).

Chart reviews for OPSCC patients were performed using a clinical information system Connect Care to obtain age, sex, date of diagnosis, date of death or last known alive, treatment types, tobacco smoking history, clinical and pathologic TNM staging (AJCC 8th edition), tumor histopathology, tumor subsite, and p16 IHC positivity. Survival

times was calculated in years from the date of diagnosis to the date last known alive by follow-up or as noted on their electronic records, or date of death. Surviving patients are censored if they are still alive at the end of follow-up.

Univariate Cox proportional hazards models were used to analyze prognostic variables for overall survival (OS), disease-specific survival (DSS) and disease-free survival (DFS) (135). The event for OS is death from any cause, the event for DSS is death specifically from the disease and the event for DFS is recurrence of the disease. The prognostic variables for OS, DSS and DFS were also analyzed using multivariate Cox Proportional hazards. Hazard ratios (HR) were calculated with 95% Confidence Interval (CI). The variables for survival analysis were p16, HPV, p16/HPV combination, HPV strain, age, sex, smoking, stage (according to TNM 8^{th} edition) and treatment type (136).

Kaplan-Meier method and log-rank tests were used for estimating and comparing the 5-year survival probability (OS, DSS, DFS) to determine statistical significance between groups (p16+ vs p16-, HPV- vs HPV+, p16+/HPV+ vs p16+/HPV-, p16-/HPV+ and p16-/HPV-), stratified by age, sex, smoking, T-stage, N-stage and treatment type. The statistical significance was defined as $p<0.05$. A post-hoc power analysis (power chi square test) was conducted to estimate the sample size needed to detect a 5% difference and estimate the sample size required for 80% power for those with observed power less than 80%.

Chapter 3: Results

3.1 Patient Characteristics

From January 2015 to June 2022, 456 participants were eligible for this study (**Figure 3.1**). A total of 300 were OPSCC with p16 IHC available and 259 were p16 positive. Participants with non-oropharyngeal HNSCC were included for comparison in addition to patients with benign tonsil disease and squamous papilloma as negative controls (N=156). The mean age of the study participants with OPSCC was 61 years with the vast majority being male (85.3%), former smokers (47.7%) or current smokers (30.0%), with a mean of 31.5 pack per years for smoking history. A significant number of the participants had cancer in the tonsillar subsite (70.3%) followed by base of tongue (27.3%) in early stages of the disease T2 (41.7%) and N2 (46.0%) according to the AJCC 8 TNM staging system and surgery + radiotherapy (S+RT) is the most common treatment (31.0%) (**Table 3.1**).

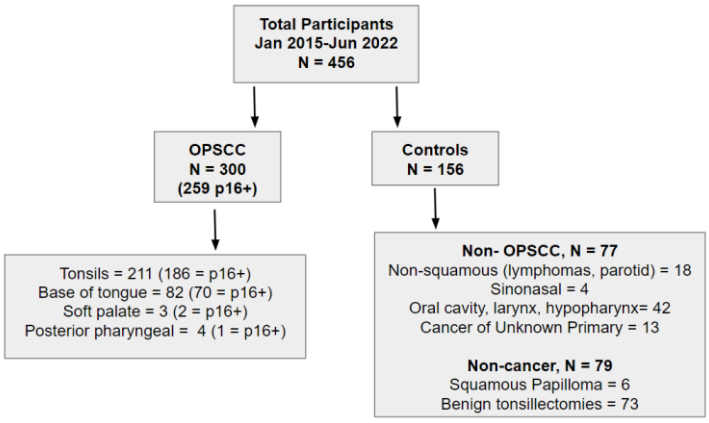


Figure 3. 1: Participants included in this study

Table 3. 1: Clinical and pathological characteristics of oropharyngeal cancer patients

Characteristic		**Participants no. (%), N=300**
Age at Diagnosis	Mean (SD)	61.1 (8.9)
	Median (range)	61 (34-84)
	30-49	28 (9.3)
	50-69	219 (73.0)
	70+	53 (17.7)
Sex	Male	256 (85.3)
	Female	44 (14.7)
Smoking status	Current smoker (87 >10py)	90 (30.0)
	Former smoker (131 >10py)	143 (47.7)
	Nonsmoker	67 (22.3)
Pack years (current and former smokers), n=233	Mean (former and current)	31.5
	Mean (former)	27.2
	Mean (current)	38.1
	<10	15 (6.4)
	>10	218 (93.6)
p16 pathology	p16+	259 (86.3)
	p16-	41 (13.7)
Tumor subsite	Tonsil	211 (70.3)
	Base of tongue	82 (27.3)
	Soft palate	3 (1.0)
	Posterior pharyngeal wall	4 (1.3)
T-stage	T1	69 (23.0)
	T2	125 (41.7)
	T3	69 (23.0)
	T4	37 (12.3)
N-stage	N0	32 (10.7)
	N1	106 (35.3)
	N2	138 (46.0)
	N3	24 (8.0)
Treatment Type	Surgery + CRT	69 (23.0)
	Surgery + RT	93 (31.0)
	CRT	45 (15.0)
	RT	7 (2.3)
	Surgery	79 (26.3)
	Palliative RT	5 (1.7)
	No treatment	2 (0.7)

3.2 Diagnostic Accuracy of HPV-ddPCR Compared to p16 IHC

HPV-ddPCR has a sensitivity of 95.8 % (93.3-98.2), specificity of 97.1% (94.3-99.9) and accuracy of 96.2% (93.7-98.8) compared to p16 IHC. The overall agreement between swabs ddPCR and p16 IHC was high at 0.92 (0.88-0.96) (**Table 3.2**). Of the 259 participants with p16+ by IHC, 201 tested positive for the disease by ddPCR utilizing oropharyngeal swabs. 3/6 HNCUP with p16+ IHC were HPV-ddPCR+, 2/4 sinonasal with p16+ IHC were HPV-ddPCR+, 1/2 OCSCC with p16+ IHC were HPV-ddPCR+. All the non-squamous cell and papilloma (24 participants) were HPV- ddPCR-. 2/73 benign tonsils were HPV- ddPCR+ **(Table 3.3)**.

Table 3. 2: Diagnostic accuracy of HPV-ddPCR + CDKN2A (p16 gene) swabs compared to p16 IHC

Statistics	HPV-ddPCR + CDKN2A value % (95% CI)
Sensitivity	95.8 (93.3 – 98.2)
Specificity	97.1 (94.3 – 99.9)
PPV*	98.4 (96.9 – 99.9)
NPV*	92.5 (88.1 – 96.7)
Accuracy	96.2 (93.7 – 98.8)
PLR	33.3 (12.7 – 87.5)
NLR	0.04 (0.02 – 0.08)
**Kappa (K)	0.92 (0.88 – 0.96)

CI, confidence interval. NLR, negative likelihood ratio; NPV; negative predictive value; PLR, positive likelihood ratio; PPV; positive predictive value. *Because the sample sizes in disease positive and disease negative groups may not reflect the true population prevalence of the disease, PPV and NPV may be inaccurate. **Agreement between tests: HPV-ddPCR+ CDKN2A has near perfect agreement compared to p16IHC.

3.3 Association Between p16 and HPV-ddPCR

P16 positivity was found in 86.3% OPSCC, 46.2% HNCUP, 100% sinonasal, 4.8 % OCSCC and 5.6% non-squamous. Participants with benign tonsillar disease, squamous papilloma and non- squamous cell did not have p16 staining available but were included in the analysis as negative controls. HPV-ddPCR positivity were found in 67.0% OPSCC, 23.1% HNCUP, 50.0% sinonasal, 14.3% OCSCC and 2.7% benign tonsils. No HPV-ddPCR positivity in non-squamous cells and opharyngeal squamous papillomas **(Table 3.3)**.

Table 3. 3: P16 in tissues and hr-HPV in swabs positivity in cancerous and non-cancerous head and neck

Diagnosis	p16 IHC positivity, # (%)	Swab HPV- ddPCR positivity, # (%)
OPSCC (N = 300)	259 (86.3)	201 (67.0)
HNCUP (n = 13)	6 (46.2)	3 (23.1)
Sinonasal SCC (n=4)	4 (100)	2 (50.0)
Oral cavity, larynx, hypopharynx SCC (n=42)	2 (4.8)	6 (14.3)
Non-squamous: lymphomas and parotid (n=18)	1 (5.6)	0
Papillomas[a] (n=6)	-	0
Benign tonsils (n=73)	-	2 (2.7)

a: benign squamous papilloma of tonsil or soft palate.

The p16 status and hHPV-ddPCR were analyzed for all OPSCC participants. There was only one OPSCC from base of tongue subsite that didn't have p16 testing available which was excluded from the study but tested for HPV and it was negative. For the OPSCC

cohort, 65.1% were p16+/HPV+, 20.9% were p16+/HPV-, 1.7% were p16-/HPV+, and 12.0% were p16-/HPV- **(Table 3.4).**

Table 3. 4: Summary of hr-HPV and p16 IHC status of all participants

Group	**Tumor subsite N=457**	**p16+ n(%)**	**p16- n(%)**	**HPV+ n(%)**	**HPV- n(%)**	**p16+/ HPV+ n(%)**	**p16+/ HPV- n(%)**	**p16-/ HPV+ n(%)**	**p16-/ HPV- n(%)**	**p16 not tested/ HPV+ n(%)**	**p16 not tested/ HPV- n(%)**
OPSCC (n=301)	All OPSCC	259 (86.3)	41 (13.7)	201 (67.0)	100 (33.3)	196 (65.3)	63 (21.0)	5 (1.7)	36 (12.0)	0	*1 (0.3)
	Tonsil (n=211)	186 (88.2)	25 (11.8)	148 (70.1)	63 (29.9)	145 (68.7)	41 (19.4)	3 (1.4)	22 (10.4)	0	0
	Base of tongue (n=83)	70 (85.4)	12 (14.6)	50 (61.0)	33 (40.2)	49 (59.8)	21 (25.6)	1 (1.2)	11 (13.4)	0	*1 (1.2)
	Soft palate (n=3)	2 (66.7)	1 (33.3)	2 (66.7)	1 (33.3)	2 (66.7)	0	0	1 (33.3)	0	0
	Post pharyngeal wall (n=4)	1 (25)	3 (75)	1 (25)	3 (75)	0	1 (25)	1 (25)	2 (50)	0	0
Control (n=156)	HNCUP (n=13)	6 (46.2)	5 (38.5)	3 (23.1)	10 (76.9)	2 (15.4)	4 (30.8)	1 (7.7)	4 (30.8)	0	2 (15.4)
	Sinonasal (n=4)	4 (100)	0	2 (50)	2 (50)	2 (50)	2 (50)	0	0	0	0
	Oral cavity, larynx, hypopharynx (n=42)	2 (4.8)	13 (31)	6 (14.3)	36 (85.7)	1 (2.4)	1 (4.5)	1 (4.5)	12 (28.6)	4 (9.5)	23 (54.8)
	Non squamous: lymphomas, parotid (n=18)	1 (5.6)	1 (5.6)	0	18 (100)	0	1 (5.6)	0	1 (5.6)	0	16 (94.1)
	Papilloma (n=6)	0	0	0	6 (100)	0	0	0	0	0	6 (100)
	Benign (n=73)	0	3 (4.1)	2 (2.7)	71 (97.3	0	0	0	3 (4.1)	2 (2.7)	68 (93.2)

*Excluded from the study

3.4 Detection of hr-HPV from Oropharyngeal Swabs Using ddPCR

The ddPCR protocol was designed to capture the 12 high-risk HPV (hrHPV-ddPCR) strains known to be oncogenic according to World Health Organization International Agency for Research on Cancer (137). The detection of specific sequences was confirmed with strain-specific nucleic acids (see methods). Of the 300 obtained oropharyngeal swabs, 201 tested positive for hr-HPV. HPV16 (85.1%) was predominant followed by HPV33 (6.0%), HPV18, 35 and 45 (3% each), 39 (2.0%), 31 (1.5%), 52, 56 and 58 (1.0% each) and 59 (0.5%) (**Table 3.5**).

Table 3. 5: Summary of hr-HPV distribution in OPSCC

hr-HPV Strain	OPSCC, no (%) n=201
HPV16	171 (85.1)
HPV18	6 (3.0)
HPV31	3 (1.5)
HPV33	12 (6.0)
HPV35	6 (3.0)
HPV39	4 (2.0)
HPV45	6 (3.0)
HPV51	0
HPV52	2 (1.0)
HPV56	2 (1.0)
HPV58	2 (1.0)
HPV59	1 (0.5)

Of the 259 p16+ OPSCC specimens tested for hr-HPV using ddPCR , 65.3% tested positive for HPV16. Among the 41 p16- OPSCC specimens, 4.9% tested positive for HPV16. For the 201 specimens tested positive for hr-HPV-ddPCR, 86.5% tested positive

for HPV16 from tonsil subsite, 84.0% from base of tongue and 50% from soft palate. There were 5% p16+ OPSCC that tested positive for 2 or more types of hr-HPV (2HPV+) (**Table 3.6)**.

Table 3. 6: Hr-HPV swab positivity in OPSCC according to tumor subsite and p16 positivity

HR-HPV Strain	**p16, no. (%)**		**Oropharyngeal Subsite, HPV+ no. (%)**				
	+ (n=259)	**- (n=41)**	**All HPV + OPSCC (n=201)**	**Tonsil (n=148)**	**Base of tongue (n=50)**	**Soft palate (n=2)**	**Posterior pharyngeal wall (n=1)**
HPV16	169 (65.3)	2 (4.9)	171 (85.1)	128 (86.5)	42 (84.0)	1 (50.0)	0
HPV18	6 (2.3)	0	6 (3.0)	3 (2.0)	3 (6.0)	0	0
HPV31	3 (1.2)	0	3 (1.5)	1 (0.7)	2 (4.0)	0	0
HPV33	11 (4.2)	1 (2.4)	12 (6.0)	6 (4.1)	4 (8.0)	1 (50.0)	1 (100)
HPV35	6 (2.3)	0	6 (3.0)	4 (2.7)	2 (4.0)	0	0
HPV39	4 (1.5)	0	4 (2.0)	3 (2.1)	1 (2.0)	0	0
HPV45	5 (1.9)	1 (2.4)	6 (3.0)	5 (3.4)	1 (2.0)	0	0
HPV51	0	0	0	0	0	0	0
HPV52	2 (0.8)	0	2 (1.0)	2 (1.4)	0	0	0
HPV56	1 (0.4)	1 (2.4)	2 (1.0)	2 (1.4)	0	0	0
HPV58	2 (0.8)	0	2 (1.0)	0	2 (4.0)	0	0
HPV59	1 (0.4)	0	1 (0.5)	1 (0.7)	0	0	0
Double positives	13 (5.0)	0	13 (6.5)	7 (4.7)	6 (12.0)	0	0

3.5 Univariate Cox's Proportional Hazard Model of Survival According to p16, HPV, p16/HPV and HPV Strains

There were 87 (29%) total deaths, 71 (23.7%) died of disease and 60 (20%) had disease recurrence (39 from patients who died of disease, 2 from patients who died of non-disease and 19 from patients who were alive). The five-year overall survival (OS), disease-specific survival (DSS) and disease-free survival (DFS) were analyzed for p16+ vs p16-, HPV+ vs HPV-, p16+/HPV+ vs p16+/HPV-, p16-/HPV+ and p16-/HPV-, HPV16 vs HPV- and other hr-HPV strains, HPV- vs hr-HPV strains, 1HPV+ vs HPV- and 2 to 3 types HPV strain (2-3HPV+) and HPV- vs 1HPV+ and 2-3HPV+.

3.5.1 Survival Outcomes According to p16 Status

There were statistically significant differences between p16+ and p16- patients for 5-year OS (73.7% vs 23.1%, p<0.001), DSS (77.4% vs 29.3%, p<0.001) and DFS (71.6% vs 40.9, p=0.002) (**Figure 3.2)** with post hoc power calculations of 99.99%, 99.98% and 87.26%, respectively **(Table 3.7).** Compared to p16-, p16+ have significantly lower hazard of death from any cause (OS: HR, 0.26; 95%CI, 0.17-0.41; p<0.001), hazard of death of disease (DSS: HR, 0.26; 95%CI, 0.16-0.44; p<0.001) and hazard of disease recurrence (DFS: HR, 0.39; 95%CI, 0.21-0.72; p=0.003) **(Table 3.7).**

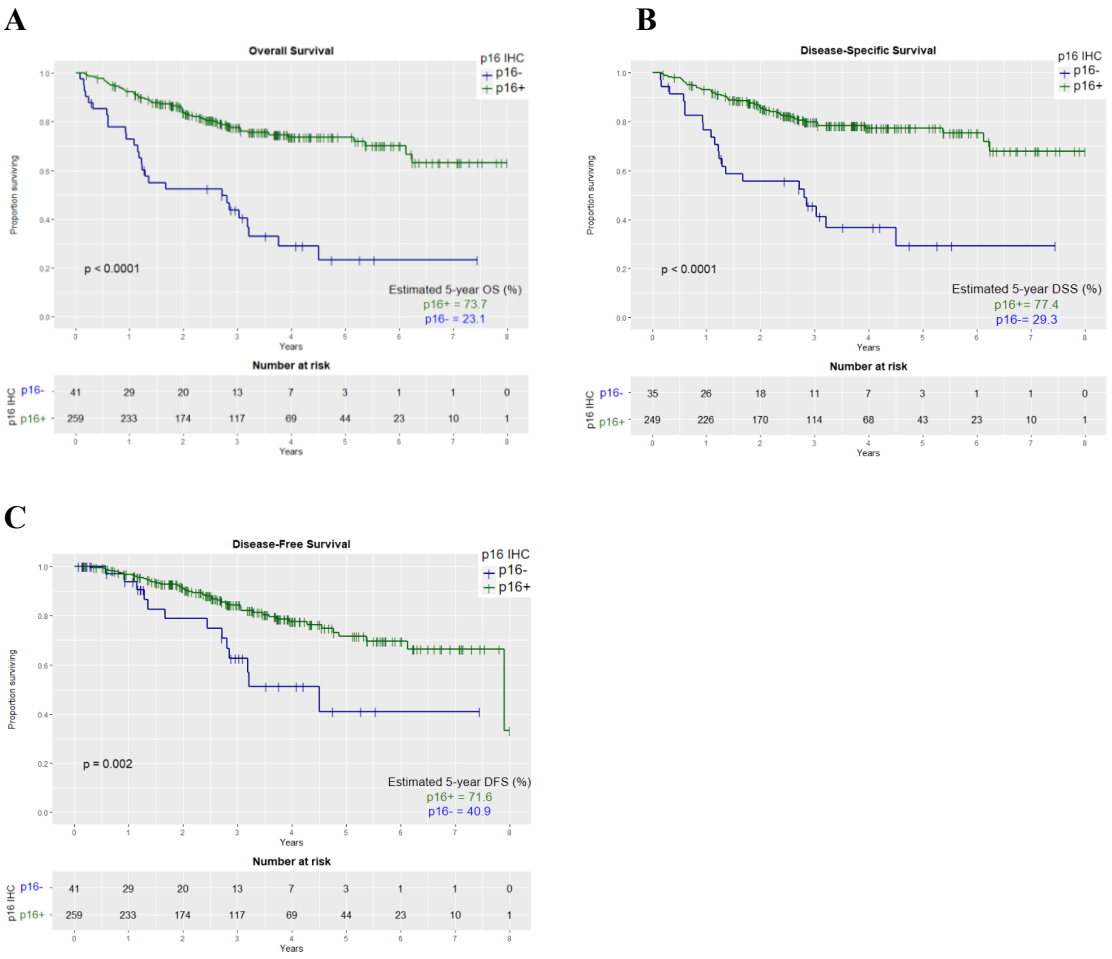


Figure 3. 2: Survival Analysis: p16+ vs p16-, A) OS B) DSS C) DFS

3.5.2 Survival Outcomes According to HPV Status

There were statistically significant differences between HPV+ patients and HPV- patients have for 5-year OS (72.2% vs 50.7, p=0.003) and DSS (76.0% vs 58%, p=0.0057) but not significant DFS (71.8% vs 59.2, p=0.051) (**Figure 3.3)** with post hoc power calculations of 84.30%, 78.73% and 49.55%, respectively. Compared to HPV-, HPV+ have significantly lower hazard of death from any cause (OS: HR, 0.53; 95%CI, 0.35-0.81; p=0.003) and hazard of death of disease (DSS: HR, 0.52; 95%CI, 0.33-0.83; p=0.007) but

not significant hazard of disease recurrence (DFS: HR, 0.60; 95%CI, 0.35-1.01; p=0.053) **(Table 3.7)**

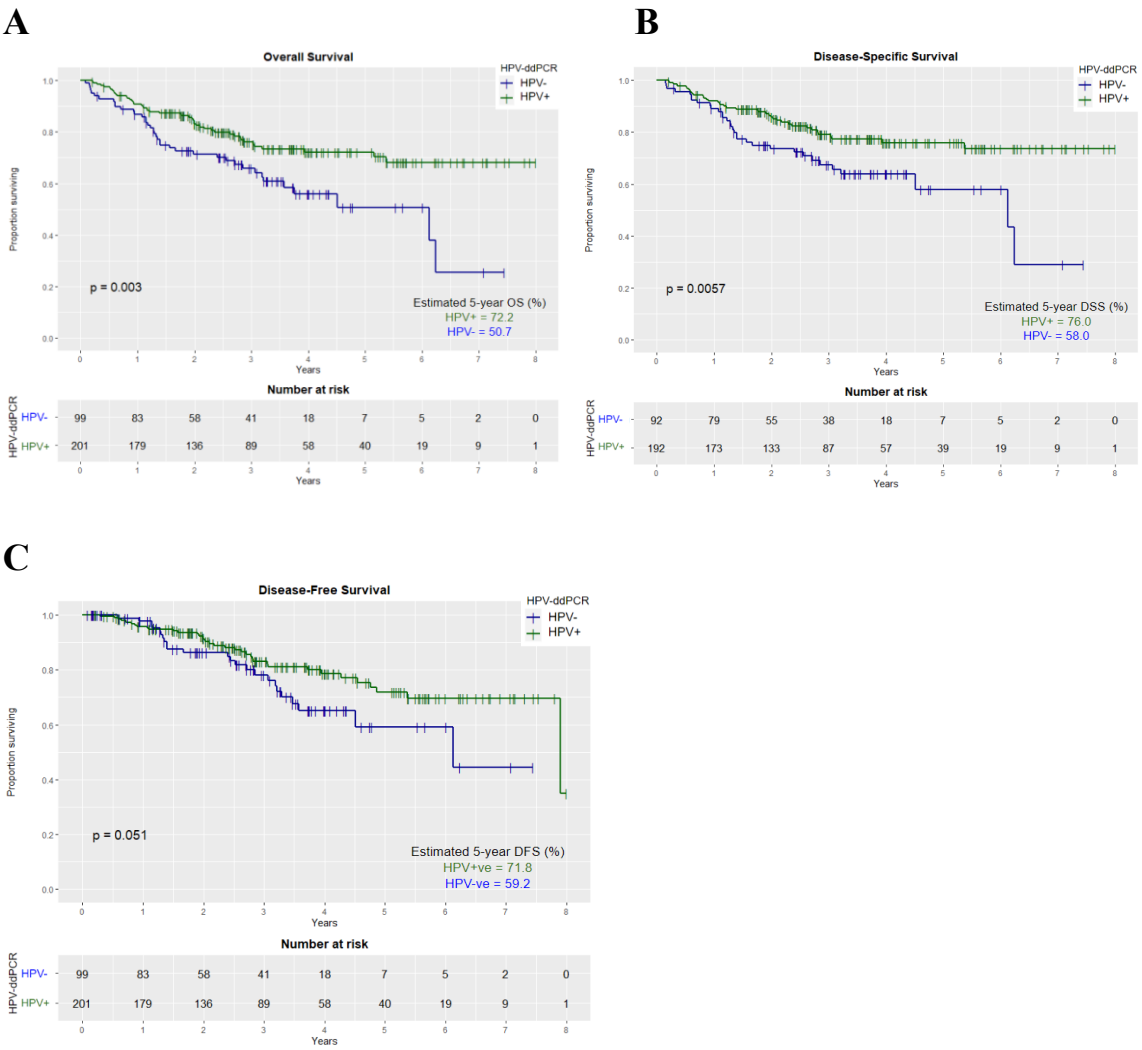


Figure 3. 3: Survival Analysis: HPV+ vs HPV -, A) OS B) DSS C) DFS

3.5.3 Survival Outcomes According to p16+/ HPV+, p16+/ HPV-, p16-/ HPV+ and p16-/ HPV-

In combining both p16 and HPV status, p16+/ HPV+ and p16+/ HPV- have the highest 5-year OS, DSS and DFS followed by p16-/ HPV+ and p16-/ HPV- have the worst

survival. There were no statistically significant differences between p16+/ HPV+ and p16+/ HPV- for OS (73.6% vs 73.3%, p=0.84), DSS (77.6% vs 76.9%, p=0.54) and DFS (72.0% vs 72.6%, p=0.63) **(Figure 3.4, Table 3.7)** with post hoc power calculations of 9.67% for DSS, and 7.36% for DFS and could not be calculated for OS **(Table 3.8)**. Compared to p16+/HPV+, p16+/ HPV- have slightly higher but not statistically significant hazard of death from any cause (OS: HR, 1.06; 95%CI, 0.59-1.91; p=0.8), hazard of death of disease (DSS: HR, 1.21; 95%CI, 0.65-2.25; p=0.5) and hazard of disease recurrence (DFS: HR, 1.17; 95%CI, 0.60-2.2; p=0.6) **(Table 3.8)**.

There were no statistically significant differences between p16+/HPV+ and p16-/HPV+ for OS (73.6% vs 30.0%, p=0.13), DSS (77.6% vs 30.0%, p=0.063) and DFS (72.0% vs 75.0%, p=0.94) (**(Figure 3.4, Table 3.7)** with post hoc power calculations of 32.91% for OS, 46.45% for DSS and could not be calculated for DFS. Compared to p16+/HPV+, p16-/ HPV+ have higher but not statistically significant hazard of death from any cause (OS: HR, 2.61; 95%CI, 0.81-8.40; p=0.11), hazard of death of disease (DSS: HR, 3.11; 95%CI, 0.95-10.1; p=0.06) and hazard of disease recurrence (DFS: HR, 1.17; 95%CI, 0.16-8.56; p=0.9) **(Table 3.8)**.

There were statistically significant differences between p16+/HPV+ and p16-/HPV- for OS (73.6% vs 21.4%, p<0.0001), DSS (77.6% vs 28.2%, p<0.0001) and DFS (72.0% vs 36.2%, p=0.00095) **(Figure 3.4, 3.7)** with post hoc power calculations of 99.99%, 99.95% and 91.02%, respectively **(Table 3.8).** Compared to p16+/HPV+, p16-/HPV- have significantly higher hazard of death from any cause (OS: HR, 4.13; 95%CI, 2.51-6.80; p<0.001), hazard of death of disease (DSS: HR, 4.22; 95%CI, 2.39-7.45; p<0.001) and the hazard of disease recurrence (DFS: HR, 2.98; 95%CI, 1.54-5.77; p=0.001) **(Table 3.8).**

To summarize, compared to p16+/HPV+, p16+/HPV- and p16-/HPV+ had lower but not statistically significant OS, DSS and DFS and p16-/HPV- had significantly lower OS, DSS and DFS.

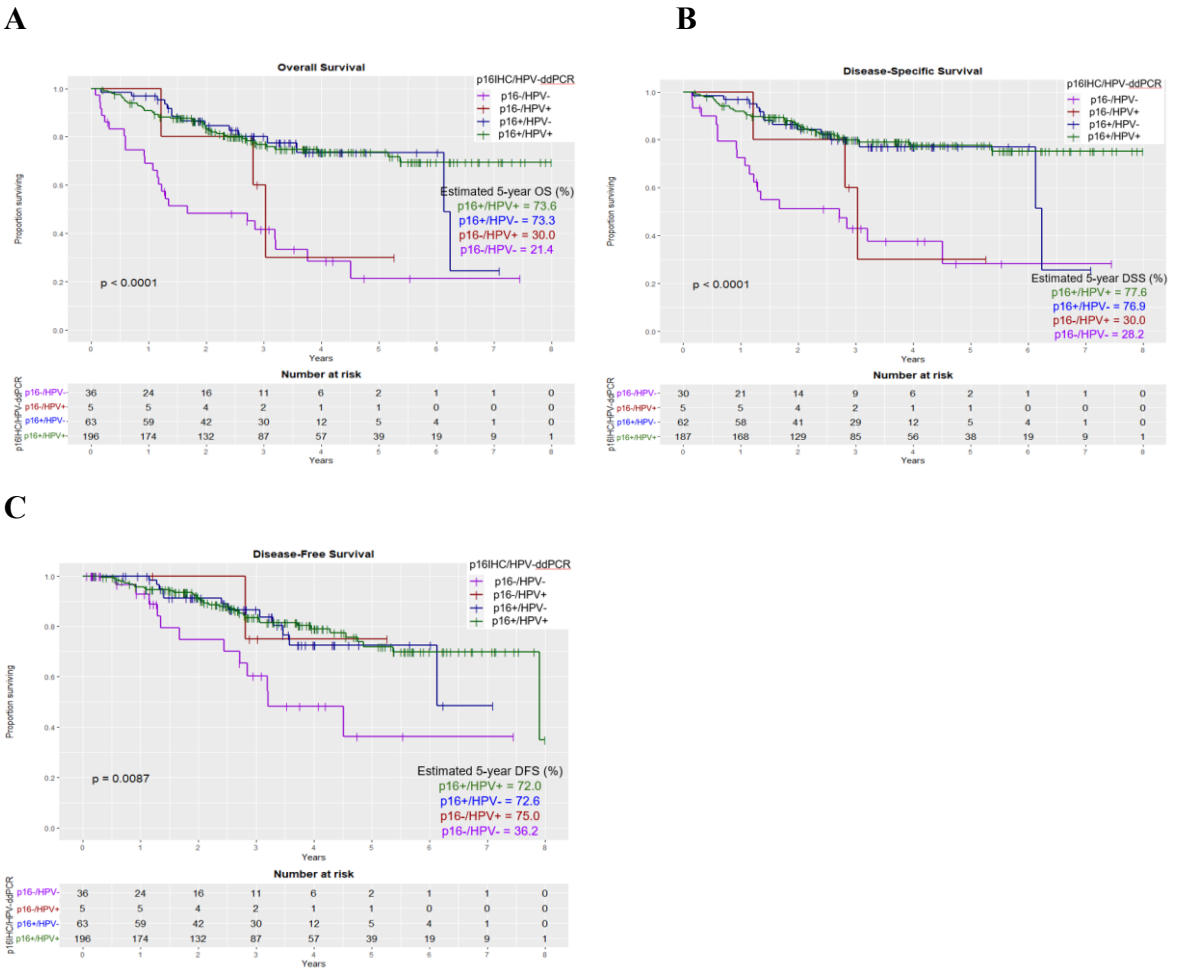


Figure 3. 4: Survival Analysis: p16+/HPV+ vs p16+/HPV-, p16/HPV+ and p16-/HPV-, A) OS B) DSS C) DFS

Table 3. 7: Summary of Proportion Surviving (OS, DSS and DFS) for p16, HPV and p16/HPV

Variables	Overall Survival		Disease-Specific Survival		Disease-Free Survival	
	Proportion Surviving (%)	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
p16 (vs p16-)						
p16-	23.1	**<0.0001**	29.3	**<0.0001**	40.9	**0.002**
p16+	73.7		77.4		71.6	
HPV (vs HPV-)						
HPV-	50.7	**0.003**	58.0	**0.0057**	59.2	0.051
HPV+	72.2		76.0		71.8	
p16/HPV (vs p16+/HPV+)						
p16+/HPV+	73.6		77.6		72.0	
p16+/HPV-	73.3	0.84	76.9	0.54	72.6	0.63
p16-/HPV+	30.0	0.13	30.0	0.063	75.0	0.94
p16-/HPV-	21.4	**<0.0001**	28.2	**<0.0001**	36.2	**0.00095**

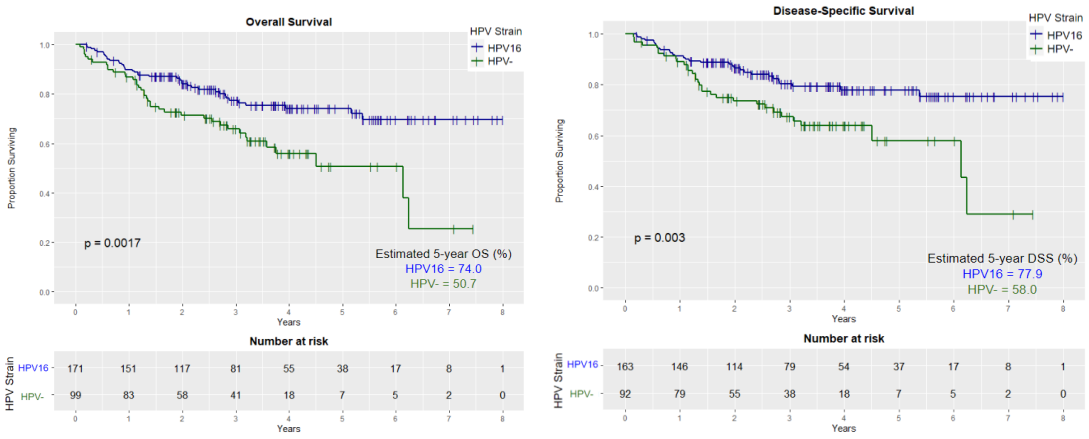
Table 3. 8: Univariate Cox Proportional Hazard Ratios and Post-hoc Power Calculations for p16, HPV, p16/HPV

Log-rank Test Comparing Survival Rates Based on p16, HPV and p16/HPV	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
p16- Reference					
OS: p16+	0.26 (0.17-0.41)	**<0.001**	300 (41+259)	**99.99**	
DSS: p16+	0.26 (0.16-0.44)	**<0.001**	284 (35+249)	**99.98**	
DFS: p16+	0.39 (0.21-0.72)	**0.003**	300 (41+259)	**87.26**	
HPV- Reference					
OS: HPV+	0.53 (0.35-0.81)	**0.003**	300 (99+201)	**84.30**	
DSS: HPV+	0.52 (0.33-0.83)	**0.007**	284 (92+192)	78.73	294
DFS: HPV+	0.60 (0.35-1.01)	0.053	300 (99+201)	49.55	621
p16+/HPV+ Reference					
OS: p16+/HPV-	1.06 (0.59-1.91)	0.8	259 (196+63)	---	---
DSS: p16+/HPV-	1.21 (0.65-2.25)	0.5	249 (187+62)	9.67	4906
DFS: p16+/HPV-	1.17 (0.60-2.27)	0.6	259 (196+63)	7.36	10012
OS: p16-/HPV+	2.61 (0.81-8.40)	0.11	201 (196+5)	32.91	686
DSS: p16-/HPV+	3.11 (0.95-10.1)	0.060	192 (187+5)	46.45	431
DFS: p16-/HPV+	1.17 (0.16-8.56)	0.9	201 (196+5)	---	---
OS: p16-/HPV-	4.13 (2.51-6.80)	**<0.001**	232 (196+36)	**99.99**	
DSS: p16-/HPV-	4.22 (2.39-7.45)	**<0.001**	217 (187+30)	**99.95**	
DFS: p16-/HPV-	2.98 (1.54-5.77)	**0.001**	232 (196+36)	**91.02**	

3.5.4 OPSCC Survival Outcomes According to HPV Strain

There were statistically significant differences between HPV16 and HPV- for 5-year OS (74.0% vs 50.7%, p=0.0017) and DSS (77.9% vs 58.0%, p=0.003) but not significant DFS (70.9% vs 59.2%, p=0.073) (**Figure 3.5, Table 3.9)** with post hoc power calculations of 88.07%, 86.28% and 43.24%, respectively **(Table 3.10)**. Compared to HPV16, HPV- have significantly higher hazard of death from any cause (OS: HR, 2.02; 95%CI, 1.29-3.15; p=0.002) and hazard of death of disease (DSS: HR, 2.09; 95%CI, 1.27-3.43; p=0.004) but not significant hazard of disease recurrence (DFS: HR, 1.62; 95%CI, 0.95-2.77; p=0.076) **(Table 3.10).**

A **B**



C

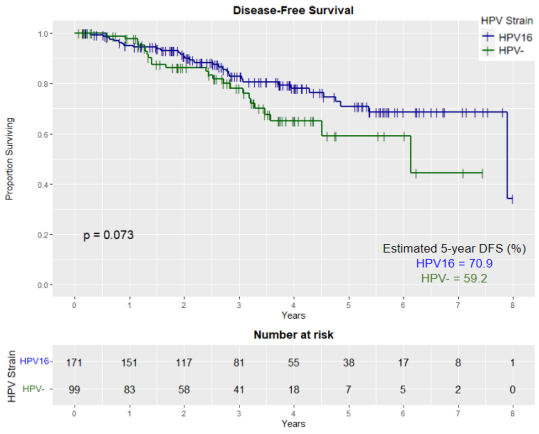


Figure 3. 5: Survival Analysis: HPV16 vs HPV-, A) OS B) DSS C) DFS

There were statistically significant differences between HPV16 and HPV33 for 5-year OS (74.0% vs 39.1%, p=0.023) and DSS (77.9% vs 43.8%, p=0.026) but not significant DFS (70.9% vs 75.8%, p=0.8) **(Figure 3.6, Table 3.9)** with post hoc power calculations of 62.58%, 60.00% and 6.16%, respectively **(Table 3.10).** Compared to HPV16, HPV33 have significantly higher hazard of death from any cause (OS: HR, 2.62; 95%CI, 1.10-6.20; p=0.029) and hazard of death of disease (DSS: HR, 2.79; 95%CI, 1.08-7.20; p=0.034) but not significant hazard of disease recurrence (DFS: HR, 1.20; 95%CI, 0.29-5.04; p=0.8) **(Table 3.10).**

There were statistically significant differences between HPV16 and HPV59 for 5-year DSS (77.9% vs 0, p=0.045) but not significant OS (74.0% vs 0, p=0.072) and DFS (70.9% vs 100%, p=0.73) **(Table 3.9)** with post hoc power calculations of 51.62%, 43.22% and 6.15% respectively **(Table 3.10).** There were no statistically significant differences between HPV16 and HPV59 in hazard of death from any cause, hazard of death of disease and hazard of disease recurrence **(Table 3.10).**

Table 3.9 summarizes the 5-year OS, DSS, and DFS for HPV16 against other HPV strains. Other than the differences outlined above, there are no other significant differences in survival rates. HPV16 has a lower but not statistically significant hazard of death from any cause, hazard of death of disease and hazard of disease recurrence compared to HPV18, HPV31, HPV39, HPV58 and HPV 59 and higher but not statistically significant compared to HPV35 and HPV45 but not significant **(Table 3.10).**

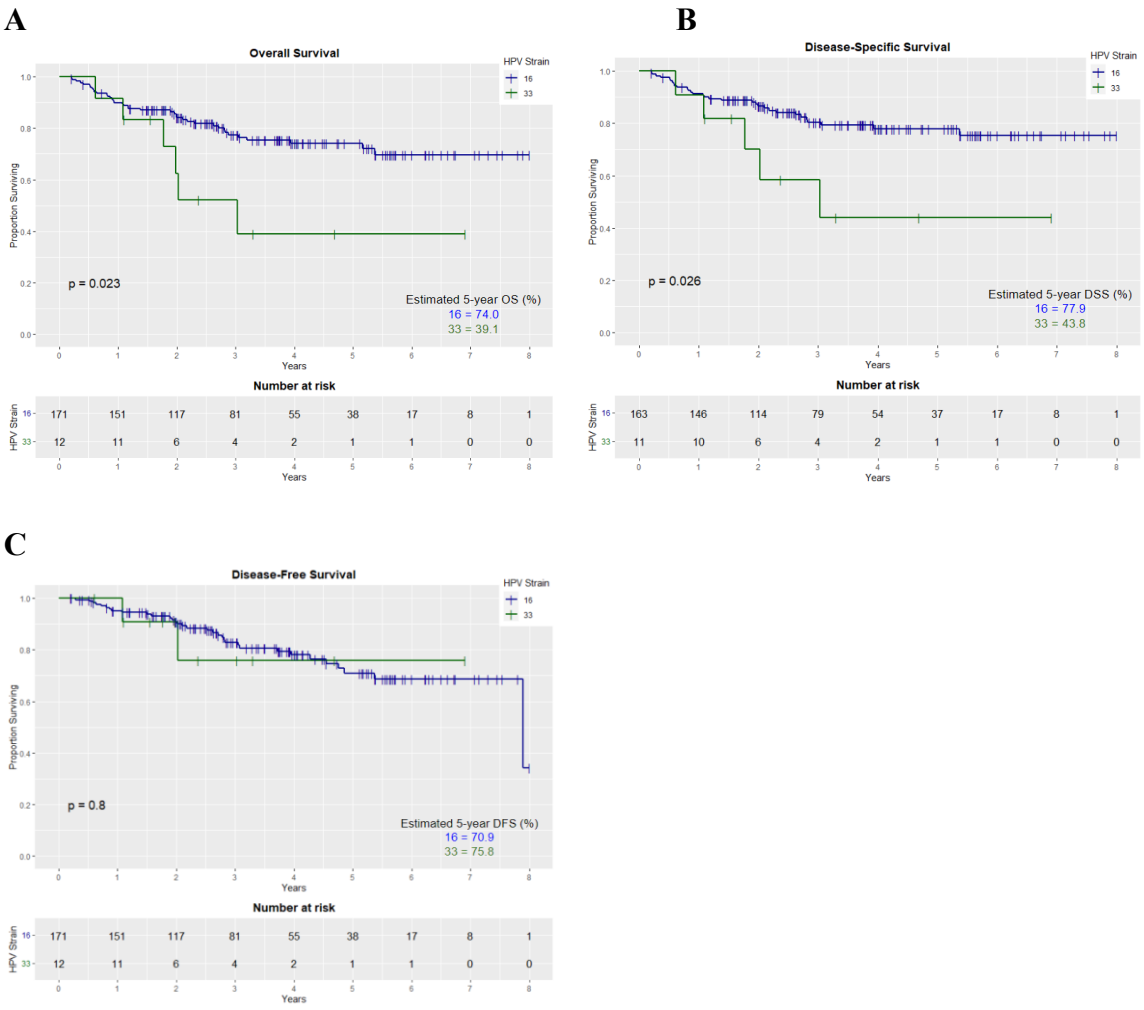


Figure 3. 6: Survival Analysis: HPV16 vs HPV33, A) OS B) DSS C) DFS

Table 3. 9: Summary of Proportion Surviving (OS, DSS and DFS) for HPV16 vs HPV- and other hr-HPV strains

	Overall Survival		Disease-Specific Survival		Disease-Free Survival	
Variables	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
HPV16	74.0		77.9		70.9	
HPV-	50.7	**0.0017**	58.0	**0.003**	59.2	0.073
HPV18	66.7 (3.5y)	0.37	80.0 (3.5y)	0.84	83.3 (3.5y)	0.72
HPV31	66.7 (2.6y)	0.39	66.7 (2.6y)	0.31	66.7 (2.6y)	0.15
HPV33	39.1	**0.023**	43.8	**0.026**	75.8	0.8
HPV35	75.0	0.75	75.0	0.89	75.0	0.96
HPV39	75.0	0.36	77.5	0.23	50.0	0.22
HPV45	83.3	0.62	83.3	0.76	100	0.24
HPV52	100 (3.7y)	0.55	100 (3.7y)	0.59	100 (3.7y)	0.63

HPV56	100 (2.9y)	0.51	100 (2.9y)	0.55	100 (2.9y)	0.59
HPV58	50.0 (4.2y)	0.26	50.0 (4.2y)	0.18	50.0 (4.2y)	0.14
HPV59	0 (2.3y)	0.072	0 (2.3y)	**0.045**	100 (2.3y)	0.73

Table 3. 10: Univariate Cox Proportional Hazard Ratios and Post-hoc Power Calculations for HPV16 vs HPV- and other hr-HPV strains

Log-rank Test Comparing Survival Rates of HPV 16 against HPV- and hr-HPV other strains	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
HPV16 Reference					
OS: HPV-	2.02 (1.29-3.15)	**0.002**	270 (171+99)	**88.07**	
DSS: HPV-	2.09 (1.27-3.43)	**0.004**	255 (163+92)	**86.28**	
DFS: HPV-	1.62 (0.95-2.77)	0.076	270 (171+99)	43.24	660
OS: HPV 18	1.91 (0.46-7.96)	0.4	174 (168+6)	14.55	1708
DSS: HPV 18	1.22 (0.17-9.00)	0.8	166 (161+5)	---	---
DFS: HPV 18	1.44 (0.19-10.7)	0.7	174 (168+6)	6.16	13627
OS: HPV 31	2.34 (0.32-17.2)	0.4	173 (170+3)	13.32	1940
DSS: HPV 31	2.73 (0.37-20.2)	0.3	165 (162+3)	16.99	1297
DFS: HPV 31	3.97 (0.53-29.9)	0.2	173 (170+3)	30.51	647
OS: HPV 33	2.62 (1.10-6.20)	**0.029**	183 (171+12)	62.58	277
DSS: HPV 33	2.79 (1.08-7.20)	**0.034**	174 (163+11)	60.00	279
DFS: HPV 33	1.20 (0.29-5.04)	0.8	183 (171+12)	6.16	14335
OS: HPV 35	0.72 (0.10-5.26)	0.7	176 (170+6)	6.15	13857
DSS: HPV 35	0.87 (0.12-6.39)	0.9	168 (162+6)	---	---
DFS: HPV 35	0.95 (0.13-6.99)	>0.9	176 (170+6)	---	---
OS: HPV 39	1.93 (0.47-8.01)	0.4	173 (169+4)	15.78	1509
DSS: HPV 39	2.34 (0.56-9.78)	0.2	165 (161+4)	21.94	926
DFS: HPV 39	2.38 (0.57-9.97)	0.2	173 (169+4)	23.18	906
OS: HPV 45	0.61 (0.08-4.41)	0.6	173 (167+6)	8.50	4527
DSS: HPV 45	0.73 (0.10-5.37)	0.8	165 (159 6)	6.15	12969
DFS: HPV 45	0 (0-inf)	>0.9	173 (167+6)	21.96	969
OS: HPV 52	0 (0-inf)	>0.9	173 (171+2)	8.50	4527
DSS: HPV 52	0 (0-inf)	>0.9	165 (163+2)	8.50	4317
DFS: HPV 52	0 (0-inf)	>0.9	173 (171+2)	7.32	6790
OS: HPV 56	0 (0-inf)	>0.9	173 (171+2)	9.69	3396
DSS: HPV 56	0 (0-inf)	>0.9	165 (163+2)	9.69	3243
DFS: HPV 56	0 (0-inf)	>0.9	173 (171+2)	8.49	4536
OS: HPV 58	2.99 (0.41-21.8)	0.3	171 (169+2)	20.72	1033
DSS: HPV 58	3.58 (0.49-26.4)	0.2	163 (161+2)	26.87	711
DFS: HPV 58	3.99 (0.54-29.5)	0.2	171 (169+2)	31.69	611
OS: HPV 59	5.15 (0.70-37.9)	0.11	171 (170+1)	43.22	420
DSS: HPV 59	6.02 (0.81-44.6)	0.079	163 (162+1)	51.62	320
DFS: HPV 59	0 (0-inf)	>0.9	171 (170+1)	6.15	13403

Table 3.11 summarizes the 5-year OS, DSS, and DFS for HPV- against other HPV strains. HPV- patients compared to HPV33 have higher but not statistically significant OS (50.7% vs 39.1, p=0.6) and DSS (58.0% vs 43.8%, p=0.61) and lower but not statistically significant DFS (59.7% vs 75.8%, p=0.71). HPV- patients compared to HPV58 have higher but not statistically significant OS (50.7% vs 50.0, p=0.67), DSS (58.0% vs 50.0%, p=0.48) and DFS (59.2% vs 50.0%, p=0.39) **(Table 3.11).**

Compared to HPV-, HPV31 and HPV58 have higher but not statistically significant hazard of death from any cause, hazard of death of disease and hazard of disease recurrence **(Table 3.12**). Compared to HPV-, HPV33 and HPV59 have higher but not statistically significant hazard of death from any cause and hazard of death of disease and higher but not statistically significant hazard of disease recurrence **(Table 3.12**).

Table 3. 11: Summary of Proportion Surviving (OS, DSS and DFS) for HPV- vs HPV16 and other hr-HPV strains

	Overall Survival		**Disease-Specific Survival**		**Disease-Free Survival**	
Variables	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
HPV-	50.7		58.0		59.2	
HPV16	74.0	**0.0017**	77.9	**0.003**	70.9	0.073
HPV18	66.7 (3.5y)	0.95	80.0 (3.5y)	0.64	83.3 (3.5y)	0.92
HPV31	66.7 (2.6y)	0.83	66.7 (2.6y)	0.74	66.7 (2.6y)	0.31
HPV33	39.1	0.6	43.8	0.61	75.8	0.71
HPV35	75.0	0.24	75.0	0.29	75.0	0.58
HPV39	75.0	0.98	77.5	0.83	50.0	0.64
HPV45	83.3	0.28	83.3	0.37	100	0.15
HPV52	100 (3.7y)	0.42	100 (3.7y)	0.47	100 (3.7y)	0.51
HPV56	100 (2.9y)	0.39	100 (2.9y)	0.4	100 (2.9y)	0.53
HPV58	50.0 (4.2y)	0.67	50.0 (4.2y)	0.48	50.0 (4.2y)	0.39
HPV59	0 (2.3y)	0.26	0 (2.3y)	0.22	100 (2.3y)	0.7

Table 3. 12: Univariate Cox Proportional Hazard Ratios and Post-hoc Power Calculations for HPV- vs HPV16 and other hr-HPV strains.

Comparing Survival Rates of HPV- against hr-HPV strains	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
HPV- Reference					
OS: HPV 16	0.50 (0.32-0.78)	**0.002**	270 (99+171)	**88.07**	
DSS: HPV 16	0.48 (0.29-0.79)	**0.004**	255 (92+163)	**84.30**	
DFS: HPV 16	0.62 (0.36-1.05)	0.076	270 (99+171)	43.24	662
OS: HPV 18	1.05 (0.25-4.36)	>0.9	105 (99+6)	---	---
DSS: HPV 18	0.63 (0.09-4.60)	0.6	97 (92+5)	7.32	3808
DFS: HPV 18	0.90 (0.12-6.67)	>0.9	105 (99+6)	---	---
OS: HPV 31	1.25 (0.17-9.18)	0.8	102 (99+3)	---	---
DSS: HPV 31	1.40 (0.19-10.3)	0.7	95 (92+3)	6.15	7477
DFS: HPV 31	2.71 (0.36-20.6)	0.3	102 (99+3)	17.00	801
OS: HPV 33	1.26 (0.53-2.99)	0.6	111 (99+12)	8.50	2905
DSS: HPV 33	1.28 (0.50-3.31)	0.6	103 (92+11)	8.51	2691
DFS: HPV 33	0.76 (0.18-3.23)	0.7	111 (99+12)	6.15	8721
OS: HPV 35	0.32 (0.04-2.38)	0.3	105 (99+6)	21.96	589
DSS: HPV 35	0.36 (0.05-2.65)	0.3	98 (92+6)	18.23	700
DFS: HPV 35	0.57 (0.08-4.28)	0.6	105 (99+6)	8.51	2743
OS: HPV 39	0.98 (0.24-4.09)	>0.9	103 (99+4)	---	---
DSS: HPV 39	1.16 (0.28-4.89)	0.8	96 (92+4)	---	---
DFS: HPV 39	1.41 (0.33-6.02)	0.6	103 (99+4)	7.32	4036
OS: HPV 45	0.35 (0.05-2.54)	0.3	105 (99+6)	19.48	687
DSS: HPV 45	0.41 (0.06-3.04)	0.4	98 (92+6)	14.54	962
DFS: HPV 45	0 (0-inf)	>0.9	105 (99+6)	29.29	413
OS: HPV 52	0 (0-inf)	>0.9	101 (99+2)	12.11	1321
DSS: HPV 52	0 (0-inf)	>0.9	94 (92+2)	11.34	1477
DFS: HPV 52	0 (0-inf)	>0.9	101 (99+2)	9.69	1984
OS: HPV 56	0 (0-inf)	>0.9	101 (99+2)	13.33	1132
DSS: HPV 56	0 (0-inf)	>0.9	94 (92+2)	13.32	1054
DFS: HPV 56	0 (0-inf)	>0.9	101 (99+2)	9.69	1984
OS: HPV 58	1.54 (0.21-11.2)	0.7	101 (99+2)	7.32	3964
DSS: HPV 58	2.02 (0.27-14.9)	0.5	94 (92+2)	10.89	1477
DFS: HPV 58	2.36 (0.32-17.6)	0.4	101 (99+2)	13.33	1132
OS: HPV 59	2.95 (0.40-21.8)	0.3	100 (99+1)	20.73	610
DSS: HPV 59	3.24 (0.44-24.0)	0.3	93 (92+1)	23.18	487
DFS: HPV 59	0 (0-inf)	>0.9	100 (99+1)	6.16	7911

3.5.5 Survival Outcomes According to 1HPV+, HPV- and 2-3HPV+

There were statistically significant differences between 1HPV+ and HPV- for 5-year OS (72.3% vs 50.7%, p=0.0021), DSS (76.0% vs 58.0%, p=0.005) and DFS (72.3% vs 59.2%, p=0.038) **(Figure 3.7)** with post hoc power calculations of 86.91%, 81.22% and 53.57%, respectively **(Table 3.8, Section 3.5.2)**. Compared to 1HPV+, HPV- have significantly higher hazard of death from any cause (OS: HR, 1.97; 95%CI, 1.27-3.04; p= 0.002), hazard of death of disease (DSS: HR, 2.00; 95%CI, 1.24-3.24; p=0.005) and hazard of disease recurrence (DFS: HR, 1.77; 95%CI, 1.04-3.03; p= 0.038) **(Table 3.13).**

There were no statistically significant differences between 1HPV+ and 2-3HPV+ for 5-year OS (72.3% vs 68.4%, p=0.3), DSS (76.0% vs 74.1%, p=0.3) and DFS (72.3% vs 67.7%, p=0.3) (Figure 3.7) with post hoc power calculations of 18.24%, 14.55 % and 20.71 %, respectively **(Table 3.13)**. Compared to 1HPV+, 2-3HPV+ have higher but not statistically significant hazard of death from any cause (OS: HR, 1.69; 95%CI, 0.67-4.28; p= 0.3), hazard of death of disease (DSS: HR, 1.68; 95%CI, 0.6-4.74; p=0.3) and hazard of disease recurrence (DFS: HR, 1.82; 95%CI, 0.64-5.16; p= 0.3) **(Table 3.13).**

There were no statistically significant differences between HPV- and 2-3HPV+ for 5-year OS (50.7% vs 68.4 %, p=0.8), DSS (58.0% vs 74.1 %, p=0.7) and DFS (59.2% vs 67.7 %, p>0.9) **(Figure 3.7)** with post hoc power calculations of 6.15% for OS and DSS and could not be calculated DFS. **(Table 3.14)**. Compared to HPV-, 2-3HPV+ have lower but not statistically significant hazard of death from any cause (OS: HR, 0.86; 95%CI, 0.34-2.18; p= 0.8) and hazard of death of disease (DSS: HR, 0.84; 95%CI, 0.3-2.38; p=0.7)

and about the same hazard of disease recurrence (DFS: HR, 1.03; 95%CI, 0.35-2.98; p>0.9) **(Table 3.14).**

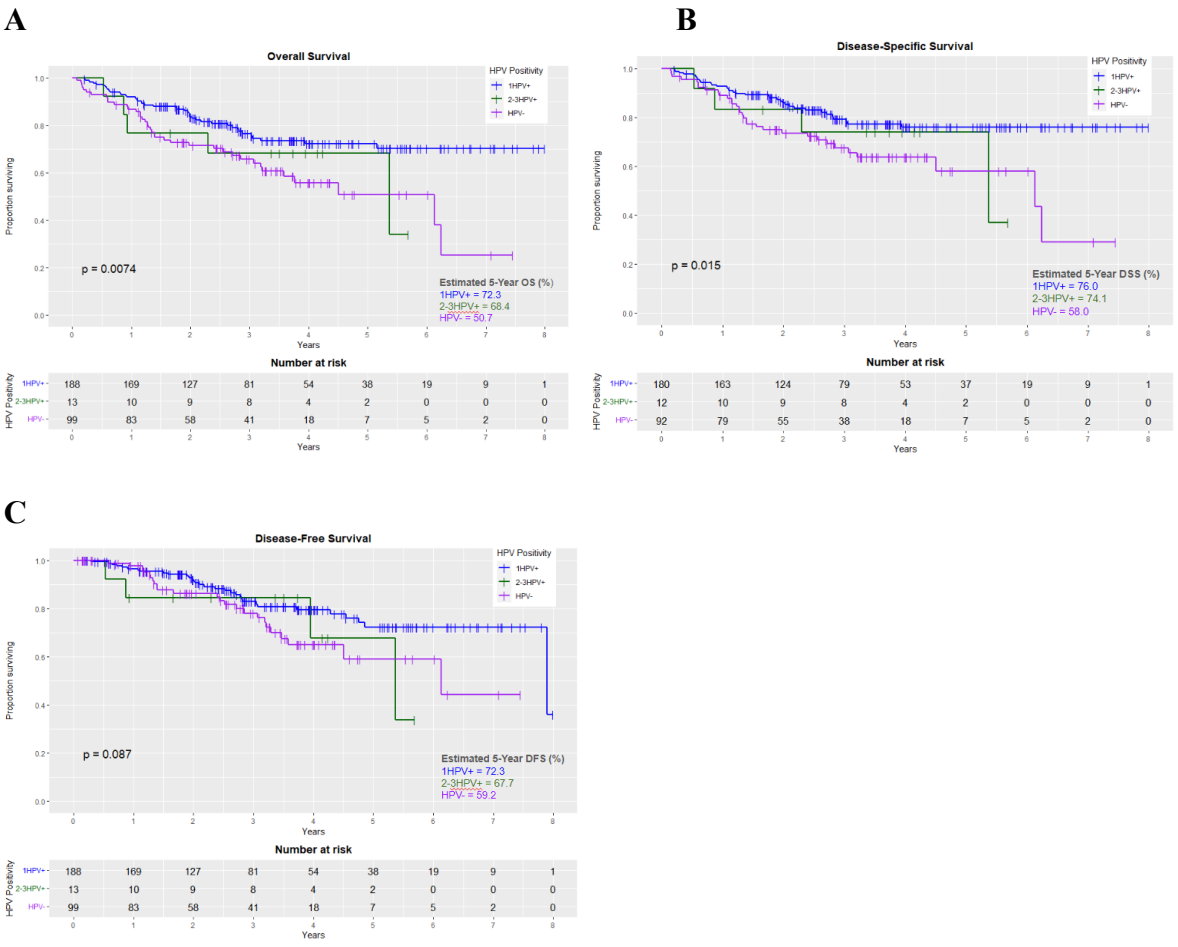


Figure 3. 7: Survival Analysis: 1HPV+ vs HPV-, 2-3HPV+ A) OS B) DSS C) DFS

Table 3. 13: Univariate Cox Proportional Hazard Ratios and Post-hoc Power Calculations for HPV+ vs HPV-, 2-3HPV+ Strains

Comparing Survival Rates of 1HPV+ against HPV-, 2-3HPV+ strains	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
1HPV+ Reference					
OS: HPV-	1.97 (1.27-3.04)	**0.002**	287 (188+99)	**86.91**	
DSS: HPV-	2.00 (1.24-3.24)	**0.005**	272 (180+92)	**81.22**	
DFS: HPV-	1.77 (1.04-3.03)	**0.038**	287 (188+99)	53.57	537
OS: 2-3HPV+	1.69 (0.67-4.28)	0.3	201 (188+13)	18.24	1435
DSS: 2-3HPV+	1.68 (0.6-4.74)	0.3	192 (180+12)	14.55	1884
DFS: 2-3HPV+	1.82 (0.64-5.16)	0.3	201 (188+13)	20.71	1214

Table 3. 14: Univariate Cox Proportional Hazard Ratios and Post-hoc Power Calculations for HPV- vs HPV+, 2-3HPV+ Strains

Comparing Survival Rates of HPV- against 1HPV+, 2-3HPV+ strains	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
HPV- Reference					
OS: 1HPV +	0.51 (0.33-0.78)	**0.002**	287 (99+188)	**86.91**	
DSS: 1HPV+	0.50 (0.31-0.81)	**0.005**	272 (92+180)	**81.22**	
DFS: 1HPV+	0.57 (0.33-0.97)	**0.038**	287 (99+188)	53.57	537
OS: 2-3HPV+	0.86 (0.34-2.18)	0.8	112 (99+13)	6.15	8791
DSS: 2-3HPV+	0.84 (0.3-2.38)	0.7	104 (92+12)	6.15	8163
DFS: 2-3HPV+	1.03 (0.35-2.98)	>0.9	112 (99+13)	---	---

3.6 Univariate Cox's Proportional Hazard Model of Survival According to Age, Sex, Smoking, Tumor Stage, Nodal Stage and Treatment Type

The proportion surviving of OPSCC cohort for univariate analysis according to age, sex, smoking, tumor stage, nodal stage and treatment type are summarized in **Table 3.15** in this section. The corresponding survival plots are in **Appendix A, Figures 1-6.**

3.6.1 Survival Outcomes According to Age

The 5-year OS, DSS and DFS of age group 30-49 were compared against age group 50-69 and age group 70+. There were no statistically significant differences between age group 30-49 and 50-69 for 5-year OS (85.2% vs 69.1%, p=0.96), DSS (85.2% vs 74.2%, p=0.18) and DFS (64.4% vs 71.4%, p=0.64) **(Table 3.15, Appx A Figure 1)** with post hoc power calculations of 38.75% for OS, 26.84% for DSS and could not be calculated for DFS. Compared to age group 30-49, age group 50-69 have higher but not statistically significant hazard of death from any cause (OS: HR, 2.58; 95%CI, 0.81-8.25; p= 0.11) and hazard of death of disease (DSS: HR, 2.19; 95%CI, 0.68-7.03; p=0.2) and lower but not statistically significant hazard of disease recurrence (DFS: HR, 0.95; 95%CI, 0.41-2.24; p>0.9) **(Table 3.16).**

There were statistically significant differences between age group 30-49 and age group 70+ for 5-year OS (85.2% vs 40.9%, p=0.0011) and DSS (85.2% vs 43.8%, p=0.0046) but not significant DFS (64.4% vs 51.3%, p=0.64) **(Table 3.15, Appx A Figure**

1) with post hoc power calculations 90.52%, 80.74% and 7.32% respectively **(Table 3.16).** Compared to age group 30-49, age group 70+ have significantly higher hazard of death from any cause (OS: HR, 5.86; 95%CI, 1.77-19.4; p= 0.004) and hazard of death of disease (DSS: HR, 4.92; 95%CI, 1.45-16.7; p=0.010) but not significant hazard of disease recurrence (DFS: HR, 1.28; 95%CI, 0.46-3.52; p= 0.6) **(Table 3.16).**

3.6.2 Survival Outcomes According to Sex

There were no statistically significant differences between males and females for 5-year OS (65.8% vs 69.1%, p=0.29), DSS (70.8% vs 73.7%, p=0.39) and DFS (66.5% vs 75.3%, p=0.19) **(Table 3.15, Appx A Figure 2)** with post hoc power calculations of 18.26%, 13.31% and 25.65%, respectively. Compared to males, females have lower but not statistically significant hazard of death from any cause (OS: HR, 0.71; 95%CI, 0.38-1.34; p= 0.3), hazard of death of disease (DSS: HR, 0.74; 95%CI, 0.37-1.49; p=0.4) and hazard of disease recurrence (DFS: HR, 0.59; 95%CI, 0.27-1.31; p= 0.2) **(Table 3.16).**

3.6.3 Survival Outcomes According to Smoking

The 5-year OS, DSS and DFS for nonsmokers were compared against former smokers with >10 pack years (former >10py) and current smokers with >10 pack years (current >10py). There were no statistically significant differences between nonsmokers and former >10py for 5-year OS (80.1% vs 71.9%, p=0.11), DSS (82.6% vs 74.3% p=0.088) and DFS (70.6% vs 79.5%, p=0.95) **(Table 3.15, Appx A Figure 3),** with post hoc power calculations of 36.64% for OS, 39.75% for DSS and could not be calculated for DFS. Compared to nonsmokers, former >10py have higher but not statistically significant

hazard of death from any cause (OS: HR, 1.75; 95%CI, 0.87-3.53; p= 0.12) and hazard of death of disease (DSS: HR, 1.93; 95%CI, (0.88-4.20); p=0.10) and lower but not statistically significant hazard of disease recurrence (DFS: HR, 0.97; 95%CI, (0.45-2.06); p>0.9) **(Table 3. 16).**

There were statistically significant differences between nonsmokers and current >10py for 5-year OS (80.1% vs 50.6%, p=0.0048) and DSS (82.6% vs 59.5% p=0.01) but not significant DFS (70.6% vs 48.8%, p=0.057) (**Table 3.15, Appx A Figure 3)** with post hoc power calculations of 80.77%, 72.84% and 47.56%, respectively **(Table 3.16).** Compared to nonsmokers, current >10py have significantly higher hazard of death from any cause (OS: HR, 2.69; 95%CI, 1.33-5.44; p= 0.006), hazard of death of disease (DSS: HR, 2.78; 95%CI, 1.26-6.14; p=0.012) and hazard of disease recurrence (DFS: HR, 2.10; 95%CI, 1.02-4.34; p= 0.045) **(Table 3. 16).**

3.6.4 Survival Outcomes According to Primary Tumor Stage

The 5-year OS, DSS and DFS for T1 stage patients were compared to T2, T3 and T4. There were no statistically significant differences between T1 and T2 for 5-year OS (75.0% vs 75.5% vs, p=0.75), DSS (79.3% vs 78.2%, p=0.62) and DFS (80.1% vs 75.3%, p=0.96) **(Table 3.15, Appx A Figure 4)** with post hoc power calculations of 6.18% for OS, 8.49% for DSS and could not be calculated for DFS. Compared to T1, T2 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.90; 95%CI, 0.47-1.70; p= 0.7), hazard of death of disease (DSS: HR, 0.83; 95%CI, 0.41-1.67; p=0.6) and hazard of disease recurrence (DFS: HR, 0.98; 95%CI, 0.44-2.21; p>0.9) **(Table 3.16).**

There was a statistically significant difference between T1 and T3 for 5-year DFS (80.1% vs 48.9%, p=0.014) but not significant OS (75.0% vs 58.3% vs, p=0.087) and DSS (79.3% vs 64.4%, p=0.13) **(Table 3.15, Appx A Figure 4),** with post hoc power calculation of 68.98% for DFS, 39.89% for OS and 31.87% for DSS **(Table 3.16).** Compared to T1, T3 has significantly higher hazard of disease recurrence (DFS: HR, 2.53; 95%CI, 1.16-5.52; p=0.020) but not significant hazard of death from any cause (OS: HR, 1.70; 95%CI, 0.89-3.25; p= 0.11) and hazard of death of disease (DSS: HR, 1.66; 95%CI, 0.82-3.34; p=0.2) **(Table 3.16).**

There were statistically significant differences between T1 and T4 for 5-year OS (75.0% vs 31.0%, p<0.0001), DSS (79.3% vs 38.2%, p<0.0001) and DFS (80.1% vs 54.3%, p=0.002) **(Table 3. 15, Appx A Figure 4)** with post hoc power calculations of 99.56%, 98.64% and 86.83%, respectively **(Table 3.16).** Compared to T1, T4 stage have significantly higher hazard of death from any cause (OS: HR, 4.12; 95%CI, 2.14-7.91; p<0.001), hazard of death of disease (DSS: HR, 4.14; 95%CI, 2.03-8.46; p<0.001) and hazard of disease recurrence (DFS: HR, 3.93; 95%CI, 1.66-9.29; p=0.002) **(Table 3.16).**

3.6.5 Survival Outcomes According to Nodal Stage

The 5-year OS, DSS and DFS for N0 stage patients were compared against N1, N2 and N3 for 5year OS, DSS and DFS. There were statistically significant differences between N0 and N1 for 5-year OS (40.8% vs 84.9%, p=0.0046) and DSS (47.0% vs 87.2%, p=0.021) but not significant DFS (63.6% vs 70.3%, p=0.98) **(Table 3.15, Appx A Figure 5)** with post hoc power calculations of 80.82% for OS, 63.55% for DSS and cannot be quantified for DFS **(Table 3.16).** Compared to N0, N1 stage have significantly lower

hazard of death from any cause (OS: HR, 0.35; 95%CI, 0.17-0.76; p= 0.007) and hazard of death of disease (DSS: HR, 0.39; 95%CI, 0.17-0.91; p=0.030) but not significant hazard of disease recurrence (DFS: HR, 1.05; 95%CI, 0.35-3.18; p>0.9) **(Table 3.16).** There were no statistically significant differences between N0 and N2 for 5-year OS (40.8% vs 62.6%, p=0.68), DSS (47.0% vs 65.8%, p=0.96) and DFS (63.6% vs 65.8%, p=0.23) **(Table 3.15, Appx A Figure 5)** with post hoc power calculations of 7.28% for OS, 23.21% for DFS and cannot be quantified for DSS **(Table 3.16).** Compared to N0, N2 stage have lower but not statistically significant hazard of death from any cause (OS: HR, 0.87; 95%CI, 0.46-1.65; p= 0.7) and hazard of death of disease (DSS: HR, 0.98; 95%CI, 0.47-2.01; p>0.9) and higher but not statistically significant hazard of disease recurrence (DFS: HR, 1.76; 95%CI, 0.62-4.99; p=0.3) **(Table 3.16).** There were no statistically significant differences between N0 and N3 for 5-year OS (40.8% vs 40.1%, p=0.26), DSS (47.0% vs 53.2%, p=0.36) and DFS (63.6% vs 49.0%, p=0.18) **(Table 3.15, Appx A Figure 5)** with post hoc power calculations of 19.40%, 14.58% and 26.80%, respectively **(Table 3.16).** Compared to N0, N3 stage have higher but not statistically significant hazard of death from any cause (OS: HR, 1.57; 95%CI, 0.72-3.44; p= 0.3), hazard of death of disease (DSS: HR, 1.60; 95%CI, 0.63-4.02; p=0.3) and hazard of disease recurrence (DFS: HR, 2.59; 95%CI, 0.77-8.69; p=0.12) **(Table 3.16).**

Since N0 has a lower 5-year OS, DSS and DFS compared to N1, N1 was also compared to N2 and N3. There were statistically significant differences between N1 and N2 for 5-year OS (84.9% vs 62.6%, p=0.0017) and DSS (87.2% vs 65.8%, p=0.003) but not significant DFS (70.3% vs 65.8%, p=0.083) **(Table 3.15, Appx A Figure 5)** with post hoc power calculations of 87.78%, 60.92% and 41.07%, respectively **(Table 3.16).**

Compared to N1, N2 have significantly higher hazard of death from any cause (OS: HR, 2.46; 95%CI, 1.38-4.41; p= 0.002) and hazard of death of disease (DSS: HR, 2.50; 95%CI, 1.34-4.68; p=0.004) but not significant hazard of disease recurrence (DFS: HR, 1.67; 95%CI, 0.91-3.09; p=0.10) **(Table 3.16).** There were statistically significant differences between N1 and N3 for 5-year OS (84.9% vs 40.1%, p<0.0001) and DSS (87.2% vs 53.2%, p=0.00034) but not significant DFS (70.3% vs 49.0%, p=0.074) **(Table 3.15, Appx A Figure 5)** with post hoc power 99.12%, 94.68% and 43.26%, respectively **(Table 3.16).** Compared to N1, N3 have significantly higher hazard of death from any cause (OS: HR, 4.43; 95%CI, 2.10-9.32; p<0.001), hazard of death of disease (DSS: HR, 4.09; 95%CI, 1.75-9.58; p=0.001) and hazard of disease recurrence (DFS: HR, 2.46; 95%CI, 1.03-5.88; p=0.044) **(Table 3.16).**

3.6.6 Survival Outcomes According to Treatment Type

There was a statistically significant difference between surgery with radiation therapy (S+RT) and surgery with chemo radiation therapy (S+CRT) for 5-year DFS (80.8% vs 61.4 %, p=0.042**)** but not significant OS (66.0% vs 66.3%, p=0.75) and DSS (76.6% vs 71.3%, p=0.59) **(Table 3.15, Appx A Figure 6),** with post hoc power calculations of 53.56% (DFS), 6.17% (OS) and 8.43% (DSS) **(Table 3.16).** Compared to S+RT, S+CRT has significantly higher hazard of disease recurrence (DFS: HR, 2.03; 95%CI, 1.02-4.04; p=0.045) but not significant hazard of death from any cause (OS: HR, 1.08; 95%CI, 0.61-1.93; p=0.8) and hazard of death of disease (DSS: HR, 1.18; 95%CI, 0.62-2.26; p=0.6) **(Table 3.16).**

There were no statistically significant differences between S+RT and CRT for 5-year OS (66.0% vs 78.3%, p=0.38), DSS (76.6% vs 78.3%, p=0.71) and DFS (80.8% vs 68.4 %, p=0.3**) (Table 3.15, Appx A Figure 6)** with post hoc power calculations of 14.51%, 6.18% and 18.16% respectively **(Table 3.16).** Compared to S+RT, CRT have lower but not statistically significant hazard of death from any cause (OS: HR, 0.62; 95%CI, 0.27-1.43; p=0.3) and hazard of death of disease (DSS: HR, 0.77; 95%CI, 0.33-1.83; p=0.6) and higher but not statistically significant hazard of disease recurrence (DFS: HR, 1.52; 95%CI, 0.64-3.64; p=0.3) **(Table 3.16).**

There were no statistically significant differences between S+RT and RT for 5-year OS (66.0% vs 57.1%, p=0.12), DSS (76.6% vs 66.7%, p=0.33) and DFS (80.8% vs 100%, p=0.47**) (Table 3.15, Appx A Figure 6)** with post hoc power calculations of 34.11%, 15.80% and 10.94% respectively **(Table 3.16).** Compared to S+RT, RT have higher but not statistically significant hazard of death from any cause (OS: HR, 2.50; 95%CI, 0.76-8.27; p=0.13) and hazard of death of disease (DSS: HR, 2.08; 95%CI, 0.48-8.93; p=0.3). Hazard of disease recurrence was calculated at 0 (95%CI: 0-inf) since RT have 100% proportion surviving for DFS analysis **(Table 3.16)**.

There were no statistically significant differences between S+RT and S for 5-year OS (66.0% vs 60.2%, p=0.34), DSS (76.6% vs 63.6%, p=0.27) and DFS (80.8% vs 59.7 %, p=0.16**) (Table 3.15, Appx A Figure 6)** with post hoc power calculations of 15.67%, 19.36% and 29.37% respectively **(Table 3.16).** Compared to S+RT, S have higher but not statistically significant hazard of death from any cause (OS: HR, 1.29; 95%CI, 0.75-2.21; p=0.4), hazard of death of disease (DSS: HR, 1.39; 95%CI, 0.76-2.56; p=0.3) and hazard of disease recurrence (DFS: HR, 1.66; 95%CI, 0.82-3.37; p=0.2) **(Table 3.16).**

Table 3. 15: Summary of Proportion Surviving (OS, DSS and DFS) for Age, Sex, Smoking, Tumor Stage, Nodal Stage and Treatment Type

	Overall Survival		Disease-Specific Survival		Disease-Free Survival	
Variables	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
Age (vs 30-49)						
30-49	85.2		85.2		64.4	
50-69	69.1	0.96	74.2	0.18	71.4	0.9
70+	40.9	**0.0011**	43.8	**0.0046**	51.3	0.64
Sex (vs Male)						
Male	65.8		70.8		66.5	
Female	69.1	0.29	73.7	0.39	75.3	0.19
Smoking (vs Nonsmoker)						
Nonsmoker	80.1		82.6		70.6	
Former (>10py)	71.9	0.11	74.3	0.088	79.5	0.95
Current (>10py)	50.6	**0.0048**	59.5	**0.01**	48.8	0.057
Primary Tumor Stage (vs T1)						
T1	75.0		79.3		80.1	
T2	75.5	0.75	78.2	0.62	75.3	0.96
T3	58.3	0.087	64.4	0.13	48.9	**0.014**
T4	31.0	**<0.001**	38.2	**<0.001**	54.3	**0.002**
Nodal Stage (vs N0)						
N0	40.8		47.0		63.6	
N1	84.9	**0.0046**	87.2	**0.021**	70.3	0.98
N2	62.6	0.68	65.8	0.96	65.8	0.23
N3	40.1	0.26	53.2	0.36	49.0	0.18
Nodal Stage (vs N1)						
N1	84.9		87.2		70.3	
N0	40.8	**0.0046**	47.0	**0.021**	63.6	0.98
N2	62.6	**0.0017**	65.8	**0.003**	65.8	0.083
N3	40.1	**<0.001**	53.2	**0.00034**	49.0	0.074
Treatment Type (vs S+RT)						
S+RT*	66.0		76.6		80.8	
S+CRT*	66.3	0.75	71.3	0.59	61.4	**0.042**
CRT	78.3	0.38	78.3	0.71	68.1	0.3
RT	57.1	0.12	66.7	0.33	100	0.47
S	60.2	0.34	63.6	0.27	59.7	0.16

***S+RT**=Surgery + Radiation Therapy**, S+CRT** = Surgery + Chemo Radiation Therapy

Table 3. 16: Univariate Cox Proportional Hazard Ratios and Post-hoc Power Calculations for Age, Sex, Smoking, Tumor Stage, Nodal Stage and Treatment Type

Log-rank Test Comparing Survival Rates based on Age, Sex, Smoking, Tumor Stage, Nodal Stage and Treatment Type	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
Age (30-49 Reference)					
OS: Age 50-69	2.58 (0.81-8.25)	0.11	247 (28+219)	38.75	693
DSS: Age 50-69	2.19 (0.68-7.03)	0.2	237 (28+209)	26.84	1035
DFS: Age 50-69	0.95 (0.41-2.24)	>0.9	247 (28+219)	---	---
OS: Age 70+	5.86 (1.77-19.4)	**0.004**	81 (28+53)	**90.52**	
DSS: Age 70+	4.92 (1.45-16.7)	**0.010**	75 (28+47)	**80.74**	
DFS: Age 70+	1.28 (0.46-3.52)	0.6	81 (28+53)	7.32	3178
Sex (Male Reference)					
OS: Female	0.71 (0.38-1.34)	0.3	300 (256+44)	18.26	2138
DSS: Female	0.74 (0.37-1.49)	0.4	284 (242+42)	13.31	3191
DFS: Female	0.59 (0.27-1.31)	0.2	300 (256+44)	25.65	1385
Smoking (Nonsmoker Reference)					
OS: former >10py	1.75 (0.87-3.53)	0.12	198 (67+131)	36.64	594
DSS: former >10py	1.93 (0.88-4.20)	0.10	191 (65+126)	39.75	519
DFS: former >10py	0.97 (0.45-2.06)	>0.9	198 (67+131)	---	---
OS: current >10py	2.69 (1.33-5.44)	**0.006**	154 (67+87)	**80.77**	
DSS: current >10py	2.78 (1.26-6.14)	**0.012**	144 (65+79)	72.84	172
DFS: current >10py	2.10 (1.02-4.34)	**0.045**	154 (67+87)	47.56	336
Primary Tumor Stage (T1 Reference)					
OS: T2	0.90 (0.47-1.70)	0.7	194 (69+125)	6.18	14838
DSS: T2	0.83 (0.41-1.67)	0.6	187 (67+120)	8.49	4906
DFS: T2	0.98 (0.44-2.21)	>0.9	194 (69+125)	---	---
OS: T3	1.70 (0.89-3.25)	0.11	138 (69+69)	39.89	374
DSS: T3	1.66 (0.82-3.34)	0.2	132 (67+65)	31.87	472
DFS: T3	2.53 (1.16-5.52)	**0.020**	138 (69+69)	68.98	180
OS: T4	4.12 (2.14-7.91)	**<0.001**	106 (69+37)	**99.56**	
DSS: T4	4.14 (2.03-8.46)	**<0.001**	99 (67+32)	**98.64**	
DFS: T4	3.93 (1.66-9.29)	**0.002**	106 (69+37)	**86.83**	
Nodal Stage (N0 Reference)					
OS: N1	0.35 (0.17-0.76)	**0.007**	138 (32+106)	**80.82**	
DSS: N1	0.39 (0.17-0.91)	**0.030**	133 (29+104)	63.55	197
DFS: N1	1.05 (0.35-3.18)	>0.9	138 (32+106)	---	---
OS: N2	0.87 (0.46-1.65)	0.7	170 (32+138)	7.28	6790
DSS: N2	0.98 (0.47-2.01)	>0.9	160 (29+131)	---	---
DFS: N2	1.76 (0.62-4.99)	0.3	170 (32+138)	23.21	889
OS: N3	1.57 (0.72-3.44)	0.3	56 (32+24)	19.40	369
DSS: N3	1.60 (0.63-4.02)	0.3	49 (29+20)	14.58	480
DFS: N3	2.59 (0.77-8.69)	0.12	56 (32+24)	26.80	245
Nodal Stage (N1 Reference)					

OS: N0	2.82 (1.32-6.03)	**0.007**	138 (106+32)	**80.82**	
DSS: N0	2.56 (1.10-6.00)	**0.030**	133 (104+29)	63.55	197
DFS: N0	0.95 (0.31-2.86)	>0.9	138 (106+32)	---	---
OS: N2	2.46 (1.38-4.41)	**0.002**	244 (106+138)	**87.78**	
DSS: N2	2.50 (1.34-4.68)	**0.004**	235 (104+131)	60.92	208
DFS: N2	1.67 (0.91-3.09)	0.10	244 (106+138)	41.07	638
OS: N3	4.43 (2.10-9.32)	**<0.001**	130 (106+24)	**99.12**	
DSS: N3	4.09 (1.75-9.58)	**0.001**	124 (104+20)	**94.68**	
DFS: N3	2.46 (1.03-5.88)	**0.044**	130 (106+24)	43.26	319
Treatment Type (S+RT Reference)					
OS: S+CRT	1.08 (0.61-1.93)	0.8	162 (93+69)	6.17	12559
DSS: S+CRT	1.18 (0.62-2.26)	0.6	152 (86+66)	8.43	4055
DFS: S+CRT	2.03 (1.02-4.04)	**0.045**	162 (93+69)	53.56	303
OS: CRT	0.62 (0.27-1.43)	0.3	138 (93+45)	14.51	1359
DSS: CRT	0.77 (0.33-1.83)	0.6	131 (86+45)	6.18	10012
DFS: CRT	1.52 (0.64-3.64)	0.3	138 (93+45)	18.16	991
OS: RT	2.50 (0.76-8.27)	0.13	100 (93+7)	34.11	327
DSS: RT	2.08 (0.48-8.93)	0.3	92 (86+6)	15.80	801
DFS: RT	0 (0-inf)	>0.9	100 (93+7)	10.94	1558
OS: S	1.29 (0.75-2.21)	0.4	172 (93+79)	15.67	1515
DSS: S	1.39 (0.76-2.56)	0.3	161 (86+75)	19.36	1062
DFS: S	1.66 (0.82-3.37)	0.2	172 (93+79)	29.37	673

3.7 Multivariate Cox's Proportional Hazard Model of Survival

The proportion surviving (OS, DSS and DFS) of OPSCC cohort for multivariate analysis for p16, HPV and p16/HPV stratified by age, sex, smoking, tumor stage, nodal stage and treatment type are summarized in a table for each section in this chapter. The corresponding survival plots are in **Appendices B-G.**

3.7.1 Survival Outcomes for p16, HPV and p16/HPV Stratified by Age

The survival for p16, HPV and p16/HPV were stratified by age groups 30-49, 50-69 and 70+. There were statistically significant differences between p16+: 30-49 and p16-: 30-49 for 5-year OS (100% vs 0, p<0.001), DSS (100% vs 0, p<0.001) and DFS (75.0 % vs 0, p<0.001) **(Table 3.17, Appx B Figure 1)** with post hoc power calculations of 99.98% (OS and DSS) and 99.15% (DFS) **(Table 3.18).** Compared to p16-: 30-49, p16+: 30-49 have significantly lower hazard of disease recurrence (DFS: HR, 0.05; 95%CI, 0.01-0.33; p=0.002) Hazard of death from any cause and hazard of death of disease were calculated at 0 (95%CI: 0-inf) since p16+: 30-49 have 100% proportion surviving for OS and DSS analysis **(Table 3.18).**

There were statistically significant differences between p16+: 50-69 and p16-:50-69 for 5-year OS (74.7% vs 33.8%, p=0.00013) and DSS (78.6% vs 42.9%, p=0.00031) but not significant DFS (73.7% vs 55.6%, p=0.061) **(Table 3.17, Appx B Figure 2)** with post hoc power calculations of 96.96%, 95.00% and 46.44%, respectively **(Table 3.18).** Compared to p16-:50-69, p16+: 50-69 have significantly lower hazard of death from any cause (OS: HR, 0.33; 95%CI, 0.19-0.60; p<0.001) and hazard of death of disease (DSS: HR, 0.32; 95%CI, 0.17-0.61; p<0.001) but not significant hazard of disease recurrence (DFS: HR, 0.49; 95%CI, 0.23-1.05; p=0.067) **(Table 3.18).**

There was a statistically significant difference between p16+: 70+ and p16-:70+ for 5-year OS (52.1% vs 13.6%, p=0.009) but not significant DSS (53.9% vs 18.8%, p=0.069) and DFS (56.1% vs 41.7%, p=0.76) **(Table 3.17, Appx B Figure 3)** with post hoc power calculations of 74.14%, 44.31% and 6.15%, respectively **(Table 3.18).** Compared to p16-:

70+, p16+: 70+ has significantly lower hazard of death from any cause (OS: HR, 0.35; 95%CI, 0.15-0.80; p=0.012) but not significant hazard of death of disease (DSS: HR, 0.42; 95%CI, 0.16-1.10; p=0.078) and hazard of disease recurrence (DFS: HR, 0.78; 95%CI, 0.16-3.72; p=0.8) **(Table 3.18).**

There were statistically significant differences between HPV+: 30-49 and HPV-: 30-49 for 5-year OS (100% vs 50.0%, p=0.0046) and DSS (100% vs 50.0%, p=0.0046) but not significant DFS (72.7 % vs 50.0, p=0.078) **(Table 3.17, Appx B Figure 4)** with post hoc power calculations of 80.74% (OS and DSS) and 42.10% (DFS) **(Table 3.18).** Compared to HPV-: 30-45, HPV+: 30-49 have lower but not statistically significant hazard of disease recurrence (DFS: HR, 0.23; 95%CI, 0.04-1.37; p=0.11). Hazard of death from any cause and hazard of death of disease weree calculated at 0 (95%CI: 0-inf) since HPV+: 30-49 have 100% proportion surviving for OS and DSS analysis **(Table 3.18)**.

There were statistically significant differences between HPV+: 50-69 and HPV-: 50-69 for 5-year OS (74.1% vs 57.1%, p=0.025) and DSS (78.2% vs 65.4%, p=0.022) but not significant DFS (73.6% vs 67.9%, p=0.18) **(Table 3.17, Appx B Figure 5)** with post hoc power calculations of 60.87%, 63.37% and 26.89%, respectively **(Table 3.18).** Compared to HPV-: 50-69, HPV+: 50-69 have significantly lower hazard of death from any cause (OS: HR, 0.56; 95%CI, 0.33-0.94, p=0.027) and hazard of death of disease (DSS: HR, 0.52; 95%CI, 0.30-0.92; p=0.024) but not significant hazard of disease recurrence (DFS: HR, 0.66; 95%CI, 0.36-1.22; p=0.2) **(Table 3.18).**

There were no statistically significant differences between HPV+: 70+ and HPV-: 70+ for 5-year OS (46.2% vs 0, p=0.31), DSS (48.4% vs 0, p=0.71) and DFS (65.1% vs 0, p=0.4) **(Table 3.17, Appx B Figure 6)** with post hoc power calculations of 17.01%,

6.15% and 13.32%, respectively**(Table 3.18)**. Compared to HPV-: 70+, HPV+: 70+ have lower but not statistically significant hazard of death from any cause (OS: HR, 0.66; 95%CI, 0.29-1.49, p=0.3), hazard of death of disease (DSS: HR, 0.83; 95%CI, 0.33-2.13; p=0.7) and hazard of disease recurrence (DFS: HR, 0.57; 95%CI, 0.15-2.17; p=0.4) **(Table 3.18).**

There were no statistically significant differences between p16+/HPV+: 30-49 and p16+/HPV-: 30-49 for 5-year OS (100% vs 100%, p=1), DSS (100% vs 100%, p=1) and DFS (72.3% vs 100%, p=0.5) **(Table 3.17, Appx B Figure 7**) with post hoc power calculations of 9.69% for DFS and cannot be quantified for OS and DSS **(Table 3.18)**. There are no statistically significant differences between p16+/HPV+: 30-49 and p16+/HPV-: 30-49 in hazard of death from any cause, hazard of death of disease and hazard of disease recurrence **(Table 3.18).** There was no p16-/HPV+ in the age group 30-49.

There were statistically significant differences between p16+/HPV+: 30-49 and p16-/HPV-: 30-49 for OS (100% vs 0, p<0.001), DSS (100% vs 0, p<0.001) and DFS (72.3% vs 0, p=0.00012) **(Table 3.17, Appx B Figure 7**) with post hoc power calculations of 99.85% (OS and DSS) and 97.04% (DFS) **(Table 3.18).** Compared to p16+/HPV+: 30-49, p16-/HPV-: 30-49 has significantly higher hazard of disease recurrence (DFS: HR, 14.8; 95%CI, 2.43-89.9; p=0.003) but not significant hazard of death from any cause (OS: HR, 7.8 e+10; 95%CI, 0-inf; p>0.9) and hazard of death of disease for (DSS: HR, 7.8 e+10; 95%CI, 0-inf; p>0.9) **(Table 3.18).**

There were no statistically significant differences between p16+/HPV+:50-69 and p16+/HPV-:50-69 for 5-year OS (76.4% vs 68.7%, p=0.36), DSS (80.2% vs 73.2, p=0.2)

and DFS (74.3% vs 73.1, p=0.45) **(Table 3.17, Appx B Figure 8)** with post hoc power calculations of 14.55%, 24.39% and 12.12%, respectively **(Table 3.18).** Compared to p16+/HPV+:50-69, p16+/HPV-:50-69 have higher but not statistically significant hazard of death from any cause (OS: HR, 1.35; 95%CI, 0.71-2.58; p=0.4), hazard of death of disease (DSS: HR, 1.55; 95%CI, 0.78-3.08; p=0.2) and hazard of disease recurrence (DFS: HR, 1.31; 95%CI, 0.63-2.74; p=0.5) **(Table 3.18)**.

There were statistically significant differences between p16+/HPV+: 50-69 and p16-/HPV+: 50-69 for 5-year OS (76.4% vs 0, p=0.04) and DSS (80.2% vs 0, p=0.018) but not significant DFS (74.3% vs 0, p=0.31) **(Table 3.17, Appx B Figure 8)** with post hoc power calculations of 53.57%, 65.78% and 17.00%, respectively **(Table 3.18)**. Compared to p16+/HPV+: 50-69, p16-/HPV+: 50-69 have significantly higher hazard of death from any cause (OS: HR, 4.52; 95%CI, 1.07-19.0; p=0.040) and hazard of death of disease (DSS: HR, 5.18; 95%CI, 1.22-22.0; p=0.026) but not significant hazard of disease recurrence (DFS: HR, 2.90; 95%CI, 0.39-21.6; p=0.3) **(Table 3.18)**.

There were statistically significant differences between p16+/HPV+: 50-69 and p16-/HPV-: 50-69 for 5-year OS (76.4% vs 38.1%, p=0.00056) and DSS (80.2% vs 49.7%, p=0.0013) but not significant DFS (74.3% vs 57.5% p=0.082) **(Table 3.17, Appx B Figure 8)** with post hoc power calculations of 93.18%, 89.44% and 41.02%, respectively **(Table 3.18).** Compared to p16+/HPV+: 50-69, p16-/HPV-: 50-69 have significantly higher hazard of death from any cause (OS: HR, 3.11; 95%CI, 1.62-5.95; p<0.001) and hazard of death of disease (DSS: HR, 3.33; 95%CI, 1.59-6.94; p=0.001) but not significant hazard of disease recurrence (DFS: HR, 2.14; 95%CI, 0.92-4.96; p=0.077) **(Table 3.18).**

There were no statistically significant differences between p16+/HPV+: 70+ and p16+/HPV-: 70+ for 5-year OS (43.7% vs 83.3%, p=0.19), DSS (45.3% vs 83.3%, p=0.22) and DFS (60.3% vs 60.0, p=0.87) **(Table 3.17, Appx B Figure 9)** with post hoc power calculations of 25.65% for OS, 23.18% for DSS and cannot be quantified for DFS **(Table 3.18)**. Compared to p16+/HPV+: 70+, p16+/HPV-: 70+ have lower but not statistically significant hazard of death from any cause (OS: HR, 0.39; 95%CI, 0.09-1.71; p=0.2) and hazard of death of disease (DSS: HR, 0.41; 95%CI, 0.09-1.86; p=0.2), and hazard of disease recurrence were the same (DFS: HR, 1.00; 95%CI, 0.19-5.28; p>0.9) **(Table 3.18)**.

There were no statistically significant differences between p16+/HPV+: 70+ and p16-/HPV+: 70+ for 5-year OS (43.7% vs 66.7%, p=0.52), DSS (45.3% vs 66.7%, p=0.64) and DFS (60.3% vs 100%, p=0.35) **(Table 3.17, Appx B Figure 9)** with post hoc power calculations of 9.69%, 7.32% and 15.77%, respectively **(Table 3.18)**. Compared to p16+/HPV+: 70+, p16-/HPV+: 70+ have lower but not statistically significant hazard of death from any cause (OS: HR, 0.52; 95%CI, 0.07-3.95; p=0.5) and hazard of death of disease (DSS: HR, 0.61; 95%CI, 0.08-4.76; p=0.6). Hazard of disease recurrence was calculated at 0 (95%CI: 0-inf) since p16-/HPV+: 70+ have 100% proportion surviving for DFS analysis **(Table 3.18)**.

There were statistically significant differences between p16+/HPV+: 70+ and p16-/HPV-: 70+ for 5-year OS (43.7% vs 0, p=0.00031) and DSS (45.3% vs 0, p=0.012) but not significant DFS (60.3% vs 0, p=0.16) **(Table 3.17, Appx B Figure 9)** with post hoc power calculations of 95.00%, 70.88% and 28.08%, respectively **(Table 3.18).** Compared to p16+/HPV+: 70+, p16-/HPV-: 70+ have significantly higher hazard of death from any cause (OS: HR, 4.44; 95%CI, 1.82-10.9; p=0.001) and hazard of death of disease (DSS:

HR, 3.65; 95%CI, 1.25-10.7; p=0.018) but not significant hazard of disease recurrence (DFS: HR, 3.06; 95%CI, 0.59-16.0; p=0.2) **(Table 3.18).**

To summarize the age results analyses p16+ compared to p16-, p16+: 30-49 had significantly higher OS, DSS and DFS, p16+: 50-69 had significantly higher OS and DSS but not significant DFS and p16+: 70+ have significantly higher OS but not significant DSS and DFS. For HPV analyses HPV+ compared to HPV-, HPV+: 30-49 and HPV+: 50-69 had significantly higher OS and DSS but not significant DFS and HPV+: 70+ had higher but not statistically significant OS, DSS and DFS. When p16 and HPV status were combined, compared to p16+/HPV+, p16-/HPV- had significantly lower OS and DSS for age 50-69 and age 70+ but not significant DFS and significantly lower DFS for age 30-49. For discordant p16+/HPV-, compared p16+/HPV, p16+/HPV-: 30-49 had similar OS and DSS, p16+/HPV-: 50-69 had lower but not statistically significant OS, DSS and DFS and p16+/HPV-: 70+ had higher but not statistically significant OS and DSS and the same DFS. For discordant p16-/HPV+, compared to p16+/HPV+, p16-/HPV+: 50-69 had significantly lower OS and DSS but not significant DFS and p16-/HPV+: 70+ had higher but not statistically significant OS, DSS and DFS.

Table 3. 17: Summary of Proportion Surviving (OS, DSS and DFS) for p16, HPV and p16/HPV stratified by Age

Covariate	Overall Survival		Disease-Specific Survival		Disease-Free Survival	
	Proportion Surviving (%)	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
p16 and Age (vs p16-)						
p16-: 30-49	0 (3.2y)	**<0.001**	0 (3.2y)	**<0.001**	0 (3.2y)	**<0.001**
p16+: 30-49	100		100		75	
p16-: 50-69	33.8	**0.00013**	42.9	**0.00031**	55.6	0.061

p16+: 50-69	74.7		78.6		73.7	
p16-: 70+	13.6	**0.009**	18.8	0.069	41.7	0.76
p16+: 70+	52.1		53.9		56.1	
HPV and Age (vs HPV-)						
HPV-: 30-49	50.0 (4.4y)	**0.0046**	50.0 (4.4)	**0.0046**	50.0 (4.4)	0.078
HPV+: 30-49	100		100		72.7	
HPV-: 50-69	57.1	**0.025**	65.4	**0.022**	67.9	0.18
HPV+: 50-69	74.1		78.2		73.6	
HPV-: 70+	0 (4.5y)	0.31	0 (4.5y)	0.71	0 (4.5y)	0.4
HPV+: 70+	46.2		48.4		65.1	
p16/HPV and Age (vs p16+/HPV+: 30-49)						
p16+/HPV+: 30-49	100		100		72.3	
p16+/HPV-: 30-49	100 (4.4y)	1	100 (4.4y)	1	100 (4.4y)	0.5
p16-/HPV+: 30-49	N/A	N/A	N/A	N/A	N/A	N/A
p16-/HPV-: 30-49	0 (3.2y)	**<0.001**	0 (3.2y)	**<0.001**	0 (3.2y)	**0.00012**
p16/HPV and Age (vs p16+/HPV+: 50-69)						
p16+/HPV+: 50-69	76.4		80.2		74.3	
p16+/HPV-: 50-69	68.7	0.36	73.2	0.2	73.1	0.45
p16-/HPV+: 50-69	0 (3y)	**0.04**	0 (3y)	**0.018**	0 (3y)	0.31
p16-/HPV-: 50-69	38.1	**0.00056**	49.7	**0.0013**	57.5	0.082
p16/HPV and Age (vs p16+/HPV+: 70+)						
p16+/HPV+: 70+	43.7		45.3		60.3	
p16+/HPV-: 70+	83.3 (4y)	0.19	83.3 (4y)	0.22	60.0 (4y)	0.87
p16-/HPV+: 70+	66.7	0.52	66.7	0.64	100	0.35
p16-/HPV-: 70+	0 (4.5y)	**0.00031**	0 (4.5y)	**0.012**	0 (4.5y)	0.16

Table 3. 18: Multivariate Cox Proportional Hazard Regression analysis and Post-hoc Power Calculations for p16, HPV and p16/HPV stratified by Age.

Log-rank Test Comparing Survival Rates based on Age and p16, HPV and p16/HPV	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
p16- Reference					
OS: p16+: 30-49	0 (0-inf)	>0.9	28 (4+24)	**99.98**	
DSS: p16+: 30-49	0 (0-inf)	>0.9	28 (4+24)	**99.98**	
DFS: p16+: 30-49	0.05 (0.01-0.33)	**0.002**	28 (4+24)	**99.15**	
OS: p16+: 50-69	0.33 (0.19-0.60)	**<0.001**	219 (26+193)	**96.96**	
DSS: p16+: 50-69	0.32 (0.17-0.61)	**<0.001**	209 (23+186)	**95.00**	
DFS: p16+: 50-69	0.49 (0.23-1.05)	0.067	219 (26+193)	46.44	492
OS: p16+: 70+	0.35 (0.15-0.80)	**0.012**	53 (11+42)	74.14	62
DSS: p16+: 70+	0.42 (0.16-1.10)	0.078	47 (8+39)	44.31	112
DFS: p16+: 70+	0.78 (0.16-3.72)	0.8	53 (11+42)	6.15	4168
HPV- Reference					
OS: HPV+: 30-49	0 (0-inf)	>0.9	28 (9+19)	**80.74**	
DSS: HPV+: 30-49	0 (0-inf)	>0.9	28 (9+19)	**80.74**	

DFS: HPV+: 30-49	0.23 (0.04-1.37)	0.11	28 (9+19)	42.10	71
OS: HPV+: 50-69	0.56 (0.33-0.94)	**0.027**	219 (70+149)	60.87	344
DSS: HPV+: 50-69	0.52 (0.30-0.92)	**0.024**	209 (66+143)	63.37	310
DFS: HPV+: 50-69	0.66 (0.36-1.22)	0.2	219 (70+149)	26.89	955
OS: HPV+: 70+	0.66 (0.29-1.49)	0.3	53 (20+33)	17.01	416
DSS: HPV+: 70+	0.83 (0.33-2.13)	0.7	47 (17+30)	6.15	3694
DFS: HPV+: 70+	0.57 (0.15-2.17)	0.4	53 (20+33)	13.32	595
p16+/HPV+: 30-49 Reference					
OS: p16+/HPV-: 30-49	1.01 (0-inf)	>0.9	24 (19+5)	---	---
DSS: p16+/HPV-: 30-49	1.01 (0-inf)	>0.9	24 (19+5)	---	---
DFS: p16+/HPV-: 30-49	0 (0-inf)	>0.9	24 (19+5)	9.69	471
OS: p16-/HPV+: 30-49	N/A				
DSS: p16-/HPV+: 30-49	N/A				
DFS: p16-/HPV+: 30-49	N/A				
OS: p16-/HPV-: 30-49	7.8 e+10 (0-inf)	>0.9	23 (19+4)	**99.85**	
DSS: p16-/HPV-: 30-49	7.8 e+10 (0-inf)	>0.9	23 (19+4)	**99.85**	
DFS: p16-/HPV-: 30-49	14.8 (2.43-89.9)	**0.003**	23 (19+4)	**97.04**	
p16+/HPV+: 50-69 Reference					
OS: p16+/HPV-: 50-69	1.35 (0.71-2.58)	0.4	193 (147+46)	14.55	1893
DSS: p16+/HPV-: 50-69	1.55 (0.78-3.08)	0.2	186 (141+45)	24.39	914
DFS: p16+/HPV-: 50-69	1.31(0.63-2.74)	0.5	193 (147+46)	12.12	2521
OS: p16-/HPV+: 50-69	4.52 (1.07-19.0)	**0.040**	149 (147+2)	53.57	279
DSS: p16-/HPV+: 50-69	5.18 (1.22-22.0)	**0.026**	143 (141+2)	65.78	201
DFS: p16-/HPV+: 50-69	2.90 (0.39-21.6)	0.3	149 (147+2)	17.00	1171
OS: p16-/HPV-: 50-69	3.11(1.62-5.95)	**<0.001**	171 (147+24)	**93.18**	
DSS: p16-/HPV-: 50-69	3.33 (1.59-6.94)	**0.001**	162 (141+21)	**89.44**	
DFS: p16-/HPV-: 50-69	2.14 (0.92-4.96)	0.077	171 (147+24)	41.02	448
p16+/HPV+: 70+ Reference					
OS: p16+/HPV-: 70+	0.39 (0.09-1.71)	0.2	42 (30+12)	25.65	194
DSS: p16+/HPV-: 70+	0.41 (0.09-1.86)	0.2	39 (27+12)	23.18	205
DFS: p16+/HPV-: 70+	1.00 (0.19-5.28)	>0.9	42 (30+12)	---	---
OS: p16-/HPV+: 70+	0.52 (0.07-3.95)	0.5	33 (30+3)	9.69	648
DSS: p16-/HPV+: 70+	0.61 (0.08-4.76)	0.6	30 (27+3)	7.32	1179
DFS: p16-/HPV+: 70+	0 (0-inf)	>0.9	33 (30+3)	15.77	288
OS: p16-/HPV-: 70+	4.44 (1.82-10.9)	**0.001**	38 (30+8)	**95.00**	
DSS: p16-/HPV-: 70+	3.65 (1.25-10.7)	**0.018**	32 (27+5)	70.88	40
DFS: p16-/HPV-: 70+	3.06 (0.59-16.0)	0.2	38 (30+8)	28.08	157

3.7.2 Survival Outcomes for p16, HPV and p16/HPV Stratified by Sex

The survival for p16, HPV and p16/HPV were stratified by sex. There were statistically significant differences between p16+: males and p16-: males for 5-year OS (73.3% vs 17.0%, p<0.0001), DSS (77.0% vs 23.0%, p<0.0001) and DFS (70.0% vs 38.4%, p<0.0001) **(Table 3.19, Appx C Figure 1)**, with post hoc power calculations of 99.99% (OS and DSS) and 97.87% (DFS) **(Table 3.20).** Compared to p16-: males, p16+: males have significantly lower hazard of death from any cause (OS: HR, 0.20; 95%CI, 0.12-0.34; p<0.0001), hazard of death of disease (DSS: HR, 0.21; 95%CI, 0.12-0.37; p<0.0001) and hazard of disease recurrence (DFS: HR, 0.28; 95%CI, 0.14-0.54; p<0.0001) **(Table 3.20).**

There were no statistically significant differences between p16+: females and p16-: females for 5-year OS (77.1% vs 45.3%, p=0.11), DSS (81.0% vs 55.0%, p=0.16) and DFS (79.1% vs 60.1%, p=0.73) **(Table 3.19, Appx C Figure 1)**, with post hoc power calculations of 36.43%, 29.29% and 6.15%, respectively **(Table 3.20).** Compared to p16-: females, p16+: females have lower but not statistically significant hazard of death from any cause (OS: HR, 0.39; 95%CI, 0.12-1.28; p=0.12), hazard of death of disease (DSS: HR, 0.40; 95%CI, 0.11-1.49; p=0.2) and hazard of disease recurrence (DFS: HR, 0.75; 95%CI, 0.14-3.88; p=0.7) **(Table 3.20).**

There were statistically significant differences between HPV+: males and HPV-males for 5-year OS (71.6% vs 54.1%, p=0.0093) and DSS (75.2% vs 61.7%, p=0.015) but not significant DFS (68.8% vs 64.4%, p=0.11) **(Table 3.19, Appx C Figure 3)** with post hoc power calculations of 74.15%, 68.03% and 36.56%, respectively **(Table 3.20).**

Compared to HPV-: males, HPV+: males have significantly lower hazard of death from any cause (OS: HR, 0.55; 95%CI, 0.35-0.87; p=0.010) and hazard of death of disease (DSS: HR, 0.54; 95%CI, 0.33-0.90; p=0.017) but not significant hazard of disease recurrence (DFS: HR, 0.63; 95%CI, 0.36-1.11; p=0.11) **(Table 3.20).**

There were no statistically significant differences between HPV+: females and HPV- females for 5-year OS (77.4% vs 44.2%, p=0.14), DSS (80.9% vs 50.3%, p=0.18) and DFS (83.5% vs 47.6%, p=0.26) **(Table 3.19, Appx C Figure 3)** with post hoc power calculations of 30.51%, 26.86% and 20.73%, respectively **(Table 3.20).** Compared to HPV-: females, HPV+: females have lower but not statistically significant hazard of death from any cause (OS: HR, 0.42; 95%CI, 0.13-1.39; p=0.2), hazard of death of disease (DSS: HR, 0.41; 95%CI, 0.11-1.56; p=0.2) and hazard of disease recurrence (DFS: HR, 0.43; 95%CI, 0.10-1.95; p=0.3) **(Table 3.20).**

There were no statistically significant differences between p16+/HPV+: males and p16+/HPV-: males for 5-year OS (73.7% vs 71.5%, p=0.78), DSS (77.6% vs 75.4%, p=0.48) and DFS (69.4% vs 37.7%, p=0.85) **(Table 3.19, Appx C Figure 5)** with post hoc power calculations of 6.15% for OS, 10.95% for DSS and could not be quantified for DFS **(Table 3.20).** Compared to p16+/HPV+: males, p16+/HPV-: males have higher but not statistically significant hazard of death from any cause (OS: HR, 1.09; 95%CI, 0.60-1.98; p=0.8), hazard of death of disease (DSS: HR, 1.25; 95%CI, 0.67-2.36; p=0.5) and hazard of disease recurrence (DFS: HR, 1.06; 95%CI, 0.53-2.13; p=0.9) **(Table 3.20).**

There were statistically significant differences between p16+/HPV+: males and p16-/HPV+: males for 5-year OS (73.7% vs 0, p=0.0065) and DSS (77.6% vs 0, p=0.0025) but not significant DFS (69.4% vs 50.0%, p=0.4) **(Table 3.19, Appx C Figure 5)** with post

hoc power calculations of 77.63%, 85.96% and 13.33%, respectively **(Table 3.20).** Compared to p16+/HPV+: males, p16-/HPV+: males have significantly higher hazard of death from any cause (OS: HR, 4.98; 95%CI, 1.53-16.2; p=0.008) and hazard of death of disease (DSS: HR, 5.79; 95%CI, 1.76-19.1; p=0.004) but not significant hazard of disease recurrence (DFS: HR, 2.48; 95%CI, 0.34-18.4; p=0.4) **(Table 3.20).**

There were statistically significant differences between p16+/HPV+: males and p16-/HPV-: males for 5-year OS (73.7% vs 19.4%, p<0.0001), DSS (77.6% vs 27.0%, p<0.0001) and DFS (69.4% vs 37.7%, p=0.00014) **(Table 3.19, Appx C Figure 5)** with post hoc power calculations of 99.99%, 99.94% and 96.65% **(Table 3.20).** Compared to p16+/HPV+: males, p16-/HPV-: males have significantly higher hazard of death from any cause (OS: HR, 5.01; 95%CI, 2.87-8.74; p<0.0001), hazard of death of disease (DSS: HR, 5.02; 95%CI, 2.65-9.50; p<0.0001) and hazard of disease recurrence (DFS: HR, 3.85; 95%CI, 1.86-7.97; p<0.0001) **(Table 3.20).**

There were no statistically significant differences between p16+/HPV+: females and p16+/HPV-: females for 5-year OS (75.9% vs 100%, p=0.43), DSS (79.5% vs 100%, p=0.49), and DFS (82.3% vs 66.7%, p=0.39) **(Table 3.19, Appx C Figure 6)** with post hoc power calculations of 12.09%, 10.89% and 13.29%, respectively **(Table 3.20)**. Compared to p16+/HPV+: females, p16+/HPV-: females have higher but not statistically significant hazard of disease recurrence (DFS: HR, 2.77; 95%CI, 0.28-27.0; p=0.4) **(Table 3.20).** Hazard of death from any cause and hazard of death of disease were calculated at 0 (95%CI: 0-inf) since p16+/HPV-: females have 100% proportion surviving for OS and DSS.

There were no statistically significant differences between p16+/HPV+: females and p16-/HPV+: females for 5-year OS (75.9% vs 100%, p=0.49), DSS (79.5% vs 100%, p=0.54) and DFS (82.3% vs 100%, p=0.58) **(Table 3.19, Appx C Figure 6)** with post hoc power calculations of 10.89%, 9.66% and 8.5%, respectively **(Table 3.20)**. Hazard risks could not be calculated between p16+/HPV+: females and p16-/HPV+: females **(Table 3.20).**

There were no statistically significant differences between p16+/HPV+: females and p16-/HPV-: females for 5-year OS (75.9% vs 35.4%, p=0.061), DSS (79.5% vs 45.0%, p=0.091) and DFS (82.3% vs 50.0%, p=0.47) **(Table 3.19, Appx C Figure 6)** with post hoc power calculations of 46.46%, 39.88% and 10.87%, respectively **(Table 3.20)**. Compared to p16+/HPV+: females, p16-/HPV-: females have higher but not statistically significant hazard of death from any cause (OS: HR, 2.97; 95%CI, 0.90-9.81; p=0.074), hazard of death of disease (DSS: HR, 2.97; 95%CI, 0.79-11.2; p=0.11) hazard of disease recurrence (DFS: HR, 1.96; 95%CI, 0.36-10.8; p=0.4) **(Table 3.20).**

To summarize the sex results analyses p16+ compared to p16-, p16+: males had significantly higher OS, DSS and DFS and p16+: females had higher but not statistically significant OS, DSS and DFS. For HPV analyses HPV+ compared to HPV-, HPV+: males have significantly higher OS and DSS but not significant DFS and HPV+: females had higher but not statistically significant OS, DSS and DFS. When p16 and HPV status were combined, p16-/HPV-: males had significantly lower OS, DSS and DFS but did not reach significance for females. For discordant p16+/HPV-, compared p16+/HPV+, p16+/HPV-: males had similar OS and DSS and lower but not statistically significant DFS and p16+/HPV-: females had higher but not statistically significant OS and DSS and lower but

not statistically significant DFS. For discordant p16-/HPV+, compared to p16+/HPV+, p16-/HPV+: males had significantly lower OS and DSS but not significant DFS and p16-/HPV+: females had higher but not statistically significant OS, DSS and DFS.

Table 3. 19: Summary of Proportion Surviving (OS, DSS and DFS) for p16, HPV and p16/HPV stratified by Sex

Covariate	Overall Survival		Disease-Specific Survival		Disease-Free Survival	
	Proportion Surviving (%)	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
p16 and Sex (vs p16-)						
p16-: Male	17.0 (4.8y)	**<0.0001**	23.0 (4.8y)	**<0.0001**	38.4 (4.8y)	**<0.0001**
p16+: Male	73.3		77.0		70.0	
p16-: Female	45.3	0.11	55.0	0.16	60.1	0.73
p16+: Female	77.1		81.0		79.1	
HPV and Sex (vs HPV-)						
HPV-: Male	54.1	**0.0093**	61.7	**0.015**	64.4	0.11
HPV+: Male	71.6		75.2		68.8	
HPV-: Female	44.2	0.14	50.3	0.18	47.6	0.26
HPV+: Female	77.4		80.9		83.5	
p16/HPV and Sex (vs p16+/HPV+: Male)						
p16+/HPV+: Male	73.7		77.6		69.4	
p16+/HPV-: Male	71.5	0.78	75.4	0.48	37.7	0.85
p16-/HPV+: Male	0	**0.0065**	0	**0.0025**	50.0 (3y)	0.4
p16-/HPV-: Male	19.4 (4.8y)	**<0.0001**	27.0 (4.8y)	**<0.0001**	37.7 (4.8y)	**0.00014**
p16/HPV and Sex (vs p16+/HPV+: Female)						
p16+/HPV+: Female	75.9		79.5		82.3	
p16+/HPV-: Female	100 (3.7y)	0.43	100 (3.7y)	0.49	66.7 (3.7y)	0.39
p16-/HPV+: Female	100	0.49	100	0.54	100	0.58
p16-/HPV+: Female	35.4	0.061	45.0	0.091	50.0	0.47

Table 3. 20: Multivariate Cox Proportional Hazard Regression analysis and Post-hoc Power Calculations for p16, HPV and p16/HPV stratified by Sex

Log-rank Test Comparing Survival Rates based on Sex and p16, HPV and p16/HPV	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
p16- Reference					
OS: p16+: Male	0.20 (0.12-0.34)	**<0.001**	256 (28+228)	**99.99**	
DSS: p16+: Male	0.21 (0.12-0.37)	**<0.001**	242 (23+219)	**99.99**	
DFS: p16+: Male	0.28 (0.14-0.54)	**<0.001**	256 (28+228)	**97.87**	
OS: p16+: Female	0.39 (0.12-1.28)	0.12	44 (13+31)	36.43	133
DSS: p16+: Female	0.40 (0.11-1.49)	0.2	42 (20+30)	29.29	165
DFS: p16+: Female	0.75 (0.14-3.88)	0.7	44 (13+31)	6.15	3450
HPV- Reference					
OS: HPV+: Male	0.55 (0.35-0.87)	**0.010**	256 (85+171)	74.15	296
DSS: HPV+: Male	0.54 (0.33-0.90)	**0.017**	242 (79+163)	68.03	323
DFS: HPV+: Male	0.63 (0.36-1.11)	0.11	256 (85+171)	36.56	770
OS: HPV+: Female	0.42 (0.13-1.39)	0.2	44 (14+30)	30.51	165
DSS: HPV+: Female	0.41 (0.11-1.56)	0.2	42 (13+29)	26.86	184
DFS: HPV+: Female	0.43 (0.10-1.95)	0.3	44 (14+30)	20.73	266
p16+/HPV+: Male Reference					
OS: p16+/HPV-: Male	1.09 (0.60-1.98)	0.8	228 (168+60)	6.15	17969
DSS: p16+/HPV-: Male	1.25 (0.67-2.36)	0.5	219 (160+59)	10.95	3407
DFS: p16+/HPV-: Male	1.06 (0.53-2.13)	0.9	228 (168+60)	---	---
OS: p16-/HPV+: Male	4.98 (1.53-16.2)	**0.008**	171 (168+3)	77.63	181
DSS: p16-/HPV+: Male	5.79 (1.76-19.1)	**0.004**	163 (160+3)	**85.96**	
DFS: p16-/HPV+: Male	2.48 (0.34-18.4)	0.4	171 (168+3)	13.33	1917
OS: p16-/HPV-: Male	5.01 (2.87-8.74)	**<0.001**	193 (168+25)	**99.99**	
DSS: p16-/HPV-: Male	5.02 (2.65-9.50)	**<0.001**	180 (160+20)	**99.94**	
DFS: p16-/HPV-: Male	3.85 (1.86-7.97)	**<0.001**	193 (168+25)	**96.65**	
p16+/HPV+: Female Reference					
OS: p16+/HPV-: Female	0 (0-inf)	>0.9	31 (28+3)	12.09	407
DSS: p16+/HPV-: Female	0 (0-inf)	>0.9	30 (27+3)	10.89	472
DFS: p16+/HPV-: Female	2.77 (0.28-27.0)	0.4	31 (28+3)	13.29	349
OS: p16-/HPV+: Female	---	---	30 (28+2)	10.89	472
DSS: p16-/HPV+: Female	---	---	29 (27+2)	9.66	574
DFS: p16-/HPV+: Female	---	---	30 (28+2)	8.50	785
OS: p16-/HPV-: Female	2.97 (0.90-9.81)	0.074	39 (28+11)	46.46	88
DSS: p16-/HPV-: Female	2.97 (0.79-11.2)	0.11	37 (27+10)	39.88	101
DFS: p16-/HPV-: Female	1.96 (0.36-10.8)	0.4	39 (28+11)	10.87	615

3.7.3 Survival Outcomes for p16, HPV and p16/HPV Stratified by Smoking

The survival for p16, HPV and p16/HPV were stratified by smoking. There were statistically significant differences between p16+: nonsmokers and p16-: nonsmokers for 5-year OS (85.5% vs 25.0%, p=0.001) and DSS (86.9% vs 33.3%, p=0.013) but not significant DFS (73.2% vs 37.5%, p=0.054) **(Table 3.21, Appx D Figure 1**), with post hoc power calculations of 90.70%, 70.24% and 48.55%, respectively **(Table 3.22).** Compared to p16-: nonsmokers, p16+: nonsmokers have significantly lower hazard of death from any cause (OS: HR, 0.14; 95%CI, 0.04-0.55; p=0.005) and hazard of death of disease (DSS: HR, 0.17; 95%CI, 0.03-0.83; p=0.029) but not significant hazard of disease recurrence (DFS: HR, 0.24; 95%CI, 0.05-1.16; p=0.076) **(Table 3.22).**

There were statistically significant differences between p16+: former smokers with >10 packyears (former >10py) and p16-: former >10py for 5-year OS (77.7% vs 32.0%, p<0.0001) and DSS (79.4% vs 36.9%, p=0.0003) but not significant DFS (80.0% vs 75.0%, p=0.077) **(Table 3.21, Appx D Figure 2**), with post hoc power calculations of 99.04%, 95.10% and 6.18%, respectively **(Table 3.22).** Compared to p16-: former >10py, p16+: former >10py have significantly lower hazard of death from any cause (OS: HR, 0.23; 95%CI, 0.11-0.48; p<0.001) and hazard of death of disease (DSS: HR, 0.25; 95%CI, 0.11-0.57; p<0.001) but not significant hazard of disease recurrence (DFS: HR, 0.81; 95%CI, 0.19-3.47; p=0.8) **(Table 3.22).**

There were statistically significant differences between p16+: current smokers with >10 packyears (current >10py) and p16-: current >10py for 5-year OS (61.6% vs 12.1%, p=0.0063), DSS (68.8% vs 19.6%, p=0.011) and DFS (56.7% vs 19.8%, p=0.009) **(Table**

3.21, Appx D Figure 3), with post hoc power calculations of 78.30%, 71.67% and 74.27%, respectively **(Table 3.22).** Compared to p16-: current >10py, p16+: current >10py have significantly lower hazard of death from any cause (OS: HR, 0.38; 95%CI, 0.19-0.78; p=0.008), hazard of death of disease (DSS: HR, 0.36; 95%CI, 0.16-0.82; p=0.015) and hazard of disease recurrence (DFS: HR, 0.35; 95%CI, 0.15-0.80; p= 0.013) **(Table 3.22).**

There were no statistically significant differences between HPV+: nonsmokers and HPV-: nonsmokers for 5-year OS (82.5% vs 77.1%, p=0.79), DSS (84.3% vs 80.8%, p=0.94) and DFS (84.3% vs 80.8%, p=0.88) **(Table 3.21, Appx D Figure 4**) with post hoc power calculations of 6.18% for OS and could not be quantified for DSS and DFS. Compared to HPV-: nonsmokers, HPV+: nonsmokers have lower but not statistically significant hazard of death from any cause (OS: HR, 0.84; 95%CI, 0.24-2.99; p=0.8), hazard of death of disease (DSS: HR, 0.94; 95%CI, 0.22-3.96; p>0.9) and hazard of disease recurrence (DFS: HR, 0.91; 95%CI, 0.25-3.22; p=0.9) **(Table 3.22).**

There were statistically significant differences between HPV+: former >10py and HPV-: former >10py for 5-year OS (77.2% vs 62.2%, p=0.05) but not significant for DSS (79.4% vs 65.0%, p=0.069) and DFS (83.6% vs 70.7%, p=0.29) **(Table 3.21, Appx D Figure 5**) with post hoc power calculations of 50.81%, 44.38% and 18.35%, respectively **(Table 3.22).** Compared to HPV-: former >10py, HPV+: former >10py have lower but not statistically significant hazard of death from any cause (OS: HR, 0.52; 95%CI, 0.27-1.01; p=0.053), hazard of death of disease (DSS: HR, 0.52; 95%CI, 0.26-1.06; p=0.074) and hazard of disease recurrence (DFS: HR, 0.63; 95%CI, 0.26-1.49; p=0.3) **(Table 3.22).**

There were statistically significant differences between HPV+: current >10py and HPV-: current >10py for 5-year OS (62.4% vs 19.3%, p=0.0093), DSS (67.9% vs 33.1%,

p=0.017) and DFS (56.0% vs 29.3%, p=0.022) **(Table 3.21, Appx D Figure 6**) with post hoc power calculations of 74.27%, 66.68% and 62.40%, respectively **(Table 3.22).** Compared to HPV-: current >10py, HPV+: current >10py have significantly lower hazard of death from any cause (OS: HR, 0.42; 95%CI, 0.21-0.82; p=0.012), hazard of death of disease (DSS: HR, 0.40; 95%CI, 0.18-0.87; p=0.021) and hazard of disease recurrence (DFS: HR, 0.41; 95%CI, 0.19-0.90; p=0.026) **(Table 3.22).**

There were no statistically significant differences between p16+/HPV+: nonsmokers and p16+/HPV-: nonsmokers for 5-year OS (82.5% vs 92.3%, p=0.33), DSS (84.3% vs 92.3%, p=0.43) and DFS (69.7% vs 82.1%, p=0.61) **(Table 3.21, Appx D Figure 7**), with post hoc power calculations of 15.86%, 12.05% and 8.50%, respectively. **(Table 3.22).** Compared to p16+/HPV+: nonsmokers, p16+/HPV-: nonsmokers have lower but not statistically significant hazard of death from any cause (OS: HR, 0.35; 95%CI, 0.04-2.95; p=0.3), hazard of death of disease (DSS: HR, 0.42; 95%CI, 0.05-3.57; p=0.4) and hazard of disease recurrence (DFS: HR, 0.65; 95%CI, 0.13-3.24; p=0.6) **(Table 3.22).** There were no patients with p16-/HPV+ nonsmokers.

There were statistically significant differences between p16+/HPV+: nonsmokers and p16-/HPV-: nonsmokers for 5-year OS (82.5% vs 25.0%, p=0.007) and DSS (84.3% vs 33.3%, p=0.04) but not significant DFS (69.7% vs 37.5%, p=0.11) **(Table 3.21, Appx D Figure 7**) with post hoc power calculations of 99.13%, 55.58% and 36.34%, respectively **(Table 3.22).** Compared to p16+/HPV+: nonsmoker, p16-/HPV-: nonsmoker have significantly higher hazard of death from any cause (OS: HR, 5.60; 95%CI, 1.39-22.6; p=0.015) but not significant hazard of death of disease (DSS: HR, 4.87; 95%CI, 0.93-25.4;

p=0.06) and hazard of disease recurrence (DFS: HR, 3.63; 95%CI, 0.72-18.2; p=0.12) **(Table 3.22).**

There were no statistically significant differences between p16+/HPV+: former >10py and p16+/HPV-: former >10py for 5-year OS (79.5% vs 72.9%, p=0.4), DSS (81.9% vs 72.9%, p=0.29) and DFS (83.1% vs 71.6%, p=0.42) **(Table 3.21, Appx D Figure 8**) with post hoc power calculations of 12.12%, 18.33% and 13.39% respectively. Compared to p16+/HPV+: former >10py, p16+/HPV-: former >10py have higher but not statistically significant hazard of death from any cause (OS: HR, 1.34; 95%CI, 0.60-3.03; p=0.5), hazard of death of disease (DSS: HR, 1.55; 95%CI, 0.67-3.59; p=0.3) and hazard of disease recurrence (DFS: HR, 1.46; 95%CI, 0.57-3.74; p=0.4) **(Table 3.22).**

There were statistically significant differences between p16+/HPV+: former >10py and p16-/HPV+: former > 10py for 5-year DSS (81.9% vs 33.3%, p=0.05) but not significant OS (79.5% vs 33.3%, p=0.098) and DFS (83.1% vs 100%, p=0.52) **(Table 3.21, Appx D Figure 8**) with post hoc power calculations of 37.49%, 49.73% and 9.69%, respectively **(Table 3.22).** Compared to p16+/HPV+: former >10py, p16-/HPV+: former > 10py have higher but not statistically significant hazard of death from any cause (OS: HR, 3.51; 95%CI, 0.81-15.3; p=0.094) and hazard of death of disease (DSS: HR, 4.31; 95%CI, 0.97-19.1; p=0.054). Hazard of disease recurrence was calculated at 0 (95%CI: 0-inf) since p16-/HPV+: former >10py have 100% proportion surviving for DFS analysis. **(Table 3.22).**

There were statistically significant differences between p16+/HPV+: former >10py and p16-/HPV-: former >10py for 5-year OS (79.5% vs 33.3%, p<0.0001) and DSS (81.9% vs 40.0%, p=0.00081) but not significant DFS (83.1% vs 66.7%, p=0.42) **(Table 3.21,**

Appx D Figure 8) with post hoc power calculations of 98.56%, 91.76% and 12.05%, respectively **(Table 3.22).** Compared to p16+/HPV+: former >10py, p16-/HPV-: former >10py have significantly higher hazard of death from any cause (OS: HR, 5.26; 95%CI, 2.26-12.2; p<0.001) and hazard of death of disease (DSS: HR, 4.71; 95%CI, 1.81-12.3; p=0.002) but not significant hazard of disease recurrence (DFS: HR, 1.93; 95%CI, 0.43-8.65; p=0.4) **(Table 3.22).**

There were no statistically significant differences between p16+/HPV+: current >10py and p16+/HPV-: current >10py for 5-year OS (63.9% vs 32.1%, p=0.23), DSS (69.7% vs 55.6%, p=0.24) and DFS (57.5% vs 42.9%, p=0.29) **(Table 3.21, Appx D Figure 9**) with post hoc power calculations of 23.30%, 22.16% and 18.20% respectively **(Table 3.22).** Compared to p16+/HPV+: current >10py, p16+/HPV-: current >10py have higher but not statistically significant hazard of death from any cause (OS: HR, 1.95; 95%CI, 0.66-5.79; p=0.2), hazard of death of disease (DSS: HR, 2.09; 95%CI, 0.60-7.33; p=0.3) and hazard of disease recurrence (DFS: HR, 1.92; 95%CI, 0.55-6.68; p=0.3) **(Table 3.22).**

There were no statistically significant differences between p16+/HPV+: current >10py and p16-/HPV+: current >10py for 5-year OS (63.9% vs 0, p=0.25), DSS (69.7% vs 0, p=0.19) and DFS (57.5% vs 0, p=0.099) **(Table 3.21, Appx D Figure 9**) with post hoc power calculations of 20.72%, 25.72% and 37.47% respectively **(Table 3.22).** Compared to p16+/HPV+: current >10py, p16-/HPV+: current >10py have higher but not statistically significant hazard of death from any cause (OS: HR, 3.81; 95%CI, 0.50-29.1; p=0.2), hazard of death of disease (DSS: HR, 4.11; 95%CI, 0.53-31.8; p=0.2) and hazard of disease recurrence (DFS: HR, 5.77; 95%CI, 0.73-45.5; p=0.10) **(Table 3.22).**

There were statistically significant differences between p16+/HPV+: current >10py and p16-/HPV-: current >10py for 5-year OS (63.9% vs 13.2%, p=0.0096), DSS (69.7% vs 22.2%, p=0.019) and DFS (57.5% vs 21.7%, p=0.018) **(Table 3.21, Appx D Figure 9**) with post hoc power calculations of 73.49%, 64.90% and 65.80%, respectively **(Table 3.22).** Compared to p16+/HPV+: current >10py, p16-/HPV- current >10py have significantly higher hazard of death from any cause (OS: HR, 2.81; 95%CI, 1.31-6.00; p=0.0.008), hazard of death of disease (DSS: HR, 2.96; 95%CI, 1.23-7.13; p=0.016) and hazard of disease recurrence (DFS: HR, 2.99; 95%CI, 1.23-7.26; p=0.016) **(Table 3.22).**

To summarize the smoking results analyses p16+ compared to p16-, p16+: nonsmoker have significantly higher OS and DSS but not significant DFS, p16+: former >10py had significantly higher OS and DSS but not significant DFS and p16+: current >10py had significantly higher OS, DSS and DFS. For HPV analyses HPV+ compared to HPV-, HPV+: nonsmoker had higher but not statistically significant OS, DSS and DFS, and HPV+: former >10py had significantly higher OS but not significant DSS and DFS and HPV+: current >10py had significantly higher OS, DSS and DFS. When p16 and HPV status were combined, compared to p16+/HPV+, p16-/HPV- had significantly lower OS and DSS for all groups and significantly lower DFS for current >10py. For discordant p16+/HPV- compared to p16+/HPV+, p16+/HPV-: nonsmokers had higher but not statistically significant OS, DSS and DFS, p16+/HPV-: former >10py had lower but not statistically significant OS, DSS and DFS and p16+/HPV-: current >10py had lower but not statistically significant OS, DSS and DFS. For discordant p16-/HPV+ compared to p16+/HPV+, p16+/HPV-: former >10py had significantly lower DSS but not statistically

significant OS and DFS and p16-/HPV+: current >10py had lower but not statistically significant OS, DSS and DFS.

Table 3. 21: Summary of Proportion Surviving (OS, DSS and DFS) for p16, HPV and p16/HPV stratified by Smoking

Covariate	Overall Survival		Disease-Specific Survival		Disease-Free Survival	
	Proportion Surviving (%)	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
p16 and Smoking (vs p16-)						
p16-: nonsmoker	25.0	**0.001**	33.3	**0.013**	37.5	0.054
p16+: nonsmoker	85.5		86.9		73.2	
p16-: former >10py	32.0	**<0.0001**	36.9	**0.0003**	75.0	0.77
p16+: former >10py	77.7		79.4		80.0	
p16-: current >10py	12.1 ((4.8y)	**0.0063**	19.6 (4.8y)	**0.011**	19.8 (4.8y)	**0.009**
p16+: current >10py	61.6		68.8		56.7	
HPV and Smoking (vs HPV-)						
HPV-: nonsmoker	77.1	0.79	80.8	0.94	80.8	0.88
HPV+: nonsmoker	82.5		84.3		84.3	
HPV-: former >10py	62.2	**0.05**	65.0	0.069	70.7	0.29
HPV+: former >10py	77.2		79.4		83.6	
HPV-: current >10py	19.3	**0.0093**	33.1	**0.017**	29.3	**0.022**
HPV+: current >10py	62.4		67.9		56.0	
p16/HPV and Smoking (vs p16+/HPV+: nonsmoker)						
p16+/HPV+: nonsmoker	82.5		84.3		69.7	
p16+/HPV-: nonsmoker	92.3	0.33	92.3	0.43	82.1	0.61
p16-/HPV+: nonsmoker	N/A	N/A	N/A	N/A	N/A	N/A
p16-/HPV-: nonsmoker	25.0	**0.007**	33.3	**0.04**	37.5	0.11
p16/HPV and Smoking (vs p16+/HPV+: former >10py)						
p16+/HPV+: former >10py	79.5		81.9		83.1	
p16+/HPV-: former >10py	72.9	0.4	72.9	0.29	71.6	0.42
p16-/HPV+: former >10py	33.3	0.098	33.3	**0.05**	100	0.52
p16-/HPV-: former >10py	33.3	**<0.0001**	40.0	**0.00081**	66.7	0.42
p16/HPV and Smoking (vs p16+/HPV+: current >10py)						
p16+/HPV+: current >10py	63.9		69.7		57.5	
p16+/HPV-: current >10py	32.1	0.23	55.6	0.24	42.9	0.29
p16-/HPV+: current >10py	0 (2.8y)	0.25	0 (2.8y)	0.19	0 (2.8y)	0.099
p16-/HPV-: current >10py	13.2 (4.8y)	**0.0096**	22.2 (4.8y)	**0.019**	21.7 (4.8y)	**0.018**

Table 3. 22: Multivariate Cox Proportional Hazard Regression analysis and Post-hoc Power Calculations for p16, HPV and p16/HPV stratified by Smoking

Log-rank Test Comparing Survival Rates based on Smoking and p16, HPV and p16/HPV	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
p16- Reference					
OS: p16+: nonsmoker	0.14 (0.04-0.55)	**0.005**	67 (4+63)	**90.70**	
DSS: p16+: nonsmoker	0.17 (0.03-0.83)	**0.029**	65 (3+62)	70.24	83
DFS: p16+: nonsmoker	0.24 (0.05-1.16)	0.076	67 (4+63)	48.55	143
OS: p16+: former >10py	0.23 (0.11-0.48)	**<0.001**	131 (15+116)	**99.04**	
DSS: p16+: former >10py	0.25 (0.11-0.57)	**<0.001**	126 (13+113)	**95.10**	
DFS: p16+: former >10py	0.81 (0.19-3.47)	0.8	131 (15+116)	6.18	10012
OS: p16+: current >10py	0.38 (0.19-0.78)	**0.008**	87 (19+68)	78.30	91
DSS: p16+: current >10py	0.36 (0.16-0.82)	**0.015**	79 (16+63)	71.67	97
DFS: p16+: current >10py	0.35 (0.15-0.80)	**0.013**	87 (19+68)	74.27	101
HPV- Reference					
OS: HPV+: nonsmoker	0.84 (0.24-2.99)	0.8	67 (22+45)	6.18	5161
DSS: HPV+: nonsmoker	0.94 (0.22-3.96)	>0.9	65 (21+44)	---	---
DFS: HPV+: nonsmoker	0.91 (0.25-3.22)	0.9	67 (22+45)	---	---
OS: HPV+: former >10py	0.52 (0.27-1.01)	0.053	131 (47+84)	50.81	263
DSS: HPV+: former >10py	0.52 (0.26-1.06)	0.074	126 (45+81)	44.38	300
DFS: HPV+: former >10py	0.63 (0.26-1.49)	0.3	131 (47+84)	18.35	928
OS: HPV+: current >10py	0.42 (0.21-0.82)	**0.012**	87 (26+61)	74.27	101
DSS: HPV+: current >10py	0.40 (0.18-0.87)	**0.021**	79 (22+57)	66.68	109
DFS: HPV+: current >10py	0.41 (0.19-0.90)	**0.026**	87 (26+61)	62.40	132
p16+/HPV+: nonsmoker Reference					
OS: p16+/HPV-: nonsmoker	0.35 (0.04-2.95)	0.3	63 (45+18)	15.86	546
DSS: p16+/HPV-: nonsmoker	0.42 (0.05-3.57)	0.4	62 (44+18)	12.05	818
DFS: p16+/HPV-: nonsmoker	0.65 (0.13-3.24)	0.6	63 (45+18)	8.50	1649
OS: p16-/HPV+: nonsmoker	N/A				
DSS: p16-/HPV+: nonsmoker	N/A				
DFS: p16-/HPV+: nonsmoker	N/A				
OS: p16-/HPV-: nonsmoker	5.60 (1.39-22.6)	**0.015**	49 (45+4)	**99.13**	
DSS: p16-/HPV-: nonsmoker	4.87 (0.93-25.4)	0.060	47 (44+3)	53.58	88
DFS: p16-/HPV-: nonsmoker	3.63 (0.72-18.2)	0.12	49 (45+4)	36.34	149
p16+/HPV+: former >10py Reference					
OS: p16+/HPV-: former >10py	1.34 (0.60-3.03)	0.5	116 (81+35)	12.12	1515
DSS: p16+/HPV-: former >10py	1.55 (0.67-3.59)	0.3	113 (78+35)	18.33	801
DFS: p16+/HPV-: former >10py	1.46 (0.57-3.74)	0.4	116 (81+35)	13.39	1291
OS: p16-/HPV+: former >10py	3.51 (0.81-15.3)	0.094	84 (81+3)	37.49	245
DSS: p16-/HPV+: former >10py	4.31 (0.97-19.1)	0.054	81 (78+3)	49.73	167
DFS: p16-/HPV+: former >10py	0 (0-inf)	>0.9	84 (81+3)	9.69	1649
OS: p16-/HPV-: former >10py	5.26 (2.26-12.2)	**<0.001**	93 (81+12)	**98.56**	
DSS: p16-/HPV-: former >10py	4.71 (1.81-12.3)	**0.002**	88 (78+10)	**91.76**	
DFS: p16-/HPV-: former >10py	1.93 (0.43-8.65)	0.4	93 (81+12)	12.05	1227
p16+/HPV+: current >10py Reference					
OS: p16+/HPV-: current >10py	1.95 (0.66-5.79)	0.2	68 (60+8)	23.30	354
DSS: p16+/HPV-: current >10py	2.09 (0.60-7.33)	0.3	63 (56+7)	22.16	349

DFS: p16+/HPV-: current >10py	1.92 (0.55-6.68)	0.3	68 (60+8)	18.20	487
OS: p16-/HPV+: current >10py	3.81 (0.50-29.1)	0.2	61 (60+1)	20.72	369
DSS: p16-/HPV+: current >10py	4.11 (0.53-31.8)	0.2	57 (56+1)	25.72	263
DFS: p16-/HPV+: current >10py	5.77 (0.73-45.5)	0.10	61 (60+1)	37.47	178
OS: p16-/HPV-: current >10py	2.81 (1.31-6.00)	**0.008**	78 (60+18)	73.49	92
DSS: p16-/HPV-: current >10py	2.96 (1.23-7.13)	**0.016**	71 (56+15)	64.90	102
DFS: p16-/HPV-: current >10py	2.99 (1.23-7.26)	**0.016**	78 (60+18)	65.80	110

3.7.4 Survival Outcomes p16, HPV and p16/HPV Stratified by Tumor Stage

The survival for p16, HPV and p16/HPV were stratified by tumor stage. There was a statistically significant difference between p16+: T1 and p16-: T1 for 5-year OS (80.0% vs 50.0%, p=0.035) but no significant differences for DSS (84.1% vs 55.6%, p=0.076) and DFS (81.5% vs 74.1%, p=0.51) **(Table 3.23, Appx E Figure 1**), with post hoc power calculations of 55.63%, 42.08% and 9.69%, respectively **(Table 3.24).** Compared to p16-: T1, p16+: T1 have significantly lower hazard of death from any cause (OS: HR, 0.33; 95%CI, 0.11-0.98; p=0.045) but not significant hazard of death of disease (DSS: HR, 0.36; 95%CI, 0.11-1.17; p=0.089) and hazard of disease recurrence (DFS: HR, 0.59; 95%CI, 0.12-2.88; p=0.5) **(Table 3.24).**

There were statistically significant differences between p16+: T2 and p16-: T2 for 5-year OS (82.9% vs 20.5%, p<0.0001) and DSS (85.0% vs 24.2%, p=0.00026) but not significant for DFS (79.3% vs 33.3%, p=0.082) **(Table 3.23, Appx E Figure 2**), with post hoc power calculations of 99.47%, 95.53% and 41.03%, respectively **(Table 3.24).** Compared to p16-: T2, p16+: T2 have significantly lower hazard of death from any cause (OS: HR, 0.18; 95%CI, 0.08-0.41; p<0.001) and hazard of death of disease (DSS: HR,

0.20; 95%CI, 0.08- 0.52; p=0.001) but not significant hazard of disease recurrence (DFS: HR, 0.35; 95%CI, 0.10-1.21; p=0.10) **(Table 3.24).**

There were no statistically significant differences between p16+:T3 and p16-: T3 for 5-year OS (63.6% vs 45.7%, p=0.12), DSS (66.1% vs 37.5%, p=0.47) and DFS (51.2% vs 40.0%, p=0.19) **(Table 3.23, Appendix E Figure 3**) with post hoc power calculations of 32.24%, 10.94% and 25.65%, respectively **(Table 3.24).** Compared to p16-: T3, p16+: T3 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.43; 95%CI, 0.15-1.28; p=0.13), hazard of death of disease (DSS: HR, 0.58; 95%CI, 0.13-2.53; p=0.5) and hazard of disease recurrence (DFS: HR, 0.44; 95%CI, 0.13-1.55; p=0.2) **(Table 3.24).**

There were statistically significant differences between p16+: T4 and p16-: T4 for 5-year OS (43.1% vs 9.1%, p=0.038) and DSS (53.9% vs 10.1%, p=0.0077) but not significant DFS (65.2% vs 22.9%, p=0.15) **(Table 3.23, Appx E Figure 4**), with post hoc power calculations of 54.55%, 75.94% and 30.46%, respectively **(Table 3.24).** Compared to p16-: T4**,** p16+: T4 have significantly lower hazard of death from any cause (OS: HR, 0.42; 95%CI, 0.19-0.97; p=0.043) and hazard of death of disease (DSS: HR, 0.30; 95%CI, 0.12-0.77; p=0.012) but not significant hazard of disease recurrence (DFS: HR, 0.44; 95%CI, 0.14-1.39; p=0.2) **(Table 3.24).**

There were no statistically significant differences between HPV+: T1 and HPV-: T1 for 5-year OS (86.4% vs 60.7%, p=0.051), DSS (86.4% vs 69.6%, p=0.12) and DFS (88.2% vs 69.4%, p=0.39) **(Table 3.23, Appx E Figure 5**) with post hoc power calculations of 49.69%, 34.01% and 14.61%, respectively **(Table 3.24).** Compared to HPV-: T1, HPV+: T1 have lower but not statistically significant hazard of death from any

cause (OS: HR, 0.36; 95%CI, 0.12-1.05; p=0.061), hazard of death of disease (DSS: HR, 0.43; 95%CI, 0.14-1.31; p=0.14) and hazard of disease recurrence (DFS: HR, 0.56; 95%CI, 0.15-2.10; p=0.4) **(Table 3.24).**

There was a statistically significant difference between HPV+: T2 and HPV-: T2 for 5-year OS (81.6% vs 57.0%, p=0.037) but not significant for DSS (84.3% vs 59.9%, p=0.059) and DFS (78.3% vs 63.7%, p=0.21) **(Table 3.23, Appx E Figure 6**), with post hoc power calculations of 54.32%, 47.42% and 24.37%, respectively **(Table 3.24).** Compared to HPV-: T2, HPV+: T2 have significantly lower hazard of death from any cause (OS: HR, 0.44; 95%CI, 0.20-0.97; p=0.042) but not significant hazard of death of disease (DSS: HR, 0.44; 95%CI, (0.18-1.05; p=0.066) and hazard of disease recurrence (DFS: HR, 0.54; 95%CI, 0.20-1.42; p=0.2) **(Table 3.24).**

There were no statistically significant differences between HPV+: T3 and HPV-: T3 for 5-year OS (64.9% vs 25.5%, p=0.23), DSS (67.9% vs 49.2%, p=0.39) and DFS (52.6% vs 42.4%, p=0.18) **(Table 3.23, Appx E Figure 7**) with post hoc power calculations of 23.07%, 13.36% and 27.00%, respectively **(Table 3.24).** Compared to HPV-: T3, HPV+: T3 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.60; 95%CI, 0.25-1.40; p=0.2), hazard of death of disease (DSS: HR, 0.66; 95%CI, 0.25-1.72; p=0.4) and hazard of disease recurrence (DFS: HR, 0.54; 95%CI, 0.21-1.35; p=0.2) **(Table 3.24).**

There were no statistically significant differences between HPV+: T4 and HPV-: T4 for 5-year OS (35.8% vs 22.2%, p=0.27), DSS (44.9% vs 27.3%, p=0.099) and DFS (64.4% vs 35.0%, p=0.28) **(Table 3.23, Appx E Figure 8**) with post hoc power calculations of 19.46%, 37.57% and 19.46%, respectively **(Table 3.24).** Compared to

HPV-: T4, HPV+: T4 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.62; 95%CI, 0.27-1.45; p=0.3), hazard of death of disease (DSS: HR, 0.46; 95%CI, 0.18-1.18; p=0.11) and hazard of disease recurrence (DFS: HR, 0.54; 95%CI, 0.17-1.70; p=0.3) **(Table 3.24).**

There were no statistically significant differences between p16+/HPV+: T1 and p16+/HPV-: T1 for 5-year OS (86.0% vs 67.9%, p=0.29), DSS (86.0% vs 80.2%, p=0.48) and DFS (87.8% vs 68.9%, p=0.61) **(Table 3.23, Appx E Figure 9**), with post hoc power calculations of 18.13%, 10.91% and 8.47% for DFS **(Table 3.24).** Compared to p16+/HPV+: T1, p16+/HPV-: T1 have higher but not statistically significant hazard of death from any cause (OS: HR, 1.92; 95%CI, 0.56-6.65; p=0.3), hazard of death of disease (DSS: HR, 1.62; 95%CI, 0.43-6.04; p=0.5) and hazard of disease recurrence (DFS: HR, 1.45; 95%CI, 0.32-6.48; p=0.3) **(Table 3.24).**

There were no statistically significant differences between p16+/HPV+: T1 and p16-/HPV+ for 5-year OS (86.0% vs 100%, p=0.7), DSS (86.0% vs 100%, p=0.7) and DFS (87.8% vs 100%, p=0.72) **(Table 3.23, Appx E Figure 9**), with post hoc power calculations of 6.14% for OS, DSS and DFS **(Table 3.24).** Hazard risks were calculated at 0 (95%CI: 0-inf) between p16+/HPV+: T1 and p16-/HPV+: T1 as there were no events in the latter group (ie. 100% proportion surviving for OS, DSS and DFS **(Table 3.24).**

There were statistically significant differences between p16+/HPV+: T1 and p16-/HPV-: T1 for OS (86.0% vs 44.4%, p=0.0095) and DSS (86.0% vs 50.0%, p=0.0095) but not significant DFS (87.8% vs 70.1%, p=0.35) **(Table 3.23, Appx E Figure 9**), with post hoc power calculations of 73.57% for OS and DSS and 15.85% for DFS **(Table 3.24).** Compared to p16+/HPV+: T1, p16-/HPV-: T1 have significantly higher hazard of death

from any cause (OS: HR, 4.57; 95%CI, 1.32-15.9; p=0.017) and hazard of death of disease (DSS: HR, 3.86; 95%CI, 1.03-14.5; p=0.045) but not significant hazard of disease recurrence (DFS: HR, 2.33; 95%CI, 0.42-12.8; p=0.3) **(Table 3.24).**

There were no statistically significant differences between p16+/HPV+: T2 and p16+/HPV-: T2 for 5-year OS (84.1% vs 80.1%, p=0.35), DSS (87.0% vs 80.1%, p=0.21) and DFS (79.7% vs 80.9%, p=0.33) **(Table 3.23, Appx E Figure 10**), with post hoc power calculations of 15.86%, 24.36% and 15.86%, respectively (**Table 3.24)**. Compared to p16+/HPV+: T2, p16+/HPV-:T2 have higher but not statistically significant hazard of death from any cause (OS: HR, 1.63; 95%CI, 0.60-4.44; p=0.3), hazard of death of disease (DSS: HR, 2.05; 95%CI, 0.71-5.94; p=0.2) and hazard of disease recurrence (DFS: HR, 1.80; 95%CI, 0.60-5.43; p=0. 3) **(Table 3.24).**

There were statistically significant differences between p16+/HPV+: T2 and p16-/HPV+: T2 for OS (84.1% vs 33.3%, p=0.0078) and DSS (87.0% vs 33.3%, p=0.0039) but not significant DFS (79.7% vs 50.0%, p=0.085) **(Table 3.23, Appx E Figure 10**), with post hoc power calculations of 75.81%, 82.12% and 40.96%, respectively (**Table 3.24)**. Compared to p16+/HPV+: T2, p16-/HPV+:T2 have significantly higher hazard of death from any cause (OS: HR, 6.25; 95%CI, 1.37-28.6; p=0.018) and hazard of death of disease (DSS: HR, 7.83; 95%CI, (1.64-37.4; p=0.010) but not significant hazard of disease recurrence (DFS: HR, 5.07; 95%CI, 0.62-41.5; p=0.13) **(Table 3.24).**

There were statistically significant differences between p16+/HPV+: T2 and p16-/HPV-: T2 for OS (84.1% vs 22.5%, p<0.001) and DSS (87.0% vs 28.1%, p=0.0014) but not significant DFS (79.7% vs 37.5%, p=0.14) **(Table 3.23, Appx E Figure 10**), with post hoc power calculations of 98.59%, 89.07% and 31.59%, respectively (**Table 3.24)**.

Compared to p16+/HPV+: T2, p16-/HPV-: T2 have significantly higher hazard of death from any cause (OS: HR, 6.59; 95%CI, 2.43-17.9; p<0.001) and hazard of death of disease (DSS: HR, 5.87; 95%CI, 1.77-19.5; p=0.004) but not significant hazard of disease recurrence (DFS: HR, 2.97; 95%CI0.64-13.8; p=0.2) **(Table 3.24).**

There were no statistically significant differences between p16+/HPV+: T3 and p16+/HPV-: T3 for 5-year OS (64.9% vs 53.6%, p=0.71), DSS (67.9% vs 53.6%, p=0.58) and DFS (52.6% vs 41.7%, p=0.43) **(Table 3.23, Appx E Figure 11**) with post hoc power calculations of 6.14%, 8.53% and 12.05%, respectively (**Table 3.24)**. Compared to p16+/HPV+: T3, p16+/HPV-: T3 have higher but not statistically significant hazard of death from any cause (OS: HR, 1.28; 95%CI, 0.43-3.87; p=0.7), hazard of death of disease (DSS: HR, 1.39; 95%CI, 0.45-4.26; p=0.6) and hazard of disease recurrence (DFS: HR, 1.58; 95%CI, 0.51-4.87; p=0.4) **(Table 3.24)**. There were no patients with p16-/HPV+: T3.

There were no statistically significant differences between p16+/HPV+: T3 and p16-/HPV-: T3 for 5-year OS (64.9% vs 45.7%, p=0.11), DSS (67.9% vs 37.5%, p=0.43) and DFS (52.6% vs 40.0%, p=0.15) **(Table 3.23, Appx E Figure 11**) with post hoc power calculations of 36.45%, 12.04% and 30.60%, respectively (**Table 3.24)**. Compared to p16+/HPV+: T3, p16-/HPV-: T3 have higher but not statistically significant hazard of death from any cause (OS: HR, 2.42; 95%CI, 0.80-7.30; p=0.12), hazard of death of disease (DSS: HR, 1.83; 95%CI, 0.41-8.09; p=0.4) and hazard of disease recurrence (DFS: HR, 2.48; 95%CI, 0.69-8.91; p=0.2) **(Table 3.24)**.

There were no statistically significant differences between p16+/HPV+: T4 and p16+/HPV-: T4 for 5-year OS (38.8% vs 100%, p=0.22), DSS (49.7% vs 100%, p=0.29) and DFS (62.3% vs 100%, p=0.39) **(Table 3.23, Appx E Figure 12**) with post hoc power

calculations of 23.15%, 18.29% and 13.32% for DFS **(Table 3.24)**. Hazard risks were calculated 0 (95%CI: 0-inf) between p16+/HPV+: T4 and p16+/HPV-: T4 as there were no events in the latter group (ie. 100% proportion surviving for OS, DSS and DFS) **(Table 3.24).**

There were no statistically significant differences between p16+/HPV+: T4 and p16-/HPV+: T4 for 5-year OS (38.8% vs 0, p=0.86), DSS (49.7% vs 0%, p=0.78) and DFS (62.3% vs 100%, p=0.5) **(Table 3.23, Appx E Figure 12**) with post hoc power calculations of 6.15% for DSS, 10.86% for DFS and cannot be quantified for OS **(Table 3.24).** Compared to p16+/HPV+: T4, p16-/HPV+: T4 have higher but not statistically significant hazard of death from any cause (OS: HR, 1.28; 95%CI, 0.17-9.87; p=0.8) and hazard of death of disease (DSS: HR, 1.40; 95%CI, 0.18-11.1; p=0.7). Hazard of disease recurrence was calculated at 0 (95%CI: 0-inf) since p16/HPV+-: T4 have 100% proportion surviving for DFS analysis. **(Table 3.24).**

There was a significant difference between p16+/HPV+: T4 and p16-/HPV-: T4 for DSS (49.7% vs 11.1%, p=0.0093) but not significant for OS (38.8% vs 10.0%, p=0.055) and DFS (62.3% vs 18.2%, p=0.11) **(Table 3.23, Appx E Figure 12**), with post hoc power calculations of 74.10% for DSS and 48.58% for OS and 35.23% for DFS **(Table 3.24)**. Compared to p16+/HPV+: T4, p16-/HPV-: T4 have significantly higher hazard of death of disease (DSS: HR, 3.47; 95%CI, 1.30-9.24; p=0.013) but not significant hazard of death from any cause (OS: HR, 2.27; 95%CI, (0.97-5.36; p=0.060) and hazard of disease recurrence (DFS: HR, 2.48; 95%CI, 0.78-7.89; p=0.12) **(Table 3.24).**

To summarize the T stage results analyses p16+ compared to p16-, p16+: T1 have significantly higher OS but not significant DSS and DFS, p16+: T2 had significantly

higher OS and DSS but not significant DFS, p16+: T3 had higher but not statistically significant OS, DSS and DFS and p16+: T4 had significantly higher OS and DSS but not significant DFS. For HPV analyses HPV+ compared to HPV-, HPV+: T2 had significantly higher OS but not significant DSS and DFS and HPV+: T1, T3 and T4 had higher but not statistically significant OS, DSS and DFS. When p16 and HPV status were combined, compared to p16+/HPV+, p16-/HPV- had significantly lower OS for T1 and T2 and significantly lower DSS for T1, T2 and T3. For discordant p16+/HPV- compared to p16+/HPV+, p16+/HPV-: T1, T2 and T3 had lower but not statistically significant OS, DSS and DFS. For discordant p16-/HPV+ compared to p16+/HPV+, p16+/HPV-: T2 had significantly lower OS and DSS but not statistically significant DFS and p16-/HPV+: T4 had lower but not statistically significant OS and DSS.

Table 3. 23: Summary of Proportion Surviving (OS, DSS and DFS) for p16, HPV and p16/HPV stratified by Tumor stage

Covariate	Overall Survival		Disease-Specific Survival		Disease-Free Survival	
	Proportion Surviving (%)	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
p16 and Tumor Stage (vs p16-)						
p16-: T1	50.0	**0.035**	55.6	0.076	74.1	0.51
p16+: T1	80.0		84.1		81.5	
p16-: T2	20.5	**<0.0001**	24.2	**0.00026**	33.3	0.082
p16+: T2	82.9		85.0		79.3	
p16-: T3	45.7 (3.5y)	0.12	37.5 (3.5y)	0.47	40.0 (3.5y)	0.19
p16+: T3	63.6		66.1		51.2	
p16-: T4	9.1 (4.1y)	**0.038**	10.1 (4.1y)	**0.0077**	22.9 (4.1y)	0.15
p16+: T4	43.1		53.9		65.2	
HPV and Tumor Stage (vs HPV-)						
HPV-: T1	60.7	0.051	69.6	0.12	69.4	0.39
HPV+: T1	86.4		86.4		88.2	
HPV-: T2	57.0	**0.037**	59.9	0.059	63.7	0.21
HPV+: T2	81.6		84.3		78.3	
HPV-: T3	25.5	0.23	49.2	0.39	42.4	0.18
HPV+: T3	64.9		67.9		52.6	
HPV-: T4	22.2 (4.1y)	0.27	27.3 (4.1y)	0.099	35.0 (4.1y)	0.28

HPV+: T4	35.8		44.9		64.4	
p16/HPV and Tumor Stage (vs p16+/HPV+: T1)						
p16+/HPV+: T1	86.0		86.0		87.8	
p16+/HPV-: T1	67.9	0.29	80.2	0.48	68.9	0.61
p16-/HPV+: T1	100	0.7	100	0.7	100	0.72
p16-/HPV-: T1	44.4	**0.0095**	50.0	**0.0095**	70.0	0.35
p16/HPV and Tumor Stage (vs p16+/HPV+: T2)						
p16+/HPV+: T2	84.1		87.0		79.7	
p16+/HPV-: T2	80.1	0.35	80.1	0.21	80.9	0.33
p16-/HPV+: T2	33.3 (2.9y)	**0.0078**	33.3 (2.9y)	**0.0039**	50.0 (2.9y)	0.085
p16-/HPV-: T2	22.5	**<0.0001**	28.1	**0.0014**	37.5	0.14
p16/HPV and Tumor Stage (vs p16+/HPV+: T3)						
p16+/HPV+: T3	64.9		67.9		52.6	
p16+/HPV-: T3	53.6	0.71	53.6	0.58	41.7	0.43
p16-/HPV+: T3	N/A	N/A	N/A	N/A	N/A	N/A
p16-/HPV-: T3	45.7 (3.5y)	0.11	37.5 (3.5y)	0.43	40.0 (3.5y)	0.15
p16/HPV and Tumor Stage (vs p16+/HPV+: T4)						
p16+/HPV+: T4	38.8		49.7		62.3	
p16+/HPV-: T4	100 (3.8y)	0.22	100 (3.8y)	0.29	100 (3.8y)	0.39
p16-/HPV+: T4	0 (3y)	0.86	0 (3y)	0.78	100 (3y)	0.5
p16-/HPV-: T4	10.0 (4.1y)	0.055	11.1 (4.1y)	**0.0093**	18.2 (4.1y)	0.11

Table 3. 24 Multivariate Cox Proportional Hazard Regression analysis and Post-hoc Power Calculations for p16, HPV and p16/HPV stratified by Tumor Stage

Log-rank Test Comparing Survival Rates based on Tumor Stage and p16, HPV and p16/HPV	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
p16- Reference					
OS: p16+: T1	0.33 (0.11-0.98)	**0.045**	69 (10+59)	55.63	123
DSS: p16+: T1	0.36 (0.11-1.17)	0.089	67 (9+58)	42.08	170
DFS: p16+: T1	0.59 (0.12-2.88)	0.5	69 (10+59)	9.69	1356
OS: p16+: T2	0.18 (0.08-0.41)	**<0.001**	125 (13+112)	**99.47**	
DSS: p16+: T2	0.20 (0.08- 0.52)	**0.001**	120 (11+109)	**95.53**	
DFS: p16+: T2	0.35 (0.10-1.21)	0.10	125 (13+112)	41.03	327
OS: p16+: T3	0.43 (0.15-1.28)	0.13	69 (7+62)	34.24	225
DSS: p16+: T3	0.58 (0.13-2.53)	0.5	65 (5+60)	10.94	1014
DFS: p16+: T3	0.44 (0.13-1.55)	0.2	69 (7+62)	25.65	319
OS: p16+: T4	0.42 (0.19-0.97)	**0.043**	37 (11+26)	54.55	68
DSS: p16+: T4	0.30 (0.12-0.77)	**0.012**	32 (10+22)	75.94	36
DFS: p16+: T4	0.44 (0.14-1.39)	0.2	37 (11+26)	30.46	139
HPV- Reference					
OS: HPV+: T1	0.36 (0.12-1.05)	0.061	69 (28+41)	49.69	143
DSS: HPV+: T1	0.43 (0.14-1.31)	0.14	67 (26+41)	34.01	220
DFS: HPV+: T1	0.56 (0.15-2.10)	0.4	69 (28+41)	14.61	673

OS: HPV+: T2	0.44 (0.20-0.97)	**0.042**	125 (42+83)	54.32	230
DSS: HPV+: T2	0.44 (0.18-1.05)	0.066	120 (40+80)	47.42	263
DFS: HPV+: T2	0.54 (0.20-1.42)	0.2	125 (42+83)	24.37	615
OS: HPV+: T3	0.60 (0.25-1.40)	0.2	69 (17+52)	23.07	364
DSS: HPV+: T3	0.66 (0.25-1.72)	0.4	65 (15+50)	13.36	726
DFS: HPV+: T3	0.54 (0.21-1.35)	0.2	69 (17+52)	27.00	300
OS: HPV+: T4	0.62 (0.27-1.45)	0.3	37 (12+25)	19.46	243
DSS: HPV+: T4	0.46 (0.18-1.18)	0.11	33 (11+21)	37.57	96
DFS: HPV+: T4	0.54 (0.17-1.70)	0.3	37 (12+25)	19.46	243
p16+/HPV+: T1 Reference					
OS: p16+/HPV-: T1	1.92 (0.56-6.65)	0.3	59 (40+19)	18.13	425
DSS: p16+/HPV-: T1	1.62 (0.43-6.04)	0.5	58 (40+18)	10.91	908
DFS: p16+/HPV-: T1	1.45 (0.32-6.48)	0.6	59 (40+19)	8.47	1558
OS: p16-/HPV+: T1	0 (0-inf)	>0.9	41 (40+1)	6.14	3269
DSS: p16-/HPV+: T1	0 (0-inf)	>0.9	41 (40+1)	6.14	3269
DFS: p16-/HPV+ T1	0 (0-inf)	>0.9	41 (40+1)	6.14	3269
OS: p16-/HPV-: T1	4.57 (1.32-15.9)	**0.017**	49 (40+9)	73.57	58
DSS: p16-/HPV-: T1	3.86 (1.03-14.5)	**0.045**	49 (40+9)	73.57	58
DFS: p16-/HPV-: T1	2.33 (0.42-12.8)	0.3	49 (40+9)	15.85	425
p16+/HPV+: T2 Reference					
OS: p16+/HPV-: T2	1.63 (0.60-4.44)	0.3	112 (80+32)	15.86	969
DSS: p16+/HPV-: T2	2.05 (0.71-5.94)	0.2	109 (77+32)	24.36	537
DFS: p16+/HPV-: T2	1.80 (0.60-5.43)	0.3	112 (80+32)	15.86	969
OS: p16-/HPV+: T2	6.25 (1.37-28.6)	**0.018**	83 (80+3)	75.81	93
DSS: p16-/HPV+: T2	7.83 (1.64-37.4)	**0.010**	80 (77+3)	**82.12**	
DFS: p16-/HPV+ T2	5.07 (0.62-41.5)	0.13	83 (80+3)	40.96	218
OS: p16-/HPV-: T2	6.59 (2.43-17.9)	**<0.001**	90 (80+10)	**98.59**	
DSS: p16-/HPV-: T2	5.87 (1.77-19.5)	**0.004**	85 (77+8)	**89.07**	
DFS: p16-/HPV-: T2	2.97 (0.64-13.8)	0.2	90 (80+10)	31.59	323
p16+/HPV+: T3 Reference					
OS: p16+/HPV-: T3	1.28 (0.43-3.87)	0.7	62 (52+10)	6.14	4905
DSS: p16+/HPV-: T3	1.39 (0.45-4.26)	0.6	60 (50+10)	8.53	1558
DFS: p16+/HPV-: T3	1.58 (0.51-4.87)	0.4	62 (52+10)	12.05	818
OS: p16-/HPV+: T3	N/A				
DSS: p16-/HPV+: T3	N/A				
DFS: p16-/HPV+ T3	N/A				
OS: p16-/HPV-: T3	2.42 (0.80-7.30)	0.12	59 (52+7)	36.45	178
DSS: p16-/HPV-: T3	1.83 (0.41-8.09)	0.4	55 (50+5)	12.04	726
DFS: p16-/HPV-: T3	2.48 (0.69-8.91)	0.2	59 (52+7)	30.60	220
p16+/HPV+: T4 Reference					
OS: p16+/HPV-: T4	0 (0-inf)	>0.9	26 (24+2)	23.15	137
DSS: p16+/HPV-: T4	0 (0-inf)	>0.9	22 (20+2)	18.29	157
DFS: p16+/HPV-: T4	0 (0-inf)	>0.9	26 (24+2)	13.32	292
OS: p16-/HPV+: T4	1.28 (0.17-9.87)	0.8	25 (24+1)	---	---
DSS: p16-/HPV+: T4	1.40 (0.18-11.1)	0.7	21 (20+1)	6.15	1649
DFS: p16-/HPV+ T4	0 (0-inf)	>0.9	25 (24+1)	10.86	395
OS: p16-/HPV-: T4	2.27 (0.97-5.36)	0.060	34 (24+10)	48.58	73
DSS: p16-/HPV-: T4	3.47 (1.30-9.24)	**0.013**	29 (20+9)	74.10	34
DFS: p16-/HPV-: T4	2.48 (0.78-7.89)	0.12	34 (24+10)	35.23	107

3.7.5 Survival Outcomes for p16, HPV and p16/HPV Stratified by Nodal Stage

The survival for p16, HPV and p16/HPV were stratified by nodal stage. There were statistically significant differences between p16+: N0 and p16-: N0 for 5-year OS (82.9% vs 0, p<0.0001, DSS (82.9% vs 0, p=0.00061, and DFS (100% vs 0, p=0.0035 **(Table 3.25, Appx F Figure 1**), with post hoc power calculations of 97.87%, 93.00% and 82.98%, respectively **(Table 3.26).** Compared to p16-: N0, p16+: N0 have significantly lower hazard of death from any cause (OS: HR, 0.08; 95%CI, 0.02-0.38; p=0.001) and hazard of death of disease (DSS: HR, 0.10; 95%CI, 0.02-0.49; p=0.005). Hazard disease recurrence was calculated at 0 (95%CI: 0-inf) since p16+: N0 have 100% proportion surviving for DFS analysis **(Table 3.26).**

There were no statistically significant differences between p16+: N1 and p16-: N1 for 5-year OS (86.0% vs 60.0%, p=0.052), DSS (87.7% vs 75.0%, p=0.41), and DFS (69.9% vs 100%, p=0.51) **(Table 3.25, Appx F Figure 2)**, with post hoc power calculations of 49.44%, 13.32% and 9.63%, respectively **(Table 3.26).** Compared to p16-: N1, p16+: N1 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.25; 95%CI, 0.06-1.13; p=0.072) and hazard of death of disease (DSS: HR, 0.44; 95%CI, 0.06-3.38; p=0.4). Hazard disease recurrence was calculated at 0 (95%CI: 0-inf) since p16+: N1 have 100% proportion surviving for DFS analysis. **(Table 3.26).**

There were statistically significant differences between p16+: N2 and p16-: N2 for OS (66.9% vs 32.3%, p=0.0099) and DSS (69.8% vs 35.4%, p=0.0052) but not significant for DFS (68.2% vs 45.5%, p=0.2) **(Table 3.25, Appx F Figure 3**), with post hoc power calculations of 73.38%, 79.75%, and 24.53%, respectively **(Table 3.26).** Compared to p16-

: N2, p16+: N2 have significantly lower hazard of death from any cause (OS: HR, 0.39; 95%CI, 0.19-0.82; p=0.013) and hazard of death of disease (DSS: HR, 0.34; 95%CI, 0.16-0.75; p=0.008) but not significant hazard of disease recurrence (DFS: HR, 0.54; 95%CI, 0.21-1.41; p=0.2) **(Table 3.26).**

There were no statistically significant differences between p16+: N3 and p16-: N3 for 5-year OS (45.0% vs 25.0%, p=0.15), DSS (67.3% vs 28.6%, p=0.15) and DFS (46.7% vs 34.3%, p=0.13) **(Table 3.25, Appx F Figure 4**) with post hoc power calculations of 30.54%, 30.50% and 31.75%, respectively **(Table 3.26).** Compared to p16-: N3, p16+: N3 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.45; 95%CI, 0.15-1.36; p=0.2), hazard of death of disease (DSS: HR, 0.39; 95%CI, 0.10-1.46; p=0.2) and hazard of disease recurrence (DFS: HR, 0.33; 95%CI, 0.07-1.51; p=0.2) **(Table 3.26).**

There were statistically significant differences between HPV+: N0 and HPV-: N0 for 5-year OS (75.1% vs 0, p=0.00072), DSS (75.1% vs 0, p=0.0061) and DFS (100% vs 0, p=0.0018) **(Table 3.25, Appx F Figure 5**), with post hoc power calculations of 92.18%, 78.26%, and 87.87%, respectively **(Table 3.26).** Compared to HPV-: N0, HPV+: N0 have significantly lower hazard of death from any cause (OS: HR, 0.13; 95%CI, 0.03-0.51; p=0.003) and hazard of death of disease (DSS: HR, 0.17; 95%CI, 0.04-0.70; p=0.014) **(Table 3.26).** Hazard of disease recurrence was calculated at 0 (95%CI: 0-inf) since HPV+: N0 have 100% proportion surviving for DFS analysis.

There were statistically significant differences between HPV+: N1 and HPV-: N1 for 5-year OS (90.3% vs 71.0%, p=0.0086) and DSS (92.7% vs 73.5%, p=0.012) but not significant DFS (67.6% vs 86.8%, p=0.85) **(Table 3.25, Appx F, Figure 6**), with post hoc

power calculations of 74.71% for OS, 71.54% for DSS and could not be calculated for DFS **(Table 3.26**). Compared to HPV-: N1, HPV+: N1 have significantly lower hazard of death from any cause (OS: HR, 0.27; 95%CI, 0.09-0.77; p= 0.014) and hazard of death of disease (DSS: HR, 0.25; 95%CI, 0.08-0.80; p= 0.019) but not significant hazard of disease recurrence (DFS: HR, 1.13; 95%CI, 0.31-4.11; p=0.8) **(Table 3.26).**

There were no statistically significant differences between HPV+: N2 and HPV-: N2 for 5-year OS (62.3% vs 63.2%, p=0.94), DSS (65.7% vs 66.1%, p=0.68) and DFS (68.8% vs 61.1%, p=0.55) **(Table 3.25, Appx F Figure 7**) with post hoc power calculations of 7.31% for DSS, 9.72% for DFS and could not be calculated for OS **(Table 3.26**). Compared to HPV-: N2, HPV+: N2 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.98; 95%CI, 0.53-1.80; p> 0.9), hazard of death of disease (DSS: HR, 0.87; 95%CI, 0.45-1.67; p= 0.7) and hazard of disease recurrence (DFS: HR, 0.80; 95%CI, 0.39-1.64; p=0.5) **(Table 3.26).**

There were no statistically significant differences between HPV+: N3 and HPV-: N3 for 5-year OS (49.5% vs 30.3%, p=0.56), DSS (60.6% vs 44.4%, p=0.58) and DFS (61.1% vs 37.5%, p=0.18) **(Table 3.25, Appx F Figure 8**) with post hoc power calculations of 8.51%, 8.47% and 26.89%, respectively **(Table 3.26**). Compared to HPV-: N3, HPV+: N3 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.73; 95%CI, 0.24-2.16; p= 0.6), hazard of death of disease (DSS: HR, 0.69; 95%CI, 0.19-2.59; p= 0.6) and hazard of disease recurrence (DFS: HR, 0.34; 95%CI, 0.07-1.77; p=0.2) **(Table 3.26).**

There were no statistically significant differences between p16+/HPV+: N0 and p16+/HPV-: N0 for 5-year OS (82.4% vs 100%, p=0.73), DSS (82.4% vs 100%, p=0.73)

and DFS (100% vs 100%, p=1) **(Table 3.25, Appx F Figure 9**) with post hoc power calculations of 6.17% for OS and DSS and could not be quantified in DFS **(Table 3.26).** Hazard risks were calculated at 0 (95%CI: 0-inf) between p16+/HPV+: N0 and p16+/HPV-: N0 as there were no events in the latter group (ie. 100% proportion surviving for OS, DSS and DFS) **(Table 3.25 and 3.26).**

There were statistically significant differences between p16+/HPV+: N0 and p16-/HPV+: N0 for 5-year OS (82.4% vs 0, p=0.02) and DSS (82.4% vs 0%, p=0.02) but not significant DFS (100% vs 100%, p=1) **(Table 3.25, Appx F Figure 9**), with post hoc power calculations of 64.25% for OS and DSS and could not be calculated for DFS **(Table 3.26).** Compared to p16+/HPV+: N0, p16-/HPV+: N0 have significantly higher hazard of death from any cause (OS: HR, 12.3; 95%CI, 1.02-148; p= 0.048) but not significant hazard of death of disease (DSS: HR, 10.2; 95%CI, 0.85-122; p= 0.066) and hazard of disease recurrence (DFS: HR, 1.04; 95%CI, 0-inf; p>0.9) **(Table 3.26).**

There were statistically significant differences between p16+/HPV+: N0 and p16-/HPV-: N0 for 5-year OS (82.4% vs 0, p=0.00017), DSS (82.4% vs 0, p=0.00018) and DFS (100% vs 0, p=002) **(Table 3.25,** Appendix F, Figure 9), with post hoc power calculations of 96.36%, 87.61% and 86.87%, respectively **(Table 3.26).** Compared to p16+/HPV+: N0, p16-/HPV-: N0 have significantly higher hazard of death from any cause (OS: HR, 12.1; 95%CI, 2.52-57.9; p= 0.002) and hazard of death of disease (DSS: HR, 9.57; 95%CI, 1.87-49.1; p= 0.007) but not significant hazard of disease recurrence (DFS: HR, 7.1e09; 95%CI, 0-inf; p>0.9) **(Table 3.26).**

There were statistically significant differences between p16+/HPV+: N1 and p16+/HPV-: N1 for 5-year OS (90.1% vs 74.3%, p=0.04) and DSS (92.6% vs 74.3%,

p=0.021) but not significant DFS (60.7% vs 85.6%, p=0.94) **(Table 3.25, Appx F Figure 10**), with post hoc power calculations of 53.60% for OS, 63.31% for DSS and could not be calculated for DFS **(Table 3.26).** Compared to p16+/HPV+: N1, p16+/HPV-: N1 have significantly higher hazard of death from any cause (OS: HR, 3.11; 95%CI, 1.00-9.64; p= 0.050) and hazard of death of disease (DSS: HR, 3.70; 95%CI, 1.13-12.1; p= 0.031) but not significant hazard of disease recurrence (DFS: HR, 0.95; 95%CI, 0.26-3.45; p>0.9) **(Table 3.26).**

There were no statistically significant differences between p16+/HPV+: N1 and p16-/HPV+: N1 for 5-year OS (90.1% vs 100%, p=0.75), DSS (92.6% vs 100%, p=0.78) and DFS (60.7% vs 100%, p=0.73) **(Table 3.25, Appx F Figure 10**), with post hoc power calculations of 6.15% for OS, DSS and DFS **(Table 3.26).** Hazard risks were calculated at 0 (95%CI: 0-inf) between p16+/HPV+: N1 and p16-/HPV+: N1 as there were no events in the latter group (ie. 100% proportion surviving for OS, DSS and DFS) **(Table 3.26).**

There was a statistically significant difference between p16+/HPV+: N1 and p16-/HPV-: N1 for 5-year OS (90.1% vs 50.0%, p=0.021) but not significant for DSS (92.6% vs 66.7%, p=0.084) and DFS (60.7% vs 100%, p=0.58) **(Table 3.25, Appx F Figure 10**), with post hoc power calculations of 86.61%, 40.96% and 8.47%, respectively **(Table 3.26).** Compared to p16+/HPV+: N1, p16-/HPV-: N1 have significantly higher hazard of death from any cause (OS: HR, 8.44; 95%CI, 1.70-42.0; p= 0.009) but not significant hazard of death of disease (DSS: HR, 5.43; 95%CI, 0.63-46.5; p= 0.12). Hazard of disease recurrence was calculated at 0 (95%CI: 0-inf) since p16-/HPV+: NI have 100% proportion surviving for DFS analysis **(Table 3.26).**

There were no statistically significant differences between p16+/HPV+: N2 and p16+/HPV-: N2 for 5-year OS (63.7% vs 76.1%, p=0.39), DSS (67.3% vs 76.1%, p=0.68) and DFS (68.7% vs 66.7%, p=0.87) **(Table 3.25, Appx Figure 11**), with post hoc power calculations of 13.22% for OS, 7.28% for DSS and could not be calculated for DFS **(Table 3.26).** Compared to p16+/HPV+: N2, p16+/HPV-: N2 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.71; 95%CI, 0.33-1.55; p= 0.4) and hazard of death of disease (DSS: HR, 0.84; 95%CI, 0.38-1.87; p= 0.7) and higher but not statistically significant hazard of disease recurrence (DFS: HR, 1.07; 95%CI,(0.47-2.43; p=0.9) **(Table 3.26)**

There were no statistically significant differences between p16+/HPV+: N2 and p16-/HPV+: N2 for 5-year OS (63.7% vs 33.3%, p=0.4), DSS (67.3% vs 33.3%, p=0.27) and DFS (68.7% vs 50.0%, p=0.76) **(Table 3.25, Appx F Figure 11**) with post hoc power calculations of 13.37%, 19.37% and 6.17%, respectively **(Table 3.26).** Compared to p16+/HPV+: N2, p16-/HPV+: N2 have higher but not statistically significant hazard of death from any cause (OS: HR, 2.00; 95%CI, 0.48-8.40; p= 0.3), hazard of death of disease (DSS: HR, 2.43; 95%CI, 0.57-10.3; p= 0.2) and hazard of disease recurrence (DFS: HR, 1.50; 95%CI, 0.20-11.2; p=0.7) **(Table 3.26).**

There were statistically significant differences between p16+/HPV+: N2 and p16-/HPV-: N2 for 5-year OS (63.7% vs 32.1%, p=0.044) and DSS (67.3% vs 36.4%, p=0.029) but not significant for DFS (68.7% vs 43.8%, p=0.24) **(Table 3.25, Appx F Figure 11**), with post hoc power calculations of 52.39%, 59.22% and 21.90%, respectively **(Table 3.26).** Compared to p16+/HPV+: N2, p16-/HPV-: N2 have significantly higher hazard of death from any cause (OS: HR, 2.45; 95%CI, 1.07-5.62; p= 0.034) and hazard of death of

disease (DSS: HR, 2.90; 95%CI, 1.18-7.16; p= 0.021) but not significant hazard of disease recurrence (DFS: HR, 2.01; 95%CI, (0.68-5.90; p=0.2) **(Table 3.26).**

There were no statistically significant differences between p16+/HPV+: N3 and p16+/HPV-: N3 for 5-year OS (49.5% vs 0, p=0.53), DSS (60.6% vs 100%, p=0.33) and DFS (61.1% vs 0, p=0.35) **(Table 3.25, Appx F Figure 12**) with post hoc power calculations of 9.69%, 15.77% and 15.76%, respectively **(Table 3.26).** Compared to p16+/HPV+: N3, p16+/HPV-: N3 have lower but not statistically significant hazard of death from any cause (OS: HR, 0.50; 95%CI, 0.06-4.22; p= 0.5) and higher but not statistically significant hazard of disease recurrence (DFS: HR, 1.75 (0.15-19.9); 95%CI, 0.15-19.9; p=0.7) **(Table 3.26).** Hazard of death of disease was calculated at 0 (95%CI: 0-inf) since p16-/HPV+: N2 have 100% proportion surviving for DSS analysis. There were no p16-/HPV+ N3 patients.

There were no statistically significant differences between p16+/HPV+: and p16-/HPV-: N3 for 5-year OS (49.5% vs 25.0%, p=0.23), DSS (60.6% vs 28.6%, p=0.28) and DFS (61.1% vs 34.3%, p=0.12) **(Table 3.25, Appx F Figure 12**) with post hoc power calculations of 23.15%, 19.45% and 35.26%, respectively **(Table 3.26).** Compared to p16+/HPV+: N2, p16-/HPV+: N2 have higher but not statistically significant hazard of death from any cause (OS: HR, 1.93; 95%CI, 0.62-6.02; p= 0.3), hazard of death of disease (DSS: HR, 2.04; 95%CI, 0.55-7.64; p= 0.3) and hazard of disease recurrence (DFS: HR, 3.47; 95%CI, 0.63-19.0; p=0.2) **(Table 3.26)**.

To summarize the N stage results analyses p16+ compared to p16-, p16+: N0 had significantly higher OS, DSS and DFS, p16+: N1 had higher but not statistically significant OS and DSS and lower but not statistically significant DFS, p16+: N2 had significantly

higher OS and DSS but not significant DFS and p16+: N3 had higher but not statistically significant OS, DSS and DFS. For HPV analyses HPV+ compared to HPV-, HPV+: N0 had significantly higher OS, DSS and DFS, HPV+: N1 had significantly higher OS and DSS but not significant DFS, HPV+: N2 had lower but not statistically significant OS and DSS and higher but not statistically significant DFS and HPV+: N3 had higher but not statistically significant OS, DSS and DFS. When p16 and HPV status were combined, compared to p16+/HPV+, p16-/HPV- had significantly lower OS, DSS and DFS for N0, significantly lower OS for N1 and significantly lower OS and DSS for N2. For discordant p16+/HPV- compared to p16+/HPV+, p16+/HPV-: N1 had significantly lower OS and DSS and p16+/HPV-: N2 had higher but not statistically significant OS and DSS. For discordant p16-/HPV+ compared to p16+/HPV+, p16-/HPV+: N0 had significantly lower OS but not significant DSS and DFS and p16-/HPV+: N2 had higher but not statistically significant OS, DSS and DFS.

Table 3. 25: Summary of Proportion Surviving (OS, DSS and DFS) for p16, HPV and p16/HPV stratified by Nodal stage

Covariate	Overall Survival		Disease-Specific Survival		Disease-Free Survival	
	Proportion Surviving (%)	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
p16 and Nodal Stage (vs p16-)						
p16-: N0	0 (4.5y)	**<0.0001**	0 (4.5y)	**0.00061**	0 (4.5y)	**0.0035**
p16+: N0	82.9		82.9		100	
p16-: N1	60.0 (4.2y)	0.052	75.0 (4.2y)	0.41	100 (4.2y)	0.51
p16+: N1	86.0		87.7		69.9	
p16-: N2	32.3	**0.0099**	35.4	**0.0052**	45.5	0.2
p16+: N2	66.9		69.8		68.2	
p16-: N3	25.0	0.15	28.6	0.15	34.3	0.13
p16+: N3	45.0		67.3		46.7	
HPV and Nodal Stage (vs HPV-)						
HPV-: N0	0 (4.5y)	**0.00072**	0 (4.5y)	**0.0061**	0 (4.5y)	**0.0018**

HPV+: N0	75.1		75.1		100	
HPV-: N1	71.0 (4.8y)	**0.0086**	73.5 (4.8y)	**0.012**	86.8 (4.8y)	0.85
HPV+: N1	90.3		92.7		67.6	
HPV-: N2	63.2	0.94	66.1	0.68	61.1	0.55
HPV+: N2	62.3		65.7		68.8	
HPV-: N3	30.3	0.56	44.4	0.58	37.5	0.18
HPV+: N3	49.5		60.6		61.1	
p16/HPV and Nodal Stage (vs p16+/HPV+: N0)						
p16+/HPV+: N0	82.4		82.4		100	
p16+/HPV-: N0	100 (2.1y)	0.73	100 (2.1y)	0.73	100 (2.1y)	1
p16-/HPV+: N0	0 (3y)	**0.02**	0 (3y)	**0.02**	100 (3y)	1
p16-/HPV-: N0	0 (4.5y)	**0.00017**	0 (4.5y)	**0.00018**	0 (4.5y)	**0.002**
p16/HPV and Nodal Stage (vs p16+/HPV+: N1)						
p16+/HPV+: N1	90.1		92.6		60.7	
p16+/HPV-: N1	74.3 (4.8y)	**0.04**	74.3 (4.8y)	**0.021**	85.6 (4.8y)	0.94
p16-/HPV+: N1	100 (2.9y)	0.75	100 (2.9y)	0.78	100 (2.9y)	0.73
p16-/HPV-: N1	50.0 (4.2y)	**0.0021**	66.7 (4.2y)	0.084	100 (4.2y)	0.58
p16/HPV and Nodal Stage (vs p16+/HPV+: N2)						
p16+/HPV+: N2	63.7		67.3		68.7	
p16+/HPV-: N2	76.1	0.39	76.1	0.68	66.7	0.87
p16-/HPV+: N2	33.3	0.4	33.3	0.27	50.0	0.76
p16-/HPV-: N2	32.1 (4.8y)	**0.044**	36.4 (4.8y)	**0.029**	43.8 (4.8y)	0.24
p16/HPV and Nodal Stage (vs p16+/HPV+: N3)						
p16+/HPV+: N3	49.5		60.6		61.1	
p16+/HPV-: N3	0 (3.6y)	0.53	100 (3.6y)	0.33	0 (3.6y)	0.35
p16-/HPV+: N3	N/A	N/A	N/A	N/A	N/A	N/A
p16-/HPV-: N3	25.0	0.23	28.6	0.28	34.3	0.12

Table 3. 26: Multivariate Cox Proportional Hazard Regression analysis and Post-hoc Power Calculations for p16, HPV and p16/HPV stratified by Nodal Stage

Log-rank Test Comparing Survival Rates based on Nodal Stage and p16, HPV and p16/HPV	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
p16- Reference					
OS: p16+: N0	0.08(0.02-0.38)	**0.001**	32 (13+19)	**97.87**	
DSS: p16+: N0	0.10 (0.02-0.49)	**0.005**	29 (10+19)	**93.00**	
DFS: p16+: N0	0 (0-inf)	>0.9	32 (13+19)	**82.98**	
OS: p16+: N1	0.25 (0.06-1.13)	0.072	106 (5+101)	49.44	220
DSS: p16+: N1	0.44 (0.06-3.38)	0.4	104 (4+100)	13.32	1168
DFS: p16+: N1	0 (0-inf)	>0.9	106 (5+101)	9.63	2110
OS: p16+: N2	0.39 (0.19-0.82)	**0.013**	138 (15+123)	73.38	163
DSS: p16+: N2	0.34 (0.16-0.75)	**0.008**	131 (14+117)	79.75	132
DFS: p16+: N2	0.54 (0.21-1.41)	0.2	138 (15+123)	24.53	673
OS: p16+: N3	0.45 (0.15-1.36)	0.2	24 (8+16)	30.54	90
DSS: p16+: N3	0.39 (0.10-1.46)	0.2	20 (7+13)	30.50	75
DFS: p16+: N3	0.33 (0.07-1.51)	0.2	24 (8+16)	31.75	86

HPV- Reference					
OS: HPV+: N0	0.13 (0.03-0.51)	**0.003**	32 (14+18)	**92.18**	
DSS: HPV+: N0	0.17 (0.04-0.70)	**0.014**	29 (11+18)	78.26	31
DFS: HPV+: N0	0 (0-inf)	>0.9	32 (14+18)	**87.87**	
OS: HPV+ N1	0.27 (0.09-0.77)	**0.014**	106 (29+77)	74.71	121
DSS: HPV+: N1	0.25 (0.08-0.80)	**0.019**	104 (28+76)	71.54	128
DFS: HPV+: N1	1.13 (0.31-4.11)	0.8	106 (29+77)	---	---
OS: HPV+: N2	0.98 (0.53-1.80)	>0.9	138 (45+93)	---	---
DSS: HPV+: N2	0.87 (0.45-1.67)	0.7	131 (44+87)	7.31	5161
DFS: HPV+: N2	0.80 (0.39-1.64)	0.5	138 (45+93)	9.72	2692
OS: HPV+: N3	0.73 (0.24-2.16)	0.6	24 (11+13)	8.51	626
DSS: HPV+: N3	0.69 (0.19-2.59)	0.6	20 (9+11)	8.47	528
DFS: HPV+: N3	0.34 (0.07-1.77)	0.2	24 (11+13)	26.89	105
p16+/HPV+: N0 Reference					
OS: p16+/HPV-: N0	0 (0-inf)	>0.9	19 (17+2)	6.17	1473
DSS: p16+/HPV-: N0	0 (0-inf)	>0.9	19 (17+2)	6.17	1473
DFS: p16+/HPV-: N0	1.05 (0-inf)	>0.9	19 (17+2)	---	---
OS: p16-/HPV+: N0	12.3 (1.02-148)	**0.048**	18 (17+1)	64.25	27
DSS: p16-/HPV+: N0	10.2 (0.85-122)	0.066	18 (17+1)	64.25	27
DFS: p16-/HPV+ N0	1.04 (0-inf)	>0.9	18 (17+1)	---	---
OS: p16-/HPV-: N0	12.1 (2.52-57.9)	**0.002**	29 (17+12)	**96.36**	
DSS: p16-/HPV-: N0	9.57 (1.87-49.1)	**0.007**	26 (17+9)	**87.61**	
DFS: p16-/HPV-: N0	7.1e09 (0-inf)	>0.9	29 (17+12)	**86.87**	
p16+/HPV+: N1 Reference					
OS: p16+/HPV-: N1	3.11 (1.00-9.64)	**0.050**	101 (76+25)	53.60	189
DSS: p16+/HPV-: N1	3.70 (1.13-12.1)	**0.031**	100 (75+25)	63.31	149
DFS: p16+/HPV-: N1	0.95 (0.26-3.45)	>0.9	101 (76+25)	---	---
OS: p16-/HPV+: N1	0 (0-inf)	>0.9	77 (76+1)	6.15	6057
DSS: p16-/HPV+: N1	0 (0-inf)	>0.9	76 (75+1)	6.15	6057
DFS: p16-/HPV+ N1	0 (0-inf)	>0.9	77 (76+1)	6.15	6057
OS: p16-/HPV-: N1	8.44 (1.70-42.0)	**0.009**	80 (76+4)	**86.61**	
DSS: p16-/HPV-: N1	5.43 (0.63-46.5)	0.12	78 (75+3)	40.96	205
DFS: p16-/HPV-: N1	0 (0-inf)	>0.9	80 (76+4)	8.47	2110
p16+/HPV+: N2 Reference					
OS: p16+/HPV-: N2	0.71 (0.33-1.55)	0.4	123 (90+33)	13.22	1396
DSS: p16+/HPV-: N2	0.84 (0.38-1.87)	0.7	117 (84+33)	7.28	4670
DFS: p16+/HPV-: N2	1.07 (0.47-2.43)	0.9	123 (90+33)	---	---
OS: p16-/HPV+: N2	2.00 (0.48-8.40)	0.3	93 (90+3)	13.37	1037
DSS: p16-/HPV+: N2	2.43 (0.57-10.3)	0.2	87 (84+3)	19.37	574
DFS: p16-/HPV+ N2	1.50 (0.20-11.2)	0.7	93 (90+3)	6.17	7208
OS: p16-/HPV-: N2	2.45 (1.07-5.62)	**0.034**	102 (90+12)	52.39	197
DSS: p16-/HPV-: N2	2.90 (1.18-7.16)	**0.021**	95 (84+11)	59.22	156
DFS: p16-/HPV-: N2	2.01 (0.68-5.90)	0.2	102 (90+12)	21.90	574
p16+/HPV+: N3 Reference					
OS: p16+/HPV-: N3	0.50 (0.06-4.22)	0.5	16 (13+3)	9.69	315
DSS: p16+/HPV-: N3	0 (0-inf)	>0.9	13 (11+2)	15.77	114
DFS: p16+/HPV-: N3	1.75 (0.15-19.9)	0.7	16 (13+3)	15.76	140
OS: p16-/HPV+: N3	N/A				
DSS: p16-/HPV+: N3	N/A				
DFS: p16-/HPV+ N3	N/A				
OS: p16-/HPV-: N3	1.93 (0.62-6.02)	0.3	21 (13+8)	23.15	111
DSS: p16-/HPV-: N3	2.04 (0.55-7.64)	0.3	18 (11+7)	19.45	118
DFS: p16-/HPV-: N3	3.47 (0.63-19.0)	0.2	21 (13+8)	35.26	66

3.7.6 Survival Outcomes for p16, HPV and p16/HPV Stratified by Treatment Type

The survival for p16, HPV and p16/HPV were stratified by treatment types. There were statistically significant differences between p16+: S+RT and p16-: S+RT for 5-year OS (77.5% vs 26.0%, p=0.0022, DSS (82.6% vs 37.4%, p=0.0016, and DFS (87.0% vs 44.4%, p=0.0021 **(Table 3.27, Appx G Figure 1**), with post hoc power calculations of 86.58%, 88.54% and 86.99%, respectively **(Table 3.28).** Compared to p16-: S+RT, p16+: S+RT have significantly lower hazard of death from any cause (OS: HR, 0.29; 95%CI, 0.12-0.67; p=0.004), hazard of death of disease (DSS: HR, 0.23; 95%CI, 0.09-0.62; p=0.004) and hazard of disease recurrence (DFS: HR, 0.20; 95%CI, 0.07-0.63; p=0.005) **(Table 3.28).**

There were statistically significant differences between p16+: S+CRT and p16-: S+CRT for 5-year OS (72.0% vs 16.7%, p=0.0008), DSS (78.0% vs 16.7%, p=0.00022) and DFS (66.8% vs 16.7%, p<0.0001) **(Table 3.27, Appx G Figure 2**), with post hoc power calculations of 91.74%, 95.80% and 97.69%, respectively **(Table 3.28).** Compared with p16-: S+CRT, p16+: S+CRT have significantly lower hazard of death from any cause (OS: HR, 0.21; 95%CI, 0.07-0.57; p=0.002), hazard of death of disease (DSS: HR, 0.17; 95%CI, 0.06-0.49; p=0.001) and hazard of disease recurrence (DFS: HR, 0.16; 95%CI, 0.06-0.45; p<0.001) **(Table 3.28).**

There were no statistically significant differences between p16+: CRT and p16-: CRT for 5-year OS (77.5% vs 100%, p=0.62), DSS (77.5% vs 100%, p=0.62) and DFS (66.5% vs 100%, p=0.53) **(Table 3.27, Appx G Figure 3**) with post hoc power calculations of 7.56% for OS and DSS and 9.67% for DFS **(Table 3.28).** Hazard risks for

OS, DSS and DFS were calculated very high at 2.6e07 (95%CI: 0-inf), 2.6e07 (95%CI: 0-inf), 7.4e07 (95%CI: 0-inf), respectively since there were no events for p16-: CRT (ie, proportion surviving for OS, DSS and DFS at 100% and only one patient) **(Table 3.28).**

There were statistically significant differences between p16+: RT and p16-: RT for 5-year OS (80.0% vs 0, p=0.0082) and DSS (80.0% vs 0, p=0.025) but not significant DFS (100% vs 100%, p=1 **(Table 3.27, Appx G Figure 4**), with post hoc power calculations of 75.36% for OS, 60.89% for DSS and could not be calculated for DFS **(Table 3.28).** Hazard of death from any cause and hazard of death of disease were calculated at 0 (95%CI: 0-inf) since p16-: RT have 0% proportion surviving for OS and DSS analysis. Hazard of disease recurrence can not be calculated because p16+: RT and p16-: RT have equal proportion surviving for DFS **(Table 3.28)**.

There were statistically significant differences between p16+: S and p16-: S for 5-year OS (70.2% vs 19.7%, p=0.0054) and DSS (72.8% vs 22.5%, p=0.017) but not significant DFS (61.7% vs 39.4%, p=0.88 **(Table 3.27, Appx G Figure 5**), with post hoc power calculations of 79.19% for OS, 65.70% for DSS and could not be calculated for DFS **(Table 3.28).** Compared to p16-: S, p16+: S have significantly lower hazard of death from any cause (OS: HR, 0.34; 95%CI, 0.15-0.76; p=0.008) and hazard of death of disease (DSS: HR, 0.36; 95%CI, 0.15-0.87; p=0.023) but not significant hazard of disease recurrence (DFS: HR, 0.90; 95%CI, 0.26-3.18; p=0.9) **(Table 3.28).**

There was a statistically significant difference between HPV+: S+RT and HPV-: S+RT for 5-year DSS (81.8% vs 65.0%, p=0.031) but no significant differences for OS (70.9% vs 56.1%, p=0.068) and DFS (85.2% vs 71.5%, p=0.076) **(Table 3.27, Appx G Figure 6**), with post hoc power calculations of 57.23% for DSS, 44.17% for OS and

42.27% DFS **(Table 3.28).** Compared to HPV-: S+RT, HPV+: S+RT have significantly lower hazard of death of disease (DSS: HR, 0.39; 95%CI, 0.16-0.95; p=0.038) but not significant hazard of death from any cause (OS: HR, 0.50; 95%CI, 0.23-1.07; p=0.074) and hazard of disease recurrence (DFS: HR, 0.40; 95%CI, 0.14-1.14; p=0.087) **(Table 3.28).**

There were statistically significant differences between HPV+: S+CRT and HPV-: S+CRT for 5-year DSS (78.9% vs 50.9%, p=0.042) and DFS (73.6% vs 33.8%, p=0.0033) but not significant for OS (75.3% vs 40.6%, p=0.056) **(Table 3.27, Appx G Figure 7**), with post hoc power calculations of 53.48% for DSS, 83.86% DFS and 48.70% for OS **(Table 3.28).** Compared to HPV-: S+CRT, HPV+: S+CRT have significantly lower hazard of death of disease (DSS: HR, 0.38; 95%CI, 0.15-1.00; p=0.050) and hazard of disease recurrence (DFS: HR, 0.27; 95%CI, 0.11-0.69; p=0.006) but not significant hazard of death from any cause (OS: HR, 0.43; 95%CI, 0.18-1.05; p=0.063) **(Table 3.28).**

There were no statistically significant differences between HPV+: CRT and HPV-: CRT for 5-year OS (76.6% vs 81.0%, p=0.73), DSS (76.6% vs 81.0%, p=0.73) and DFS (61.7% vs 75.6%, p=0.41) **(Table 3.27, Appx G Figure 8**), with post hoc power calculations of 6.15% for OS and DSS and 13.36% for DFS. Compared to HPV-: CRT, HPV+: CRT have higher but not statistically significant hazard of death from any cause (OS: HR, 1.30; 95%CI, 0.29-5.86; p=0.7), hazard of death of disease (DSS: HR, 1.30; 95%CI, 0.29-5.86; p=0.7) and hazard of disease recurrence (DFS: HR, 1.81; 95%CI, 0.43-7.64; p=0.4) **(Table 3.28)**

There were no statistically significant differences between HPV+: RT and HPV-: RT for 5-year for 5-year OS (75.0% vs 33.3%, p=0.19), DSS (75.0% vs 50.0%, p=0.45) and DFS (100% vs 100%, p=1) **(Table 3.27, Appx G Figure 9),** with post hoc power

calculations of 25.66% for OS, 12.10% for DSS and could not be calculated for DFS. Compared to HPV-: RT, HPV+: RT have lower but not statistically significant hazard of death from any cause (OS: HR, 0.22; 95%CI, 0.02-2.57; p=0.2) and hazard of death of disease (DSS: HR, 0.35; 95%CI, 0.02-5.89; p=0.5). Hazard of disease recurrence could not be calculated because HPV-: RT, HPV+: RT have equal proportion surviving for DFS **(Table 3.28).**

There were no statistically significant differences between HPV+: S and HPV-: S for 5-year OS (66.1% vs 43.6%, p=0.2), DSS (68.8% vs 47.9%, p=0.33) and DFS (59.0% vs 59.0%, p=0.79) **(Table 3.27, Appx G Figure 10)** with post hoc power calculations of 24.33% 16.91% and 6.18%, respectively. Compared to HPV-: S, HPV+: S have lower but not statistically significant hazard of death from any cause (OS: HR, 0.60; 95%CI, 0.27-1.32; p=0.2) and hazard of death of disease (DSS: HR, 0.65; 95%CI, 0.27-1.55; p=0.3) and higher but not statistically significant hazard of disease recurrence (DFS: HR, 1.16; 95%CI, 0.38-3.58; p=0.8) **(Table 3.28)**

There were no statistically significant differences between p16+/HPV+: S+RT and p16+/HPV- S+RT for 5-year OS (78.2% vs 75.1%, p=0.36), DSS (85.1% vs 75.1%, p=0.1) and DFS (87.0% vs 87.4%, p=0.48) **(Table 3.28, Appx G Figure 11**), with post hoc power calculations of 9.67%, 37.58% and 10.96%, respectively. Compared with p16+/HPV+: S+RT, p16+/HPV-: S+RT have higher but not statistically significant hazard of death from any cause (OS: HR, 1.56; 95%CI, 0.59-4.10; p=0.4), hazard of death of disease (DSS: HR, 2.37; 95%CI, 0.82-6.83; p=0.11) and hazard of disease recurrence (DFS: HR, 1.65; 95%CI, 0.41-6.60; p=0.5) **(Table 3.28).**

There was a statistically significant difference between p16+/HPV+: S+RT and p16-/HPV+: S+RT for 5-year DSS (85.1% vs 33.3%, p=0.019) but no significant differences for OS (78.2% vs 33.3%, p=0.085) and DFS (87.0% vs 50.0%, p=0.16) **(Table 3.27, Appx G Figure 11**), with post hoc power calculations of 65.01% for DSS, 40.93% for OS and 29.25% DFS **(Table 3.28).** Compared to p16+/HPV+: S+RT, p16-/HPV+: S+RT have significantly higher hazard of death of disease (DSS: HR, 5.97; 95%CI, 1.24-28.8; p=0.026) but not significant hazard of death from any cause (OS: HR, 3.80; 95%CI, 0.84-17.1; p=0.082) and hazard of disease recurrence (DFS: HR, 4.38; 95%CI, 0.52-37.3; p=0.2) **(Table 3.28).**

There were statistically significant differences between p16+/HPV+: S+RT and p16-/HPV-: S+RT for 5-year OS (78.2% vs 20.6%, p=0.0065), DSS (85.1% vs 40.0%, p=0.0065) and DFS (87.0% vs 42.9%, p=0.0028) **(Table 3.27, Appx G, Figure 11**), with post hoc power calculations of 77.69%, 77.64% and 84.68%, respectively **(Table 3.28).** Compared to p16+/HPV+: S+RT, p16-/HPV-: S+RT have significantly higher hazard of death from any cause (OS: HR, 3.95; 95%CI, 1.48-10.6; p=0.006), hazard of death of disease (DSS: HR, 5.63; 95%CI, 1.65-19.2; p=0.006) and hazard of disease recurrence (DFS: HR, 6.11; 95%CI, 1.68-22.3; p=0.006) **(Table 3.28).**

There were no statistically significant differences between p16+/HPV+: S+CRT and p16+/HPV-: S+CRT for 5-year OS (75.3% vs 51.3%, p=0.59), DSS (78.9% vs 75.0%, p=0.67) and DFS (73.6% vs 41.0%, p=0.11) **(Table 3.27, Appx G Figure 12**) with power calculations 8.50%, 7.34% and 36.38%, respectively. Compared to p16+/HPV+: S+CRT, p16+/HPV-: S+CRT have higher but not statistically significant hazard of death from any cause (OS: HR, 1.36; 95%CI, 0.43-4.29; p=0.6), hazard of death of disease (DSS: HR,

1.33; 95%CI, 0.36-4.91; p=0.7) and hazard of disease recurrence (DFS: HR, 2.40; 95%CI, 0.78-7.34; p=0.13) **(Table 3.28).** There were no patients with p16-/HPV+: S+CRT.

There were statistically significant differences between p16+/HPV+: S+CRT and p16-/HPV-: S+CRT for 5-year OS (75.3% vs 16.7%, p=0.0014), DSS (78.9% vs 16.7%, p=0.00044) and DFS (73.6% vs 16.7%, p<0.0001) **(Table 3.27, Appx G Figure 12**), with post hoc power calculations of 89.44%, 94.06% and 98.11%, respectively **(Table 3.28).** Compared to p16+/HPV+: S+CRT, p16-/HPV-: S+CRT have significantly higher hazard of death from any cause (OS: HR, 5.25; 95%CI, 1.81-15.2; p=0.002), hazard of death of disease (DSS: HR, 6.17; 95%CI, 2.05-18.6; p=0.001) and hazard of disease recurrence (DFS: HR, 8.11; 95%CI, 2.63-25.0; p<0.001) **(Table 3.28).**

There were no statistically significant differences between p16+/HPV+: CRT and p16+/HPV-: CRT for 5-year OS (76.6% vs 79.5%, p=0.8), DSS (76.6% vs 79.5%, p=0.8) and DFS (61.7% vs 73.2%, p=0.49) **(Table 3.27, Appx G Figure 13**) with post hoc power calculations of 6.17%, for OS and DSS and 10.95% for DFS. Compared to p16+/HPV+: CRT, p16+/HPV-: CRT have lower but not statistically significant hazard of death from any cause (OS: HR, 0.82; 95%CI, 0.18-3.69; p=0.8), hazard of death of disease (DSS: HR, 0.82; 95%CI, 0.18-3.69; p=0.8) and hazard of disease recurrence (DFS: HR, 0.61; 95%CI, 0.14-2.55; p=0.5) **(Table 3.28).** There were no patients with p16-/HPV+: CRT.

There were no statistically significant differences between p16+/HPV+: CRT and p16-/HPV-: CRT for 5-year OS (76.6% vs 100%, p=0.61), DSS (76.6% vs 100%, p=0.61) and DFS (61.7% vs 100%, p=0.5) **(Table 3.27, Appx G Figure 13**) with post hoc power calculations of 8.53%, for OS and DSS and 10.86% for DFS. Hazard risks was calculated at 0 (95%CI: 0-inf) between p16+/HPV+: CRT and p16-/HPV-: CRT as there were no

events in the latter group (ie. 100% proportion surviving for OS, DSS and DFS) **(Table 3.28).**

There were no statistically significant differences between p16+/HPV+: RT and p16+/HPV-: RT for 5-year OS (75.0% vs 100%, p=0.52) and DSS (75.0% vs 100%, p=0.62) but not significant DFS (100% vs 100%, p=1) **(Table 3.27, Appx G Figure 14**) with power calculations of 7.32% for OS and DSS and could not be calculated for DFS. Hazard of death from any cause and hazard of death of disease were calculated at 0 (95%CI: 0-inf) since p16+/HPV-: RT have 100% proportion surviving for OS and DSS analysis. Hazard of disease recurrence could not be calculated since p16+/HPV+: RT and p16+/HPV-: RT have equal proportion surviving for DFS **(Table 3.28).** There were no patients with p16-/HPV+: RT.

There were statistically significant differences between p16+/HPV+: RT and p16-/HPV-: RT for 5-year OS (75.0% vs 0, p=0.018) and DSS (75.0% vs 0, p=0.046) but not significant DFS (100% vs 100%, p=1) **(Table 3.27, Appx G Figure 14**), with post hoc power calculations of 65.86% for OS, 51.56% for DSS and could not be calculated for DFS **(Table 3.28).** Hazard risks for OS and DSS were calculated very high at 12.7 e09 (95%CI: 0-inf) and 53.1 e09 (95%CI: 0-inf), respectively since there were 0% proportion surviving (OS and DSS) for p16-/HPV-: RT. Hazard of disease recurrence could not be calculated because p16+/HPV+: RT and p16-/HPV-: RT have equal proportion surviving for DFS **(Table 3.28).**

There were no statistically significant differences between p16+/HPV+: S and p16+/HPV-: S for 5-year OS (67.7% vs 85.7%, p=0.31), DSS (70.6% vs 85.7%, p=0.39) and DFS (58.3% vs 83.3%, p=0.4) **(Table 3.27, Appx G Figure 15**) with post hoc power

calculations of 17.00%, 14.63% and 13.26%, respectively **(Table 3.28).** Compared to p16+/HPV+: S, p16+/HPV-: S have lower but not statistically significant hazard of death from any cause (OS: HR, 0.37; 95%CI, 0.05-2.80; p=0.3), hazard of death of disease (DSS: HR, 0.42; 95%CI, 0.05-3.19; p=0.4) and hazard of disease recurrence (DFS: HR, 0.43; 95%CI, 0.06-3.28; p=0.4) **(Table 3.28).**

There were no statistically significant differences between p16+/HPV+: S and p16-/HPV+: S for 5-year OS (67.7% vs 0, p=0.68), DSS (70.6% vs 0, p=0.58) and DFS (58.3% vs 100%, p=0.5) **(Table 3.27, Appx G Figure 15**) with post hoc power calculations of 7.34% 8.55% and 10.83%, respectively **(Table 3.28).** Compared to p16+/HPV+: S, p16+/HPV-: S have higher but not statistically significant hazard of death from any cause (OS: HR, 1.56; 95%CI, 0.20-11.9; p=0.7) and hazard of death of disease (DSS: HR, 1.78; 95%CI, 0.23-13.7; p=0.6). Hazard of disease recurrence was calculated at 0 (95%CI: 0-inf) since p16-/HPV+: S has 100% proportion surviving for DFS analysis. **(Table 3.28).**

There were statistically significant differences between p16+/HPV+: S and p16-/HPV-: S for 5-year OS (67.7% vs 20.8%, p=0.011) and DSS (70.6% vs 24.3%, p=0.034) but not significant DFS (58.3% vs 36.5%, p=0.77) **(Table 3.27, Appx G Figure 15**), with post hoc power calculations of 72.18% for OS, 56.36% for DSS and 6.13% for DFS **(Table 3.28).** Compared to p16+/HPV+: S, p16-/HPV-: S have significantly higher hazard of death from any cause (OS: HR, 2.86; 95%CI, 1.25-6.55; p=0.013) and hazard of death of disease (DSS: HR, 2.66; 95%CI, 1.06-6.67; p=0.038) but not significant hazard of disease recurrence (DFS: HR, 1.20; 95%CI, 0.34-4.23; p=0.5) **(Table 3.28).**

To summarize the treatment type results analyses p16+ compared to p16-, p16+: S+RT and p16+: S+CRT had significantly higher OS, DSS and DFS, p16+: RT and p16+:S

had significantly higher OS and DSS and p16+: CRT had lower but not statistically significant OS DSS and DFS. For HPV analyses HPV+ compared to HPV-, HPV+: S+RT had significantly higher DSS, HPV+: S+CRT had significantly higher DSS and DFS but not significant OS, HPV+: CRT had lower but not statistically significant OS, DSS and DFS, HPV+: RT and S had higher but not statistically significant OS and DSS. When p16 and HPV status were combined, compared to p16+/HPV+, p16-/HPV- had significantly lower OS, DSS and DFS for S+RT and S+CRT and significantly lower OS and DSS for RT and S. For discordant p16+/HPV- compared to p16+/HPV+, p16+/HPV-: S+RT and S+CRT had lower but not statistically significant OS DSS and DFS and p16+/HPV-: CRT and S had higher but not statistically significant OS, DSS and DFS. For discordant p16-/HPV+ compared to p16+/HPV+, p16-/HPV+: S+RT had significantly lower DSS but not significant OS and DFS and p16-/HPV+: S had higher but not statistically significant OS and DSS.

Table 3. 27: Summary of Proportion Surviving (OS, DSS and DFS) for p16, HPV and p16/HPV stratified by Treatment Type

Covariate	Overall Survival		Disease-Specific Survival		Disease-Free Survival	
	Proportion Surviving (%)	**p-value**	**Proportion Surviving (%)**	**p-value**	**Proportion Surviving (%)**	**p-value**
p16 and Treatment Type (vs p16-)						
p16-: S+ RT	26.0	**0.0022**	37.4	**0.0016**	44.4	**0.0021**
p16+: S+ RT	77.5		82.6		87.0	
p16-: S+CRT	16.7 (4.8y)	**0.0008**	16.7 (4.8y)	**0.00022**	16.7 (4.8y)	**<0.0001**
p16+: S+CRT	72.0		78.0		66.8	
p16-: CRT	100 (4.1y)	0.62	100 (4.1y)	0.62	100 (4.1y)	0.53
p16+: CRT	77.5		77.5		66.5	
p16-: RT	0 (0.6y)	**0.0082**	0 (0.6y)	**0.025**	100 (0.6y)	1
p16+: RT	80.0		80.0		100	
p16-: S	19.7	**0.0054**	22.5	**0.017**	39.4	0.88
p16+: S	70.2		72.8		61.7	

HPV and Treatment Type (vs HPV-)						
HPV-: S+ RT	56.1	0.068	65.0	**0.031**	71.5	0.076
HPV+: S+ RT	70.9		81.8		85.2	
HPV-: S+CRT	40.6 (4.8y)	0.056	50.9 (4.8y)	**0.042**	33.8 (4.8y)	**0.0033**
HPV+: S+CRT	75.3		78.9		73.6	
HPV-: CRT	81.0	0.73	81.0	0.73	75.6	0.41
HPV+: CRT	76.6		76.6		61.7	
HPV-: RT	33.3 (1.9y)	0.19	50.0 (1.9y)	0.45	100 (1.9y)	1
HPV+: RT	75.0		75.0		100	
HPV-: S	43.6	0.2	47.9	0.33	59.0	0.79
HPV+: S	66.1		68.8		59.0	
p16/HPV and Treatment Type (vs p16+/HPV+: S+RT)						
p16+/HPV+: S+ RT	78.2		85.1		87.0	
p16+/HPV-: S+ RT	75.1	0.36	75.1	0.1	87.4	0.48
p16-/HPV+: S+ RT	33.3	0.085	33.3	**0.019**	50.0	0.16
p16-/HPV-: S+ RT	20.6	**0.0065**	40.0	**0.0065**	42.9	**0.0028**
p16/HPV and Treatment Type (vs p16+/HPV+: S+CRT)						
p16+/HPV+: S+CRT	75.3		78.9		73.6	
p16+/HPV-: S+CRT	51.3 (4.4y)	0.59	75.0 (4.4y)	0.67	41.0 (4.4y)	0.11
p16-/HPV+: S+CRT	N/A	N/A	N/A	N/A	N/A	N/A
p16-/HPV-: S+CRT	16.7 (4.8y)	**0.0014**	16.7 (4.8y)	**0.00044**	16.7 (4.8y)	**<0.0001**
p16/HPV and Treatment Type (vs p16+/HPV+: CRT)						
p16+/HPV+: CRT	76.6		76.6		61.7	
p16+/HPV-: CRT	79.5	0.8	79.5	0.8	73.2	0.49
p16-/HPV+: CRT	N/A	N/A	N/A	N/A	N/A	N/A
p16-/HPV-: CRT	100 (4.1y)	0.61	100 (4.1y)	0.61	100 (4.1y)	0.5
p16/HPV and Treatment Type (vs p16+/HPV+: RT)						
p16+/HPV+: RT	75.0		75.0		100	
p16+/HPV-: RT	100 (1.9y)	0.52	100 (1.9y)	0.62	100 (1.9y)	1
p16-/HPV+: RT	N/A	N/A	N/A	N/A	N/A	N/A
p16-/HPV-: RT	0 (0.6y)	**0.018**	0 (0.6y)	**0.046**	100 (0.6y)	1
p16/HPV and Treatment Type (vs p16+/HPV+: S)						
p16+/HPV+: S	67.7		70.6		58.3	
p16+/HPV-: S	85.7	0.31	85.7	0.39	83.3	0.4
p16-/HPV+: S	0 (3y)	0.68	0 (3y)	0.58	100 (3y)	0.5
p16-/HPV-: S	20.8	**0.011**	24.3	**0.034**	36.5	0.77

Table 3. 28: Multivariate Cox Proportional Hazard Regression analysis and Post-hoc Power Calculations for p16, HPV and p16/HPV stratified by Treatment Type

Log-rank Test Comparing Survival Rates based on Treatment Type and p16, HPV and p16/HPV	Hazard Ratio (95% CI)	p-value	Sample Size N (reference + test)	Power (%)	Estimated Sample Size for 80% Power
p16- Reference					
OS: p16+: S+RT	0.29 (0.12-0.67)	**0.004**	93 (14+79)	**86.58**	
DSS: p16+: S+RT	0.23 (0.09-0.62)	**0.004**	86 (12+74)	**88.54**	
DFS: p16+: S+RT	0.20 (0.07-0.63)	**0.005**	93 (14+79)	**86.99**	

OS: p16+: S+CRT	0.21 (0.07-0.57)	**0.002**	69 (6+63)	**91.74**	
DSS: p16+: S+CRT	0.17 (0.06-0.49)	**0.001**	66 (6+60)	**95.80**	
DFS: p16+: S+CRT	0.16 (0.06-0.45)	**<0.001**	69 (6+63)	**97.69**	
OS: p16+: CRT	2.6e07 (0-inf)	>0.9	45 (1+44)	7.56	1602
DSS: p16+: CRT	2.6e07 (0-inf)	>0.9	45 (1+44)	7.56	1602
DFS: p16+: CRT	7.4e07 (0-inf)	>0.9	45 (1+44)	9.67	889
OS: p16+: RT	0 (0-inf)	>0.9	7 (2+5)	75.36	8
DSS: p16+: RT	0 (0-inf)	>0.9	6 (1+5)	60.89	10
DFS: p16+: RT	--	---	7 (2+5)	---	---
OS: p16+: S	0.34 (0.15-0.76)	**0.008**	79 (16+63)	79.19	81
DSS: p16+: S	0.36 (0.15-0.87)	**0.023**	75 (14+61)	65.70	106
DFS: p16+: S	0.90 (0.26-3.18)	0.9	79 (16+63)	---	---
HPV- Reference					
OS: HPV+: S+RT	0.50 (0.23-1.07)	0.074	93 (30+63)	44.17	223
DSS: HPV+: S+RT	0.39 (0.16-0.95)	**0.038**	86 (28+58)	57.23	148
DFS: HPV+: S+RT	0.40 (0.14-1.14)	0.087	93 (30+63)	42.27	235
OS: HPV+: S+CRT	0.43 (0.18-1.05)	0.063	69 (19+50)	48.70	146
DSS: HPV+: S+CRT	0.38 (0.15-1.00)	**0.050**	66 (18+48)	53.48	124
DFS: HPV+: S+CRT	0.27 (0.11-0.69)	**0.006**	69 (19+50)	**83.86**	
OS: HPV+: CRT	1.30 (0.29-5.86)	0.7	45 (21+24)	6.15	3554
DSS: HPV+: CRT	1.30 (0.29-5.86)	0.7	45 (21+24)	6.15	3554
DFS: HPV+: CRT	1.81 (0.43-7.64)	0.4	45 (21+24)	13.36	503
OS: HPV+: RT	0.22 (0.02-2.57)	0.2	7 (3+4)	25.66	33
DSS: HPV+: RT	0.35 (0.02-5.89)	0.5	6 (2+4)	12.10	79
DFS: HPV+: RT	---	---	7 (3+4)	---	---
OS: HPV+: S	0.60 (0.27-1.32)	0.2	79 (23+56)	24.33	390
DSS: HPV+: S	0.65 (0.27-1.55)	0.3	75 (21+54)	16.91	594
DFS: HPV+: S	1.16 (0.38-3.58)	0.8	79 (23+56)	6.18	6057
p16+/HPV+: S+RT Reference					
OS: p16+/HPV-: S+RT	1.56 (0.59-4.10)	0.4	79 (60+19)	9.67	1558
DSS: p16+/HPV-: S+RT	2.37 (0.82-6.83)	0.11	74 (55+19)	37.58	216
DFS: p16+/HPV-: S+RT	1.65 (0.41-6.60)	0.5	79 (60+19)	10.96	1227
OS: p16-/HPV+: S+RT	3.80 (0.84-17.1)	0.082	63 (60+3)	40.93	166
DSS: p16-/HPV+: S+RT	5.97 (1.24-28.8)	**0.026**	58 (55+3)	65.01	83
DFS: p16-/HPV+: S+RT	4.38 (0.52-37.3)	0.2	63 (60+3)	29.25	248
OS: p16-/HPV-: S+RT	3.95 (1.48-10.6)	**0.006**	71 (60+11)	77.69	76
DSS: p16-/HPV-: S+RT	5.63 (1.65-19.2)	**0.006**	64 (55+9)	77.64	68
DFS: p16-/HPV-: S+RT	6.11 (1.68-22.3)	**0.006**	71 (60+11)	**84.68**	
p16+/HPV+: S+CRT Reference					
OS: p16+/HPV-: S+CRT	1.36 (0.43-4.29)	0.6	63 (50+13)	8.50	1649
DSS: p16+/HPV-: S+CRT	1.33 (0.36-4.91)	0.7	60 (48+12)	7.34	2334
DFS: p16+/HPV-: S+CRT	2.40 (0.78-7.34)	0.13	63 (50+13)	36.38	191
OS: p16-/HPV+: S+CRT	N/A				
DSS: p16-/HPV+: S+CRT	N/A				
DFS: p16-/HPV+: S+CRT	N/A				
OS: p16-/HPV-: S+CRT	5.25 (1.81-15.2)	**0.002**	56 (50+6)	**89.44**	
DSS: p16-/HPV-: S+CRT	6.17 (2.05-18.6)	**0.001**	54 (48+6)	**94.06**	
DFS: p16-/HPV-: S+CRT	8.11 (2.63-25.0)	**<0.001**	56 (50+6)	**98.11**	
p16+/HPV+: CRT Reference					
OS: p16+/HPV-: CRT	0.82 (0.18-3.69)	0.8	44 (24+20)	6.17	3407
DSS: p16+/HPV-: CRT	0.82 (0.18-3.69)	0.8	44 (24+20)	6.17	3407
DFS: p16+/HPV-: CRT	0.61 (0.14-2.55)	0.5	44 (24+20)	10.95	686
OS: p16-/HPV+: CRT	N/A				

DSS: p16-/HPV+: CRT	N/A				
DFS: p16-/HPV+: CRT	N/A				
OS: p16-/HPV-: CRT	0 (0-inf)	>0.9	25 (24+1)	8.53	649
DSS: p16-/HPV-: CRT	0 (0-inf)	>0.9	25 (24+1)	8.53	649
DFS: p16-/HPV-: CRT	0 (0-inf)	>0.9	25 (24+1)	10.86	395
p16+/HPV+: RT Reference					
OS: p16+/HPV-: RT	0 (0-inf)	>0.9	5 (4+1)	7.32	197
DSS: p16+/HPV-: RT	0 (0-inf)	>0.9	5 (4+1)	7.32	197
DFS: p16+/HPV-: RT	---	---	---	---	---
OS: p16-/HPV+: RT	N/A				
DSS: p16-/HPV+: RT	N/A				
DFS: p16-/HPV+: RT	N/A				
OS: p16-/HPV-: RT	12.7 e09 (0-inf)	>0.9	6 (4+2)	65.86	9
DSS: p16-/HPV-: RT	53.1 e09 (0-inf)	>0.9	5 (4+1)	51.56	10
DFS: p16-/HPV-: RT	---	---	---	---	---
p16+/HPV+: S Reference					
OS: p16+/HPV-: S	0.37 (0.05-2.80)	0.3	63 (54+9)	17.00	495
DSS: p16+/HPV-: S	0.42 (0.05-3.19)	0.4	61 (52+9)	14.63	594
DFS: p16+/HPV-: S	0.43 (0.06-3.28)	0.4	63 (54+9)	13.26	712
OS: p16-/HPV+: S	1.56 (0.20-11.9)	0.7	56 (54+2)	7.34	2181
DSS: p16-/HPV+: S	1.78 (0.23-13.7)	0.6	54 (52+2)	8.55	1396
DFS: p16-/HPV+: S	0 (0-inf)	>0.9	56 (54+2)	10.83	889
OS: p16-/HPV-: S	2.86 (1.25-6.55)	**0.013**	68 (54+14)	72.18	83
DSS: p16-/HPV-: S	2.66 (1.06-6.67)	**0.038**	64 (52+12)	56.36	112
DFS: p16-/HPV- : S	1.20 (0.34-4.23)	0.8	68 (54+14)	6.13	5436

Chapter 4: Discussion

4.1 Accuracy of HPV-ddPCR

HPV-related head and neck cancers are rapidly growing with more attention towards OPSCC tumors as the virus status is incorporated in the current staging system which determines the suitable treatment for these cancers and predicts the treatment outcomes (26,138). Therefore, determining the HPV status in OPSCC is the current standard of care (138). There is a lack of consensus on what the most accurate HPV testing tool is in clinical settings; however, utilizing IHC techniques to demonstrate the protein expression of p16 in tumor specimens has gained popularity and become the preferred clinical test for various reasons. Although it is widely used due to its availability for testing HPV status, HPV-p16 IHC does not provide information about the virus genotype which can be specifically important moving forward in virus-targeting treatment regimens for cancers (138). Moreover, p16 IHC techniques require a considerable amount of tissue biopsy/tumor surgical specimen to show the protein marker expression and it is not necessarily as cost-effective as previously thought (25,26) and can lack sensitivity with up to 25% (133) to 36% false positive rates (139). In a previous study from our center, we showed the actual cost of performing p16-IHC which can be on average of ≥ $62.10/patient (26). To our knowledge, the screening tool presented in this study is the most sensitive mean of diagnosing the high-risk oncogenic HPV types to date.

The gold standard for determining HPV positivity in OPSCC is the identification of E6 and E7 of HPV in fresh tissue using RT-qPCR (140). Besides the high associated cost and specialized required techniques, the other limitations of utilizing RT-qPCR include the

need for an adequate nucleic acid sample which can be only attainable from a tissue biopsy. Real- time quantitative PCR (RT-qPCR) has been used nowadays to diagnose HPV-related head and neck cancers more than the novel new insight ddPCR. Although RT-qPCR requires 20 to 50 times more samples to process in comparison to the low magnitude of sample needed to perform ddPCR (1 ng of RNA/reaction) (26). Taylor et al (118) compared ddPCR against qPCR and ddPCR provided more precise results. ddPCR is a highly sensitive method to test for HPV mRNA in the diagnosis of HPV-related oropharyngeal cancers. This novel technique does not require tissue sampling or biopsy. It is a non-invasive technique utilizing a very small amount of RNA for amplification through oropharyngeal swabs/ salivary sampling from mainly the tonsils and base of tongue. ddPCR has also been proven to be cost-effective in comparison to HPV-IHC method with an estimated total cost of $20.45 per patient sample including technical labour (26).

Smith et al (141) reviewed the current biomarkers and their robustness for the detection of oncogenic HPV in OPSCC saliva samples and showed that while the HPV DNA detection method is more accurate than the RNA method, they both require time-consuming procedures. A meta-analysis of the diagnostic accuracy of oral HPV detection in 2017 among people diagnosed with HNSCC showed a sensitivity of 72% (95%CI: 45-89%) and specificity of 92% (95%CI: 82- 97%) for HPV + HNSCC tumor (142). When research was limited to cases of oropharyngeal cancer with oral rinse samples or testing for HPV16 DNA testing (rather than any other oncogenic HPV) in oral swabs, the findings were similar (142). Other studies in the literature have shown various sensitivity results regarding the detection of high-risk HPV strains in oropharyngeal cancer using rinses/gargle and/or brush biopsy/ oropharyngeal swabs. **Table 4.1** provides a summary of

the outlined studies. Zhao et al (143) demonstrated a high specificity rate (97-100%) utilizing real-time quantity PCR (RT-qPCR) methods in identifying HPV-16 positive OPSCC patients from oral rinses with a variable sensitivity rate between 14 and 33%. Agrawal et al (144) had shown similar sensitivity and specificity (30% and 97% respectively) rates using multiplex RT-PCR detecting 37 HPV types in oral rinses. Rettig et al (145) and Wang et al (146) showed similar sensitivity and specificity (43% and 100% vs 40% and 100% respectively) rates using RT-PCR in detecting multiple HPV types in oral rinse (Rettig) and HPV 16 in saliva (Wang). Ahn et al (147) showed slightly higher sensitivity (52.8%) and similar specificity (100%) using RT-qPCR detecting HPV 16 in saliva. Al-Soneider et al (148) and Tachezy et al (149) demonstrated higher sensitivity but lower specificity (62% and 89% vs 62.3% and 88-96%) rates using PCR in detecting multiple HPV genera in oral rinses and brush (Al-Soneider) and PCR in detecting multiple types (Tachezy). Broglie et al (150) and Fakhry et al (151) had comparable sensitivity and specificity (83% and 94% vs 80.6% and 100%) rates using DNA PCR in brush cytology and tissue (Broglie) and multiplex PCR in oral rinse (Fakhry). Castillo et al (152) demonstrated high sensitivity (88%) and specificity (100%) rates in detecting HPV in brush cytology by Cobas HPV test, a commercially approved method for HPV detection in cervical samples. Isaac et al (25) and Biron et al (26) have shown similar high sensitivity and specificity (92% and 98% vs 91.3% and 100%) rates in detecting HPV-16 strain both by ddPCR utilizing oropharyngeal swabs (Isaac) and swabs, tissue and FNA (Biron). Chai et al (153) demonstrated high sensitivity (92.9%) and specificity (100%) rates in detecting HPV 16 by qPCR in oral rinse. Snietura et al (154) and Saito et al (155) demonstrated the highest sensitivity and specificity (100% and 96.2% vs 96% and 100%) in detecting

multiple HPV types in oral brushes using RT-PCR (Snietura) and salivary rinses using genomic sequencing for qPCR.

For the first outcome measure, our results demonstrated a high sensitivity of 95.8% and specificity of 97.1% in detecting high-risk oncogenic HPV by oropharyngeal salivary swabs without invasive measures compared to p16-IHC. This study yielded an excellent accuracy result of 96.2% overall in diagnosing hr-HPV oropharyngeal cancers. Snietura et al. (154) had higher sensitivity (100%), Saito et al (155), Chai et al (153) and Castillo et al (152) have higher specificity (all 100%) compared to our study, but they all have small OPSCC cohort (90, 72, 39 and 75 respectively) and Chai and Saito only detected HPV16. Mattox et al. (102) had lower sensitivity (75%) using oral rinse from 66 OPSCC and Hanna et al. (107) had lower sensitivity (87%) and specificity (67%) using saliva from 21 OPSCC, compared to our swab study. Several reasons could explain the different sensitivities despite using ddPCR for HPV detection in the studies. First, the sample differences used: oral rinse, saliva, and swabs. De Souza et al. (156) compared oral saline rinse and saliva for HPV detection, from 96 individuals and they found that the overall agreement was fair or poor between samples, but the saliva collection kit had the highest repeatability. Donà et al. (157) demonstrated that there is little agreement regarding HPV status between rinses and brushings, with oral rinses having a higher detection rate. Their findings may be partially explained by the lower concentration of DNA/RNA in brushings than in rinses, which makes highly sensitive detection methods helpful. Cells can be collected from specific sites by brushing/swabbing directly onto the tumor site, whereas the source of HPV-infected cells in the oral rinse may not be known, so swab sampling may be useful for screening and/or monitoring individuals at risk of OPSCC to evaluate suspicious lesions or confirm

HPV status (150,157). Second, different extraction methods could also influence the findings which could affect the DNA quality, sensitivity and number of HPV types detected (156). Mattox and Hanna both used QIAamp Circulating Nucleic Acid Kit – used for plasma and serum (Qiagen) and we used RNeasy Plus Mini Kit (Qiagen) for extraction. Third, different PCR amplification processes may also affect the accuracy of the test. Mattox used SYBR dye which binds to any double-stranded DNA and may generate false positive signals while Hanna used probe assay, like our method, which is highly specific since probes detect the specific amplification products. Method of collecting oral samples, DNA extraction and amplification processes should be considered for comparison studies.

Our bigger sample size of 300 OPSCC gave us an advantage over the above studies improving the accuracy of our results. Like Hanna, Snietura and Castillo's study, we also detected multiple hr-HPV which is beneficial since studies have shown that non-HPV16 have lower survival rates compared to HPV16 (158,159), however, we also detected the biomarker expression of the CDKN2A gene. An extensive investigation has revealed that various tumors contain high levels of CDKN2A and their expression correlates positively with prognosis and immune cell infiltration (160). In HPV- HNSCC, low copy number of CDKN2A is associated with poor survival independent of other patient and treatment factors and may be a helpful prognostic indicator in clinical settings (161). The CDKN2A gene in melanoma was quantified using ddPCR and it was found that ddPCR was a reliable technique for CNV detection with 94.4% sensitivity and 90% specificity compared to chromosomal microarray analysis (162). CDKN2A as a biomarker can be used to evaluate the prognosis of various cancers and may also be a useful therapeutic target for the treatment of tumors (160) and ddPCR can be employed as a method of detection. Our

technique of detecting multiple hr-HPV and CDKN2A gene simultaneously, using non-invasive swabs is highly accurate and can be useful for diagnosis and post-treatment surveillance. However, the sensitivity and specificity need to be interpreted with caution because they were calculated based on the results from p16 IHC, which is itself known as a surrogate marker; however, we wanted to test a swab-based assay against the clinical standard tissue-based test for HPV status determination (25).

Table 4. 1: Summary of studies using non-invasive samples to detect high-risk HPV in OPSCC

Study Name, Year	Location	Participants (N)	Sample Type	Detection Technique	HPV type detected	HPV type (frequency)	Sensitivity (%)	Specificity (%)
Current Study	Canada	456 (300 OPSCC, 77 non-OPSCC, 79 non-cancers	Opx swabs	ddPCR	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59	16 (85.1%) 33 (6.0%) 18, 35, 45 (3.0% each)	95.8	97.1
Mattox, 2022 (102)	USA	66 OPSCC (HPV +)	Oral rinse	NGS, ddPCR, RT-qPCR	16	-	NGS=75, ddPCR=8.3, RT-qPCR=2.1	-
Hanna, 2019 (107)	USA	21 OPSCC (HPV+)	Saliva, plasma	ddPCR	16, 18, 31, 45		87	67
Snietura, 2015 (154)	Poland	90 HNSCC (53 OPSCC)	Opx brush	RT-PCR	16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	16 (96%)	100	96.2
Saito, 2020 (155)	USA	72 OPSCC (28 HPV+, 15 controls)	Salivary rinses	Genomic HPV-HNSCC sequencing data from a single institutional and a TCGA cohort for qPCR	16	16 (82%)	96	100
Chai, 2016 (153)	Australia	82 HNSCC, (39 OPSCC)	Salivary oral rinse	Endpoint qPCR	16		92.9	100
Isaac, 2017 (25)	Canada	122: 36 p16+ OPSCC, 16 p16− OPSCC, 4 patients p16+	Opx swabs	ddPCR	16	16 (91.6%)	92	98

		OCSCC, 41 p16− OCSCC, and 25 healthy controls						
Biron, 2016 ((26)	Canada	68: 29 OPSCC, 29 non-OPSCC, 10 non-carcinomas	Oral and Opx swabs and tissues	ddPCR	16	16 (72.4%)	91.3	100
Castillo, 2021 (152)	Spain	75 oral OPSCC; 60 brush cytology	Brush cytology	Cobas HPV test	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 68	16 (32%)	88	100
Broglie, 2015 (150)	Switzerland	51 OPSCC (41 HPV test, 22 HPV+)	Brush cytology, tissue	HPV DNA PCR	16, 33	16 (86.4%), 33 (13.6%)	83	94
Kofler, 2017 (163)	Austria	93 HNSCC	Tumor brush	RT-PCR, Reverse line-blot hybridization	40 HPV types	16 (39.8%), 35 (4.3%), 58 (2.2%)	83-86 (78 vs p16)	85-89 (79 vs p16)
Martin-Gomez, 2019 (138)	USA	204 OPSCC, (171 oral gargle) (Men only) (168 HPV+)	Oral gargle	RHA Kit HPV SP10-LiPA25	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 68	16 (63.1%), 18 (3.9%)	82.2	
Nordfors, 2014 (164)	Sweden	76; 29 tonsillar cancer, 18 bot, 19 other HN cancer, 10 benign; 37 healthy controls	Oral rinses and Opx swabs	qPCR for L1 DNA for multiple HPV types and HPV16 E6 DNA	16, 18, 31, 33, 45, 56	16 (66%), 33 (8.5%), 18, 31, 35 (2.1% each)	82 tonsillar 50 bot,	90-92
Fakhry, 2019 (151)	USA	396 HNSCC (202 HPV+)	Oral rinse	Multiplex-PCR, Linear Array HPV Genotyping Test	37 HPV types	16 (92.3%)	80.6	100
Koslabova, 2013 (165)	Czech Republic	141 HNSCC, (75 HPV + OPSCC)	Oral rinse	PCR L1 DNA for multiple HPV types	Oncogenic multiple types	16 (77.3%), 18, 33 (2.7%each	77.1	84.5
Tachezy, 2009 (149)	Czech Republic	86 HNSCC (53 HPV+), 107 benign	Oral rinses	PCR for L1 DNA	multiple HPV types	16 (92%) 33 (6%) 26 (2%)	62.3	87.9-96.4
Al-Soneidar, 2022 (148)	Canada	396 HNC, 439 controls for α-HPV	Oral rinse, brush	Linear Array assay, PCR	α-HPV (16), β-HPV, γ-HPV	16	62	89
Ahn, 2014 (147)	USA	93 OPSCC and CUP: 81 HPV+	Saliva plasma	RT-qPCR qPCR for HPV16 E6 DNA and E7 DNA	16	16 (87%)	52.8	100
Rettig 2015 (145)	USA	124 OPC	Oral rinses	Reverse line-blot hybridization, RT-qPCR	16, 18, 31, 33, 35, 39, 45, 51,	16 (54%), 59 (1.6%), 33, 39, 45, 51, 52	43	100

					56, 58, 59, 68,73	(0.8% each)		
Wang, 2015 (146)	USA	93 HNSCC (30 HPV + OPSCC), 10 controls	Saliva, plasma	PCR for HPV16 E7	16	16 (100%)	40	100
Agrawal, 2008 (144)	USA	135 HNSCC (44 HPV+)	Oral rinses	Multiplex RT-PCR	37 HPV types	16 (38.6%)	30	97
Zhao, 2005 (143)	USA	92 HNSCC, 604 controls w/o HNSC	Salivary rinses	RT-qPCR for HPV16 E6 and E7 DNA	16	16 (32.6%)	14-32.6	97-100

*Opx = oropharynx

HPV-ddPCR was also highly accurate in our "controls". We did not detect HPV in squamous papillomas nor in non-squamous cells such as in lymphomas or parotid. Head and neck squamous papillomas are generally considered to be caused by lr-HPV and not associated with hr-HPV (166). HPV-related head and neck lymphomas are rare and the relationship between HPV and lymphomas remains largely unknown (167). Some studies detected hr-HPV in parotid (168–170) with contradicting results indicating that hr-HPV infection is associated with parotid gland tumors development (168) and HPV is not a causative agent in the development of these tumors (169). We detected HPV in 2 benign tonsils, one was known to have previous oncogenic infection (HPV16) and the other one had recurrent infection (HPV56). Kreimer et al (Kreimer 2010) conducted a systematic review of HPV in healthy individuals and they found that 3.5% had carcinogenic HPV and 1.3% had HPV16. Tam et al (171) also carried out a systematic review and meta-analysis of oral HPV infection in healthy populations and they found that 1.4% had HPV16. It is important to note that most individuals clear HPV infection without causing cancer. Persistent infection is considered a necessary but not sufficient event for cancer development as there are several molecular mechanisms involved (38). It is therefore important to monitor these patients for persistent infection before carcinogenesis occurs so

it can be caught early and treated promptly. We detected HPV in sinonasal, which was not surprising as sinonasal is the second anatomic subsite in head and neck for HPV infection (33,34). Some studies show that HPV-related SNSCC is associated with better outcomes, while others have reported that HPV status is not a significant prognostic factor (36). Since sinonasal is rare and favorable HPV prognosis is unresolve, more research studies are essential to better understand the role of hr- HPVs in sinonasal carcinoma. We detected HPV in HNCUP which was expected as many of these are known to be HPV+ (172). HPV related HNCUP display better prognosis compared to non-HPV related HNCUP therefore it is important to determine HPV positivity (172). We detected HPV from oral cavity: oral tongue, floor of mouth, retromolar trigone, mandible and tongue cribriform adenocarcinoma of salivary origin. Katirachi et al (173) executed a systematic review on the prevalence of HPV in oral cavity worldwide and their results showed that the oral tongue were the most common subsite raising the issue that they could have originated from base of tongue. The second most prevalent subsite were from the floor of mouth and several studies also found HPV in retromolar trigone and mandible (173). Cribriform adenocarcinoma of salivary origin is rare and p16 overexpression in these tumors has not been clearly defined (174). Studies that have been conducted produced conflicting results. Katirachi et al found that all the studies but one showed no association between HPV and OCSCC. The only study that showed HPV and OCSCC association included the soft palate as a part of the oral cavity (173). There are contradicting results regarding the prognosis for HPV-related OCSCC (175,176). To better understand the risk factors for oral cancer, additional prospective studies focusing on larger samples of distinct anatomical locations and tumor entities are required (176). In our study, ddPCR is highly accurate in detecting

HPV in swabs from various head and neck cancer subsites and can be a useful tool for diagnosis. To our knowledge, our method of simultaneously testing multiple 12 hr-HPV types and the CDKN2A gene is the first and largest prospective study to date for HPV testing using non-invasive swabs in a large OPSCC cohort.

4.2 HPV Strain Distribution in OPSCC

For our second outcome measure, we analyzed the distribution of oncogenic HPV types in OPSCC, and HPV16 was the most prevalent like all the other studies (177). We found that HPV33 was the second most prevalent type like other studies (149,150,164), while others had various HPV types as the second most prevalent: HPV18 (138), HPV35 (163) and HPV59 (145). Across all studies, HPV 16 is the most prevalent strain, but the second most prevalent strain varies based on geographical location. The association between HPV-16 and head and neck carcinoma, OPSCC in particular, is well established in the literature; however, the role of the other hr-HPV types: 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59, in the onset of the disease remain unclear despite some reports highly suggest the likelihood of these strains' involvement in the disease's process (158,159). Several studies suggested the likelihood of non-HPV 16 strains association and risk of HNSCC with varying results: associations for HPV 18, 33 and 52¸ (158) associations for HPV 31, 33, 35, 52, and 58 (178) and associations for HPV18 and 52 but not 33 (179). As some HPV strains may be more aggressive than others, it may be necessary to create a customized treatment plan for escalation and this will incur higher costs in the areas where aggressive HPV strains are present.

4.3 Univariate Survival Analysis

4.3.1 Survival Analysis for p16, HPV and p16/HPV

For the third outcome measure, the survival outcomes (5-year OS, DSS and DFS) according to p16, HPV and p16/HPV in OPSCC were determined. The p16 status is an important predictor of survival from many studies (180) and our results showed that it is significant for OS, DSS, DFS and all the analyses were sufficiently powered. HPV status is also an important predictor of survival (181) and our results showed that it is significant for OS, DSS but not significant for DFS and only sufficiently powered for OS **(Table 3.7 and 3.8)**. Several studies showed that combining p16 and HPV status provides more accurate diagnosis (136,182,183). Although p16 IHC and hr-HPV status generally show a high degree of concordance in OPSCC, between 4% to 30% of cases have shown discrepant results potentially affecting prognosis, (182) and we had 22% (20.9% p16+/HPV- and 1.7% p16-/HPV+) discordant patients. Mehanna et al (136) published the largest, multicenter, multinational and sufficiently powered study determining the prognosis of discordant patients and they showed that p16+/HPV- or p16-/HPV+ had significantly worse prognosis than patients with p16+/HPV+ and significantly better prognosis than p16-/HPV-. Our results showed p16+/HPV+ have better prognosis compared to discordant p16+/HPV- and p16-/HPV+ but did not reach statistical significance like Shinn study (183) and p16-/HPV- have significantly worst prognosis. Although p16-/HPV+ OPSCC is technically HPV-related, management plans should consider that most clinical trials have used p16 IHC to determine HPV status in de-escalation protocols and thereby it is important to identify the prognostic uncertainty associated with p16/HPV discordance (182). Mehanna's study had insufficient numbers of discordant patients from North

America to draw conclusions and regular HPV testing along with p16 IHC will help to further understand the prognostic implications of discordant patients here in Canada.

4.3.2 Survival Analysis for hr-HPV Strains and Multiple HPV Strains Positivity

HPV type is an important predictor of survival and patients with HPV16 had significantly better prognosis compared to HPV-, HPV33 and HPV 59, and better than HPV18, HPV31 and HPV 58 but not significant **(Table 3.8).** Like Michaud et al (158) study, they found HPV16 have higher survival compared to HPV33 and Mazul et al (159) found that HPV16 has better overall survival compared to other non-HPV16 strain. In our cohort, there is a trend for other non-HPV 16 strains to have lower survival but not statistically significant and this may be limited by number of strains and therefore lower power limiting statistical comparisons, therefore definitive conclusions cannot be drawn. It is important to identify specific HPV strains because some may be more aggressive than others and may require treatment escalation. HPV genotyping can also be a valuable prognostic factor for OPSCC patients and future survival studies should incorporate HPV genotype into their analyses.

When considering only HPV positivity and disregarding multiple strains present, HPV+ have statistically significantly higher OS and DSS but not significant DFS compared to HPV- **(Section 3.5.2)**. But when HPV status was categorized by the number of HPV strains present (ie. 1HPV+ and 2-3HPV+), 1HPV+ compared to HPV- had statistically significantly higher OS, DSS and DFS **(Section 3.5.5**). 2-3HPV+ have slightly better prognosis compared to HPV- and worst prognosis compared to 1HPV+ but not statistically

significant (**Section 3.5.5**). Both analyses were not sufficiently powered as there were only 13 patients who were 2-3HPV+. There were 12 patients with 2HPV+ and one 3HPV+ patient (55y, male smoker with HPV16, 31 and 58) with only 0.5 years survival **(Table 3.13 and 3.14)**. Treatment escalation may be necessary in cases with more than one HPV strain, as multiple strains are associated with decreased survival rate. Michaud et al (158) found that the increased numbers of hr-HPV infections dramatically increased the magnitude of associations for HNSCC. Multiple hr-HPV strains can interact with different viral proteins that have different effects on the host and increases tumor development (158). We need further research on how multiple strains affect OPSCC patients' survival, hence it is important to determine the amount of HPV strains present as multiple strains may predict highly aggressive disease which will influence treatment.

4.3.3 Survival Analysis for Age

The mean age at diagnosis with our OPSCC cohort was 61 years of age which was within the age range shown in a systematic review on HPV-related OPSCC global prevalence by Mariz et al (184). HPV-related OPSCCs were originally thought to be a disease of younger adults, but there have been rapid increases among the elderly population (185). Most of our participants were aged 50-69 but a significant amount was aged 70+. Age is an important predictor of survival as shown in several studies (186,187) and our results showed that younger patients 30-49 have favorable prognosis compared to 50-69 and older 70+ patients. Both younger and older individuals are expected to be affected by rising incidence rates and oral infections are less likely to clear among older men (187) and hence they could be at even higher risk for carcinogenesis if persistent infection is not

cleared by the immune system. This has clinical implications as multimodality treatment was associated with improved survival and increased cost in elderly participants (188).

4.3.4 Survival Analysis for Sex

Several studies showed that sex is an important predictor of survival and females have better survival than males (189–191) while Roberts et al (192) showed that there was no survival advantage for females. It is widely recognized that females are at a lower risk of developing cancer in their lifetime because they have less high-risk activities and exhibit increased awareness of health issues among other things which may increase earlier cancer detection contributing to their higher survival (193). Our results showed that females compared to males have better prognosis but did not reach statistical significance. The increasing incidence rates of HPV-related OPSCC in the United States (187) and Canada (194) is currently predominant among men. Our cohort are mostly males, and the survival analysis were not sufficiently powered so it is difficult to draw any definitive conclusions as to whether patients' sex is an important predictor of survival. Further research is needed with more female cohorts.

4.3.5 Survival Analysis for Smoking

Several studies showed that smoking is an important predictor of survival (135,195–197) and that nonsmokers have significantly better prognosis compared to former and current smokers (135,198). Despite HPV-driven OPSCC being considered a disease of nonsmokers (199,200), there is evidence associated with higher rates of HPV+ OPSCC

among former or current smokers suggesting that smoking is a risk factor in both HPV+ and HPV- (6). Our results showed that nonsmokers have significantly favorable prognosis compared to current smokers with >10py, but not significant compared to former smokers with >10py. Current smokers with >10py had the worst OS, DSS and DFS, however, the wide CIs in DSS and DFS limit the certainty of the true effect due to small sample size. Similarly, while former smokers have improved prognosis, the analyses lacked the power to draw firm conclusions. A study showed that current smokers have the worst survival compared to nonsmokers and former smokers, and that smoking cessation is associated with improved survival in oropharyngeal cancer treated with CRT (201). According to their results, cessation for 4 weeks prior to starting therapy is associated with improved survival in the HPV+ group, but there were too few patients to confirm this association more conclusively in the HPV- group (201). Another study showed that current smokers and those with higher smoking exposure had poorer prognosis compared to nonsmokers and former smokers irrespective of their treatment (202). Although quitting smoking may reduce the risk of carcinogenesis, it is unclear how long abstinence is required to have this effect (197). Most of our OPSCC participants were former and current smokers and smoking is associated with higher risk of death and treatment failure. The high prevalence of smokers with HPV+ OPSCC makes it crucial to determine the HPV status in OPSCC patients with a history of smoking to determine proper treatment and efforts should be made to achieve smoking cessation as it may improve survival.

4.3.6 Survival Analysis for Tumor Stage

Several studies showed that T stage is an important predictor of survival (203,204) which was demonstrated in our cohort. Our results showed that lower T1/T2 stage have favorable prognosis compared to higher T3/T4 and T4 has the worst prognosis like Haughey et al study (135). The significance of T stage on survival increased overtime across all T stages (204), and therefore accurate staging is crucial for clinical management and prognostication.

4.3.7 Survival Analysis for Nodal Stage

N stage is an important predictor of survival as shown in several studies (203,204) which was demonstrated in our cohort. N0/N1 has favorable prognosis compared to higher N2/N3 stage. Surprisingly, when comparing N0 and N1, N1 had better prognosis. Keane at al (204) conducted a study on the prognostic significance of nodal disease and they showed that the relationship between N stage and survival was not linear with modest differences of hazard risk between various stages N0 to N2. HPV-related OPSCCs tend to present with larger, cystic cervical lymph nodes, explaining the better outcomes for patients with higher nodal disease and the decreasing association of the N stage with survival (204). It is still crucial to accurately stage nodal disease for clinical management and prognostication as three or more nodes significantly increase hazard risks (135).

4.3.8 Survival Analysis for Treatment Type

For survival outcomes according to treatment modality, there were no significant differences observed across treatment groups like the results from retrospective studies by Dhanireddy et al (205). In our cohort, treatment modality alone is not an important predictor of survival. This has clinical implications as other treatment modality (eg. transoral robotic surgery) can be a viable option for de-escalation because it can reduce or eliminate adjuvant therapy, especially in patients with comorbidities (205).

4.4 Multivariate Survival Analysis

The p16, HPV and p16/HPV are important predictors of survival and were tested in multivariate analysis stratified according to variables that were also predictors of survival such as age, sex, smoking, T-stage, N-stage, and treatment types (135,136).

4.4.1 Multivariate Survival Analysis for p16, HPV and p16/HPV Stratified by Age

In our OPSCC cohort, age is an important predictor of survival as shown in univariate analysis showing that younger patients aged 30-49 have favorable prognosis compared to 50-69 and older 70+ patients. When age groups were stratified according to p16 status, compared to p16-, p16+ had better prognosis with higher OS across all age groups like Windon et al (186) but p16+: 30-49 also had significantly higher DSS and DFS and age 50-69 also had significantly higher DSS. When age groups were stratified according to HPV status, HPV+ compared to HPV- had better prognosis with significantly higher OS and

DSS in age groups 30-49 and 50-69. The survival benefit from HPV positivity was seen in age group 70+ but did not reach significance. Research has demonstrated that the survival benefit associated with having an HPV+ tumor is reduced in older patients when compared to younger or middle-aged individuals may be because older patients have higher mortality risks and other comorbidities (186). When p16 and HPV status were combined, increasing age is associated with worse survival in p16-/HPV- for OS like Shinn et al (156) study**.** The survival benefits from either p16 or HPV positivity were shown in p16+/HPV- age 30-49 and 70+ and p16-/HPV+: 70+ but did not reach significance. Treatment escalation may benefit p16+/HPV-: 50-69 to improve their outcomes. Although those aged 70+ have the worst survival in univariate analysis, they benefit the most from either having p16 or HPV positivity. This has clinical implications because the determination of both p16 and HPV could lead to treatment de-escalation and lower costs in elderly participants with multimodality treatment.

4.4.2 Multivariate Survival Analysis for p16, HPV and p16/HPV Stratified by Sex

As shown in univariate analysis, sex is not an important predictor of survival but when males were stratified according to p16 status, p16+ compared to p16- had better prognosis in terms of OS, DSS and DFS. Similarly, HPV+ males compared to HPV- have better prognosis in terms of OS and DSS but not significant DFS. Although p16+ and HPV+ females have higher OS, DSS and DFS compared to their p16- and HPV- counterparts, the analyses did not reach statistical significance as there were a small number of female participants. The survival benefits from either p16 or HPV positivity were shown in p16+/HPV- males for OS and DFS and females for OS and DSS but did not

reach significance. It was also shown in p16-/HPV+ females for OS, DSS and DFS but did not reach significance. Mehanna et al (136) study on p16 and HPV discordance found that there were no significant differences in sex observed. None of the analyses for females reached significance due to a small sample size and wide CIs, limiting the certainty of the true effect and preventing definitive conclusions. For males, however, p16 and HPV status play a significant role in their prognosis and may benefit from treatment escalation.

4.4.3 Multivariate Survival Analysis for p16, HPV and p16/HPV Stratified by Smoking

Smoking is an important predictor of survival from univariate analysis showing that nonsmokers had better prognoses compared to former with >10py and current smokers with >10py. When smoking status was stratified according to p16, the survival benefit from p16 positivity was statistically significant in all groups with significantly higher OS and DSS but only the current smokers with >10py also had significantly higher DFS. When stratified according to HPV, the survival benefit from HPV positivity were statistically significant in former with >10py for OS and current smokers with >10py for OS, DSS and DFS. Grønhøj et al (206) showed that HPV positivity had no impact on survival for nonsmokers, but they noted that they only had a few HPV- nonsmokers like ours, but highly significant for smokers of which they combined former and current smokers. When p16 and HPV status were combined, the survival benefit from either p16 or HPV positivity was only shown in p16+/HPV-: nonsmokers but did not reach significance. Compared to p16+/HPV+, the discordant p16+/HPV- and p16-/HPV+ former and current smokers had worst prognosis. Compared to p16+/HPV+, p16+/HPV-: nonsmokers had higher but not

statistically significant OS, DSS and DFS and Mehanna (136) et al showed that there were no differences between p16+/HPV+ and p16+/HPV- nonsmokers. Compared to p16+/HPV+ former and current smokers, the discordant p16+/HPV- and p16-/HPV+ former and current smokers had worse prognosis like Mehanna's (136) findings, although our analyses were not sufficiently powered. Former and current smokers with p16+/HPV- have worse survival than p16+/HPV+ but better outcomes compared to p16-/HPV+ and p16-/HPV-. Non-smokers with p16+/HPV– tumors mostly likely have HPV-mediated tumors, but might have lower copy numbers than p16+/HPV+ patients which makes them detectable by highly sensitive methods (136). For former and current smokers, p16 overexpression might be unrelated to HPV and this might impact counselling, treatment decision making and outcomes (136). Treatment escalation may benefit former and current smokers to improve their prognosis.

4.4.4 Multivariate Survival Analysis for p16, HPV and p16/HPV Stratified by Tumor Stage

T stage is an important predictor of survival from univariate analysis showing that lower stage T1 and T2 had significantly better prognoses compared to higher stage T3 and T4. Based on T stage, the survival benefit from p16 positivity was statistically significant in T1 for OS, and T2 and T4 for OS and DSS. The survival benefit from HPV positivity was only statistically significant in T2 for OS. When p16 and HPV status were combined, the survival benefit from either p16 or HPV positivity was not shown in any p16+/HPV- groups. Compared to p16+/HPV+, p16+/HPV- T1, T2 and T3 had lower survival but did not reach significance. To decide on the most effective treatment for a patient, both p16

and HPV should be considered. Using only p16 is valuable for p16+/HPV+ group with better prognoses and may benefit from treatment de-escalation. However, for p16+/HPV- groups with poorer outcomes, treatment escalation might be necessary.

4.4.5 Multivariate Survival Analysis for p16, HPV and p16/HPV Stratified by Nodal Stage

N stage is an important predictor of survival from univariate analysis showing that lower stage N0/N1 had better prognosis compared to higher stage N2/N3. Based on N stage, the survival benefit from p16 positivity were statistically significant in N0 for OS, DSS and DFS and N2 for OS and DSS. The survival benefit from HPV positivity were statistically significant in N0 for OS, DSS and DFS and N1 for OS and DSS. When p16 and HPV status were combined, the survival benefit from either p16 or HPV positivity were shown in p16+/HPV- for N0 and N2 but did not reach significance. Compared to their p16+/HPV+ counterparts, p16+/HPV- N0, N2 had higher OS and DSS but did not reach significance. Compared to p16+/HPV+, p16+/HPV-: N1 had significantly lower OS and DSS. This has clinical implications and therefore both p16 and HPV should be considered. Both p16+/HPV- N1 and N3 groups may benefit from treatment escalation to improve their outcomes.

4.4.6 Multivariate Survival Analysis for p16, HPV and p16/HPV Stratified by Treatment Type

Treatment type alone is not a predictor of survival in our cohort as shown in univariate analysis as there were no statistically significant differences amongst the

different treatments. However, in multivariate analysis, the survival benefit from p16 positivity were statistically significant in S+RT and S+CRT for OS, DSS, DFS and in RT and S for OS and DSS. The survival benefit from HPV positivity were statistically significant in S+RT for DSS and in S+CRT for DSS and DFS. When p16 and HPV status were combined, the survival benefit from either p16 or HPV positivity were shown in p16+/HPV- for CRT and S but did not reach significance. Compared to p16+/HPV+, p16+/HPV- S+RT and S+CRT had lower survival but did not reach significance. The differences between p16+/HPV+ and p16+/HPV- within treatments S+RT and S+CRT show that p16 alone is insufficient and does not guarantee improved prognosis.

4.4.7 Discordant p16 and HPV Survival

In our cohort, the prognosis of patients with discordant p16+/HPV- depends on age, particularly 50-69, former and current smokers, T1-T3 stage, N1 stage and treatment S+RT and S+CRT. P16+/HPV-: 50-69 had worse survival than p16+/HPV+ but better than p16-/HPV-. P16+/HPV-: former and current smokers had worse survival than p16+/HPV+: former and current smokers but better than p16-/HPV-: former and current smokers. P16+/HPV-: T1-T3 had worse survival than p16+/HPV+ but better than p16-/HPV-. P16+/HPV-: N1 had significantly worse survival than p16+/HPV+ but better than p16-/HPV-. P16+/HPV-: treated S+RT and S+CRT had worse survival than p16+/HPV+ but better than p16-/HPV-.

Mehanna (136) et al showed that OPSCC has at least two subtypes with significantly differing prognosis has important implications, therefore it is important to categorize these patients accurately for optimal patient selection for de-escalation or

escalation of treatments. It is important to note that Mehanna's study (136), a multicenter, multinational and the largest OPSCC cohort, has sufficient power to indicate the prognosis for discordant patients, but it does not have sufficient data from North America to draw definitive conclusions which they mentioned in their limitations. The only study they included from North America was from Toronto with a total of 186 OPSCC (133 p16+/HPV+, 2 p16+/HPV-, 18 p16-/HPV+ and 33 p16-/HPV-) and Canada was the region with the highest HPV attributable fraction (p16+/HPV+) and lowest p16+/HPV- patients. Mehanna's results showed that there were no significant differences between p16+/HPV+ and p16-/HPV- and p16-/HPV+ had similar characteristics to p16-/HPV- in North America. We have a total of 300 OPSCC (196 p16+/HPV+, 63 p16+/HPV-, 5 p16-/HPV+ and 36 p16-/HPV-) in our study and we observed significant differences and sufficiently powered post hoc analyses. We found that p16+/HPV+ had better prognosis compared to p16+/HPV- and p16-/HPV+ and significantly better compared to p16-/HPV- and the discordant p16+/HPV- and p16-/HPV+ had better prognosis compared to p16-/HPV-. Although most of the analyses among discordant groups were not statistically significant, we showed that p16 and HPV discordance exists and affect prognosis in terms of OS, DSS and DFS. The discordant p16+/HPV- patients might have lower copy numbers or a separate genetic subgroup of HPV- tumors with better prognosis (136) and therefore it is important to use a highly sensitive detection method. Our ddPCR detection method can be useful in this situation. In p16+/HPV- smokers who have worse outcomes, the p16 overexpression might not be HPV related and misclassification can have considerable implications for counselling, treatment decision making and outcomes and therefore de-escalation strategies might be unsuitable (136). The discordant p16-/HPV+ had worse survival compared to

p16+/HPV+ and p16+/HPV- but they had better outcomes compared to p16-/HPV-. Patients who are p16-/HPV+ may be a viable candidate for treatment escalation procedures because of their improved prognosis. However, a deeper understanding of the mechanism of disease in these tumors requires more molecular research (136). It is important to determine both p16 and HPV in OPSCC and the result of our study may provide additional information about discordant patients in North America.

4.5 Strengths and Limitations

There are several strengths of our study. First is the prospective design that allowed us to collect complete and accurate patient clinical and pathologic information over time. Second, the large OPSCC cohort provides higher accuracy. Third, which can be both a strength and a limitation is the involvement of various clinicians in sample collection, reflecting a real-world environment. Despite the variability of clinicians collecting samples, most of the swabs were of excellent quality and we were able to detect the CDKN2A and/or HPV. Fourth, using a highly accurate ddPCR method of detecting HPV in non-invasive swabs instead of tissue. Being able to detect HPV in swabs can have several important implications such as a screening tool during post-treatment surveillance. A study showed that HPV16 was detected in saliva over 3 months before clinical detection of recurrence (207) and a larger study combining saliva and plasma detected HPV16 post-treatment and predicted recurrence within 3 years (147). Saliva, oral rinses, and swabs detect HPV at similar rates (208) but swab sampling may be more specific to the site since the swab is brushed directly on the tumor site (157). Non-invasive testing can be useful to detect recurrence earlier leading to more prompt treatment. Swab testing can also be useful

for cases where a tissue biopsy may be difficult or prohibitive, such as patients with significant comorbidities, and in resource-limited settings. Additionally, it can be used as a screening tool like cervical pap smears. Fifth, determining both multiple hr-HPV types and the CDKN2A simultaneously. Detection of CDKN2A can be utilized to assess the prognosis of variety cancers and valuable therapeutic target for the treatment of tumors (161). In OPSCC, patients that have no HPV but have high levels of CDKN2A may be eligible for treatment de-escalation, and vice versa, patients with HPV but low levels of CDKN2A may need treatment escalation. HPV genotyping has significant prognostic value and may aid in understanding the role that the virus plays in the disease development. Consequently, varying HPV genotypes, in addition to p16 status, should be considered in future OPSCC survival research. HPV genotyping can also be useful for strain-directed therapies being designed with novel therapeutic vaccines. The viral etiology of HPV-related HNSCC, which targets tumor-specific proteins E2, E5, E6 and E7 enables the development of customized immune-based treatments (209). Indeed, a variety of vaccines have been described in a few studies that target the HPV16 E6 and/or E7 (210–212) and HPV16 E2 and/or E5 proteins (213). However, HPV immunotherapy trials are still in the early stages of clinical development and preliminary data show that response rates are less than 20% (209). The reason for this may be a variety of tumor suppressive mechanisms that selectively induce adaptive immune resistance to the administered therapy and therefore, a combination of therapeutic approaches is likely to be required to counteract this problem (209).

The limitations of our study include a single institution cohort resulting in less robust results and hence future studies should involve provincial and national

collaborations. We have a small number of non-HPV16 strains, p16-/HPV+ group and females which limits the statistical power of comparisons and regular testing for hr-HPVs in OPSCC will help us gather more information. As mentioned above, the variability of clinicians collecting samples is a limitation since ddPCR like other detection techniques, depends on the quality of samples and a specimen with insufficient cells could lead to false-negative results. Furthermore, some patients were unable to open their mouths completely, making it difficult to properly swab the oropharynx resulting in a lack of cells being collected, which may also produce false-negative results. Finally, the lack of a standard molecular HPV test for direct comparison of our study makes it challenging to validate our findings. Out of 300 OPSCC, there were only 18 (6%) HPV molecular testing performed on fresh or frozen tissue or FFPE using either RT-qPCR or MassARRAY. On the molecular testing that was done, 15 tests agreed, and 3 tests disagreed (1 test using RT-qPCR did not detect HPV but ddPCR detected HPV16 and 2 tests using MassARRAY: 1 test detected HPV16 but not detected by ddPCR and another did not detect HPV but ddPCR detected HPV39). The test that ddPCR did not detect HPV could be a false-negative result due to lack of cells collected and the high sensitivity of ddPCR was demonstrated in detecting HPV with the other two tests. Studies compared ddPCR against RT-qPCR and ddPCR provided more precise results (25,118,121) and ddPCR compared to MassARRAY is more accurate at low-concentration targets (115,116). Since our goals are limited to the detection of known variants, ddPCR is a better option for the detection or diagnosis of malignancies with distinct and reliable genetic markers, like HPV types.

Chapter 5: Conclusion and Future Directions

5.1 Conclusions

Oropharyngeal swabs analyzed by ddPCR is highly accurate method for minimally invasive oncogenic HPV detection in a large OPSCC cohort. To our knowledge, our method of simultaneously testing multiple 12 hr-HPV types and the CDKN2A gene using non-invasive swabs in a large OPSCC cohort is the first and largest prospective study to date for HPV testing. Detection of CDKN2A as a biomarker can be used to evaluate the prognosis of various cancers and may also be a useful therapeutic target for the treatment of tumors. It is important to determine the specific oncogenic HPV type and the number of strains present as some strains may predict extremely aggressive disease and having multiple HPV strains reduces survival. Accurate oncogenic HPV subtyping can be useful for improving patient-centered treatments, early diagnostic tool, and high potential for post-treatment surveillance. Routine HPV testing should be required regardless of p16 status to identify the various subtypes and provide appropriate treatment because p16 and HPV discordance may have an impact on patients' prognoses.

5.2 Future Directions

As our study is limited to a single institution cohort, the next stages should involve provincial and national collaborations. HPV-ddPCR swab testing can be used as a screening tool during post-treatment surveillance. Regular assessment of hr-HPV DNA using swabs during follow-up may help detect locoregional or distant metastasis prior to onset of clinical symptoms enabling more prompt and effective treatment. It can be used as

a non-invasive early diagnostic tool to be utilized during the initial presentation at the clinic or when a tissue biopsy is not available or in resource-limited settings followed by a biopsy to confirm carcinoma diagnosis. This can be useful for testing HPV from self-sampled swab for cervical cancer screening from women that do not have access to routine cervical exams or for the analysis of cervical swab samples to determine the relationship between cervical and oropharyngeal HPV in women.

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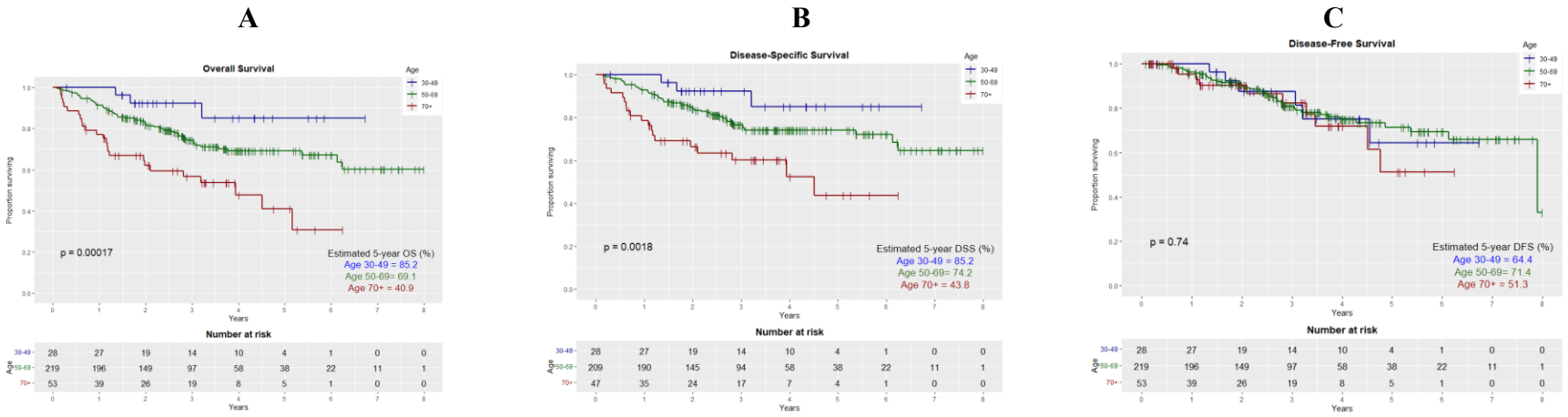
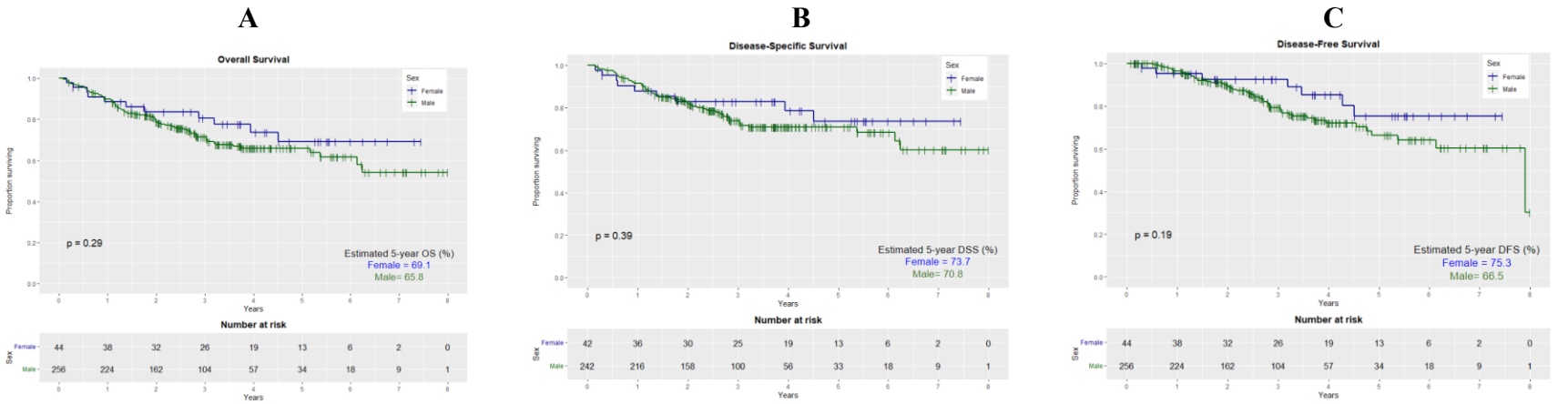
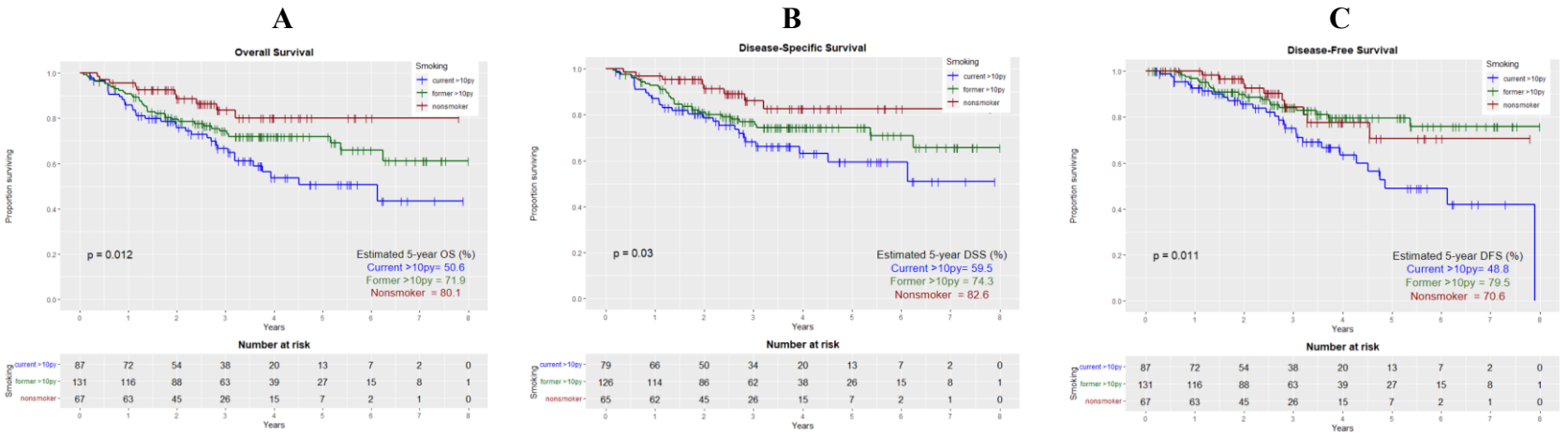
Appendix A: Univariate Survival Analysis



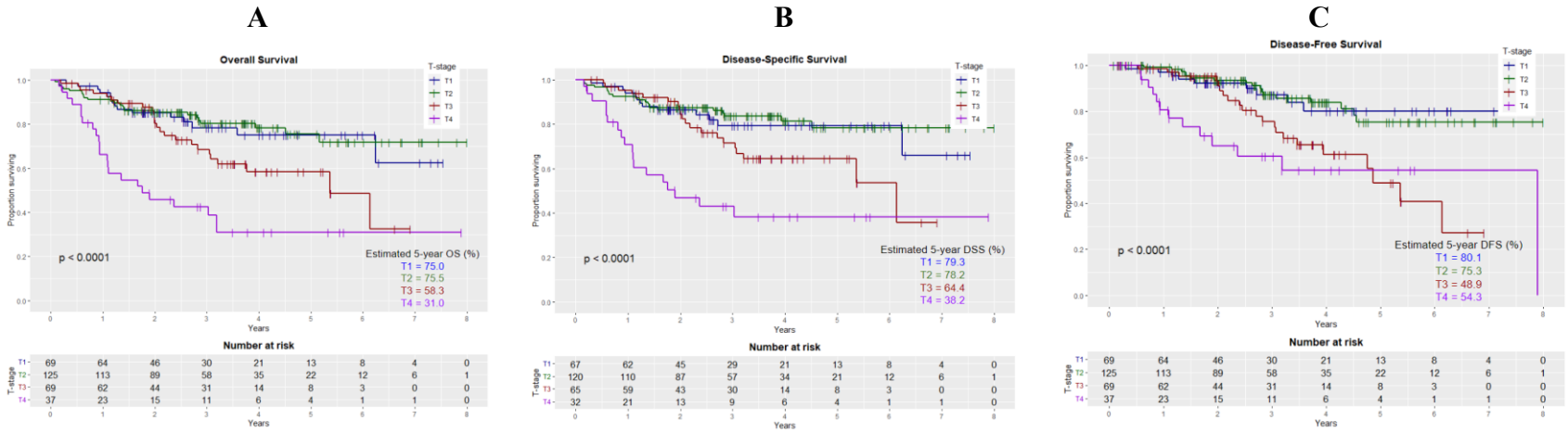
Appx A Figure 1: Survival Analysis according to age groups: 30-49, 50-69 and 70+, A) OS B) DSS C) DFS



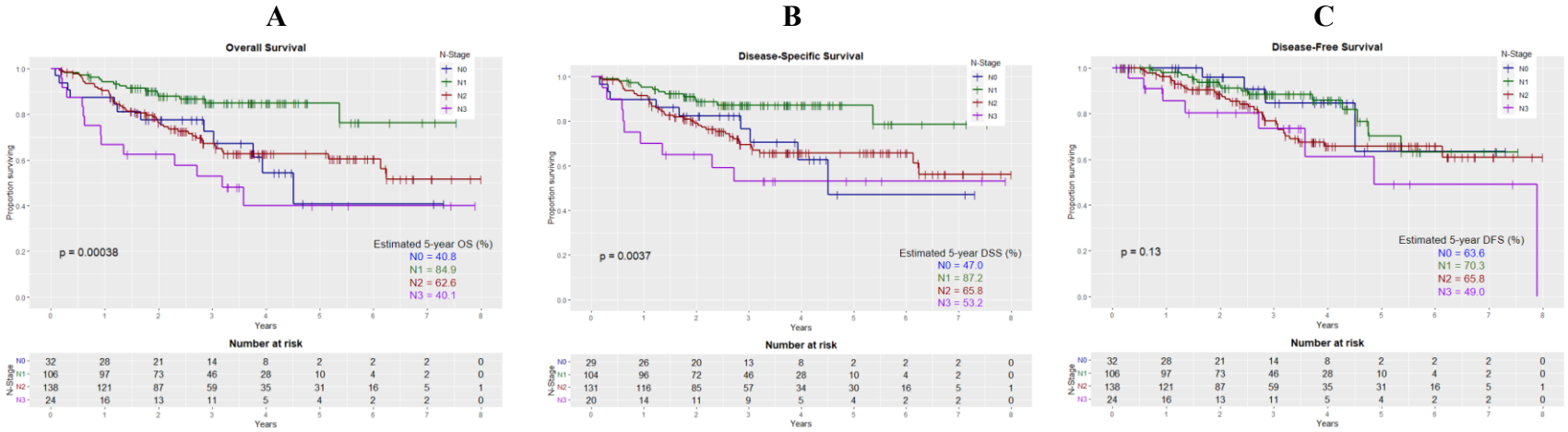
Appx A Figure 2: Survival Analysis according to Sex: Males and Females A) OS B) DSS C) DFS



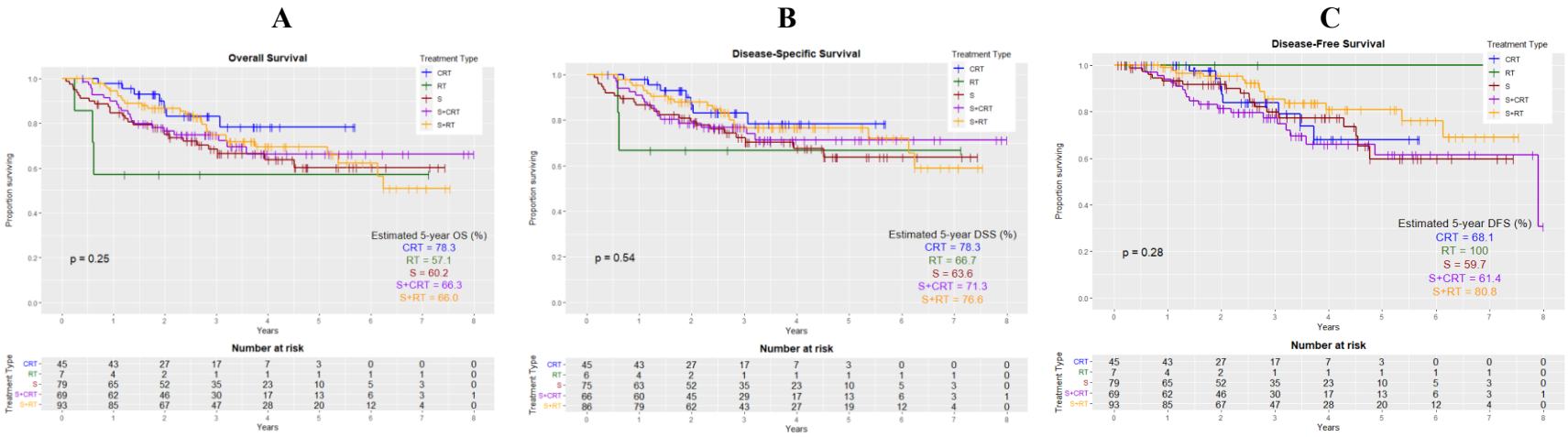
Appx A Figure 3: Survival Analysis according to Smoking Status: nonsmoker, former >10py and current >10py A) OS B) DSS C) DFS



Appx A Figure 4: Survival Analysis according to Primary Tumor Stage: T1, T2, T3 and T4 A) OS B) DSS C) DFS

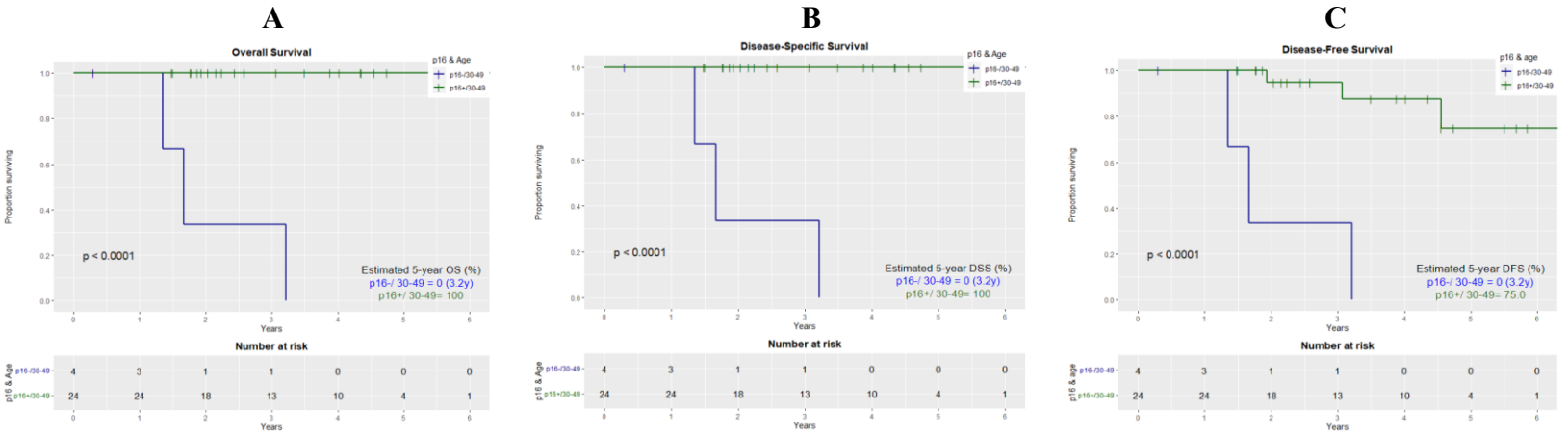


Appx A Figure 5: Survival Analysis according to Nodal stage: N0, N1, N2, N3 A) OS B) DSS C) DFS

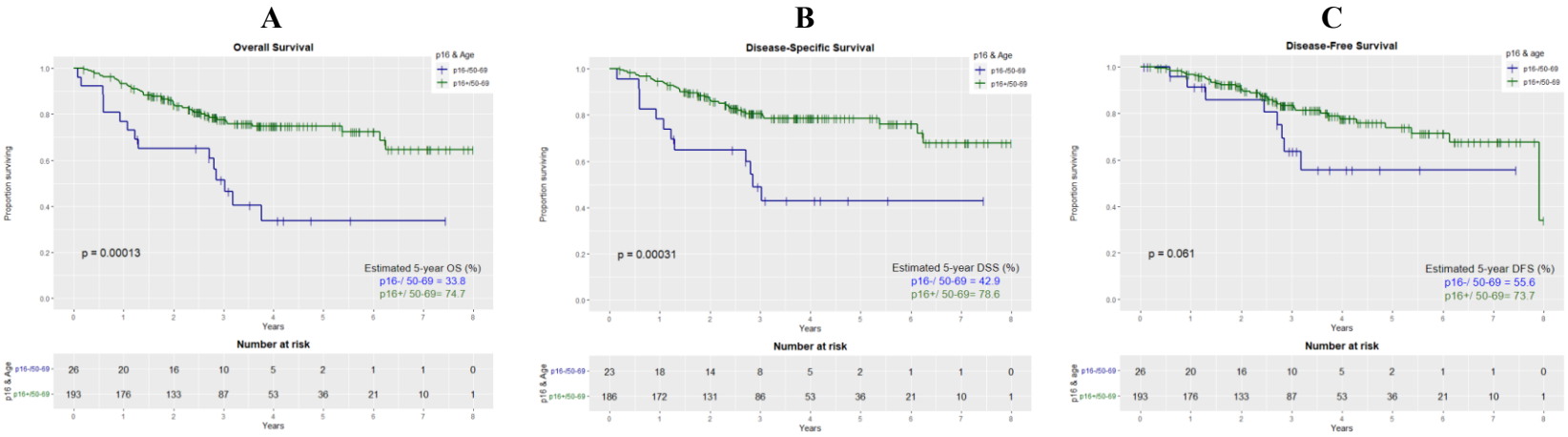


Appx A Figure 6: Survival Analysis according to Treatment type: S+CRT, S+RT, CRT, RT and S A) OS B) DSS C) DFS

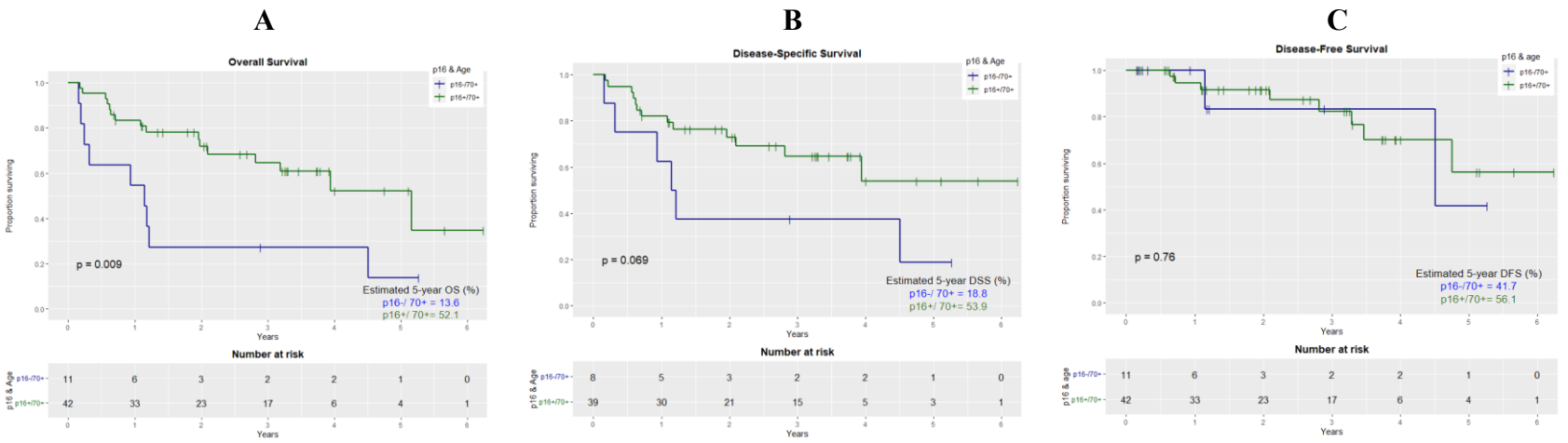
Appendix B: Multivariate Survival Analysis Stratified by Age



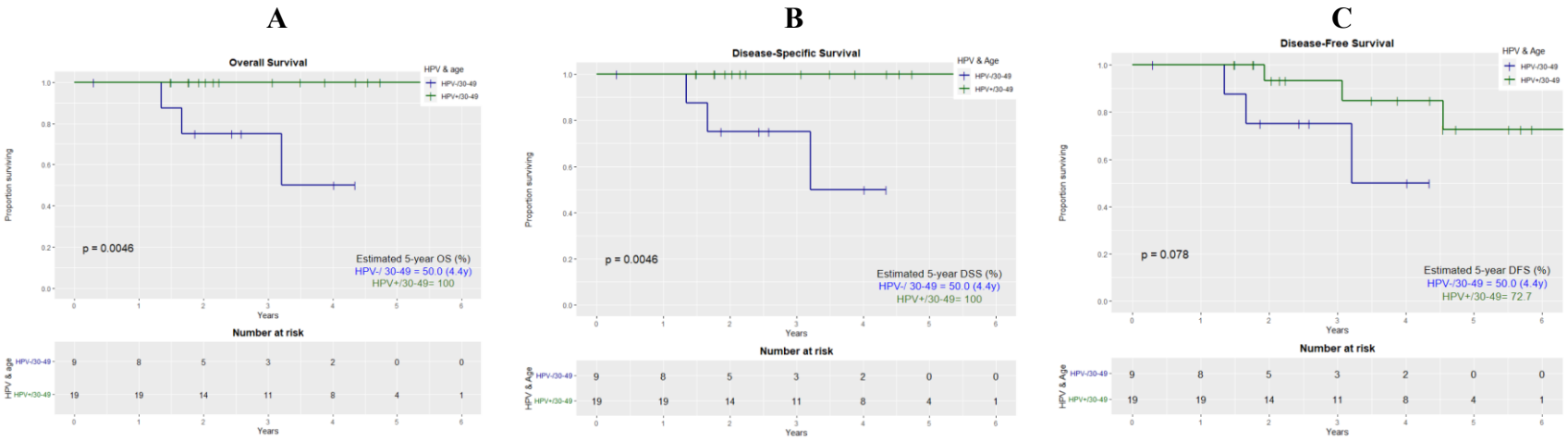
Appx B Figure 1: Survival Analysis of p16+ vs p16- Age 30-49: A) OS B) DSS C) DFS



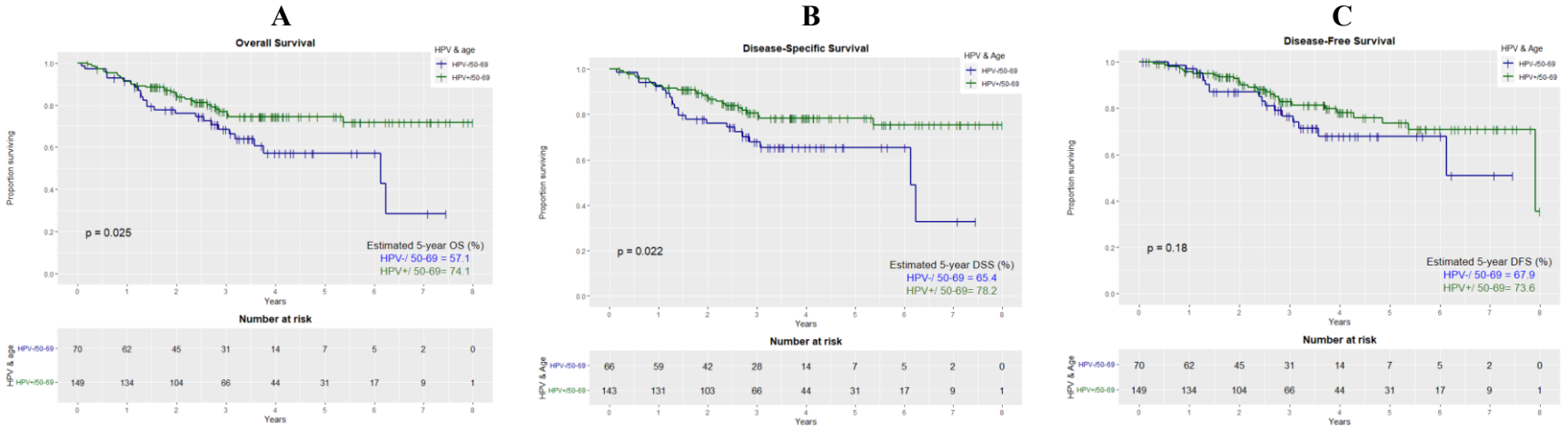
Appx B Figure 2: Survival Analysis of p16+ vs p16- Age 50-69: A) OS B) DSS C) DFS



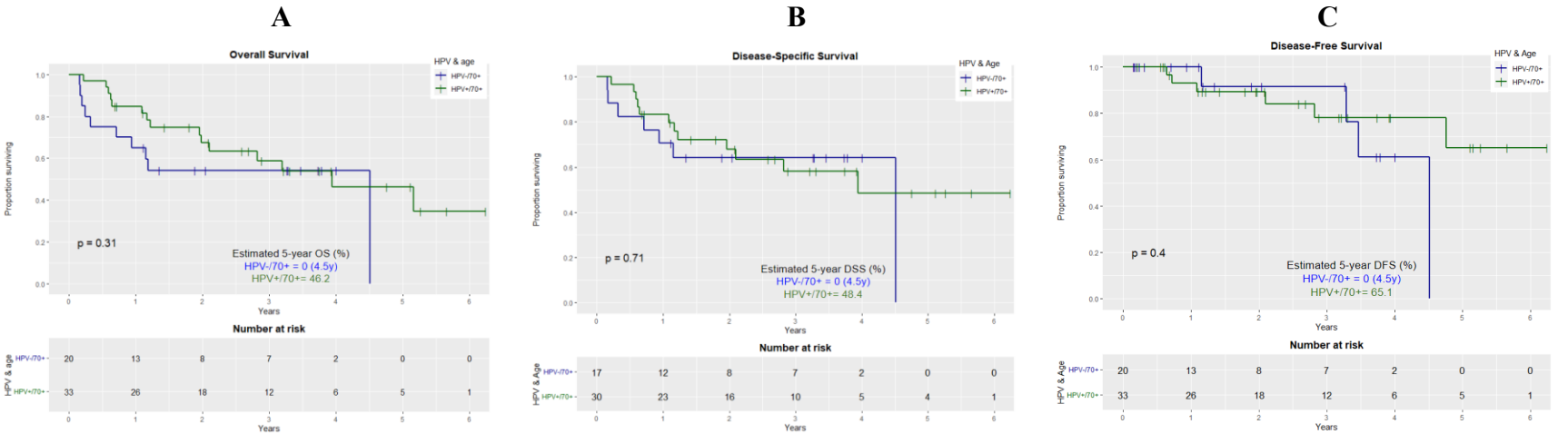
Appx B Figure 3: Survival Analysis of p16+ vs p16- Age 70+: A) OS B) DSS C) DFS



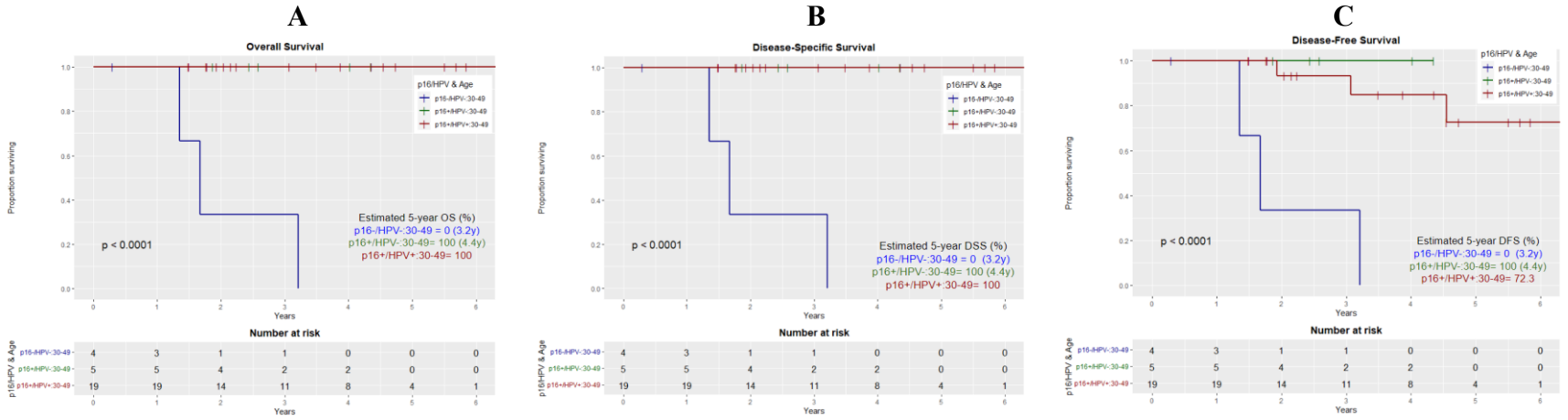
Appx B Figure 4: Survival Analysis of HPV+ vs HPV- Age 30-49: A) OS B) DSS C) DFS



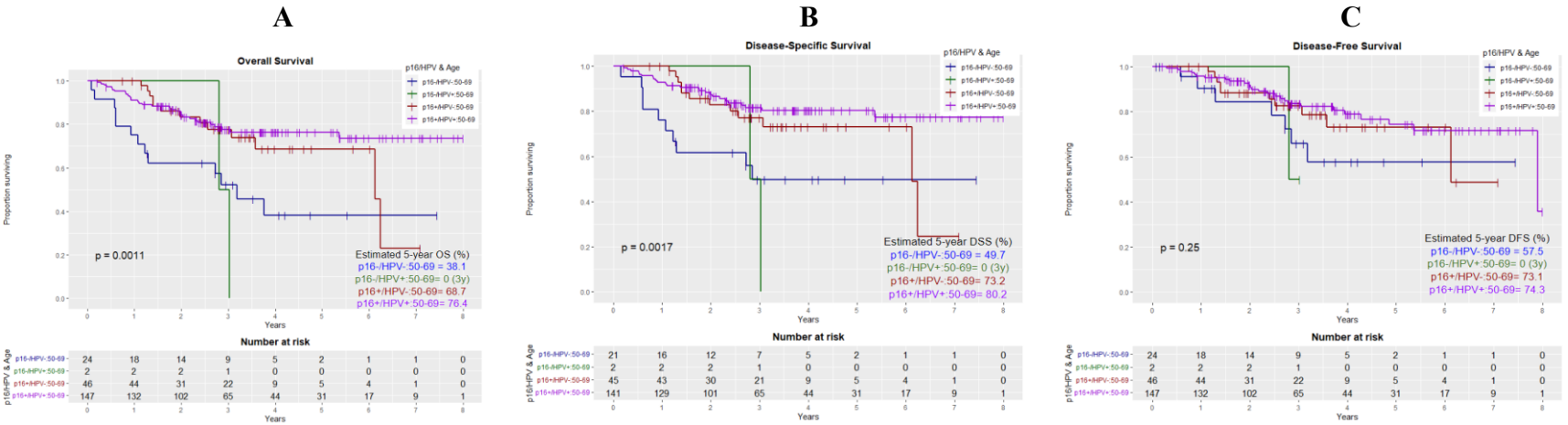
Appx B Figure 5: Survival Analysis of HPV+ vs HPV- Age 50-69: A) OS B) DSS C) DFS



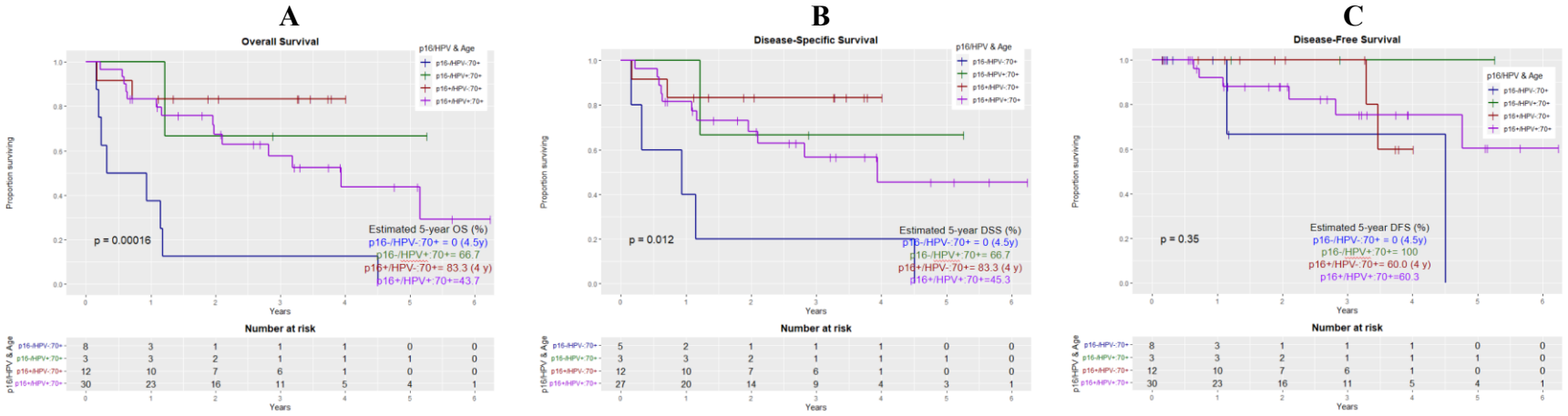
Appx B Figure 6: Survival Analysis of HPV+ vs HPV- Age 70+: A) OS B) DSS C) DFS



Appx B Figure 7: Survival Analysis of p16/HPV Age 30-49: A) OS B) DSS C) DFS

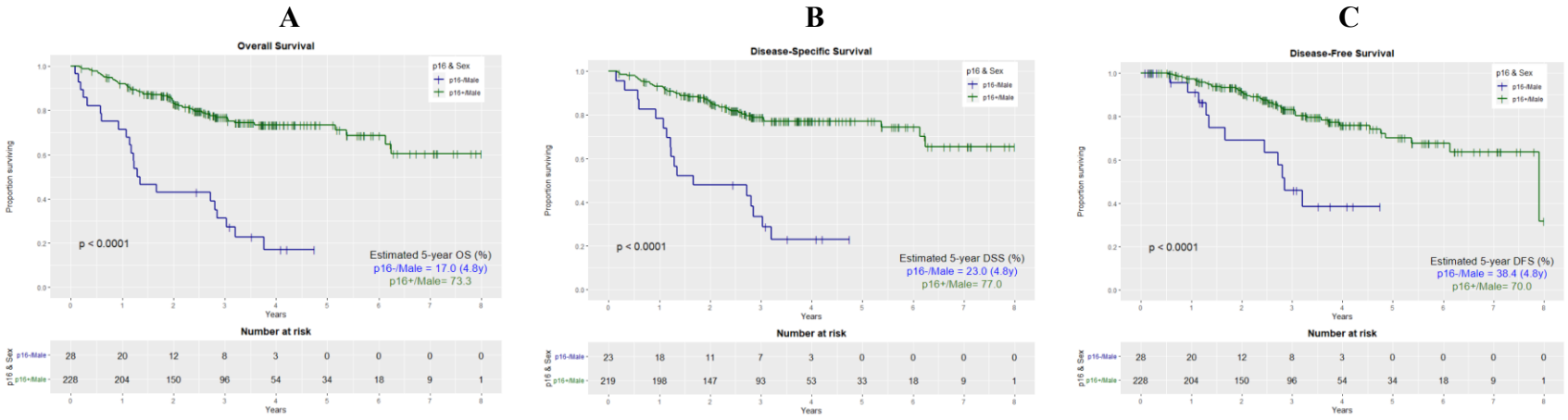


Appx B Figure 8: Survival Analysis of p16/HPV and Age 50-69: A) OS B) DSS C) DFS

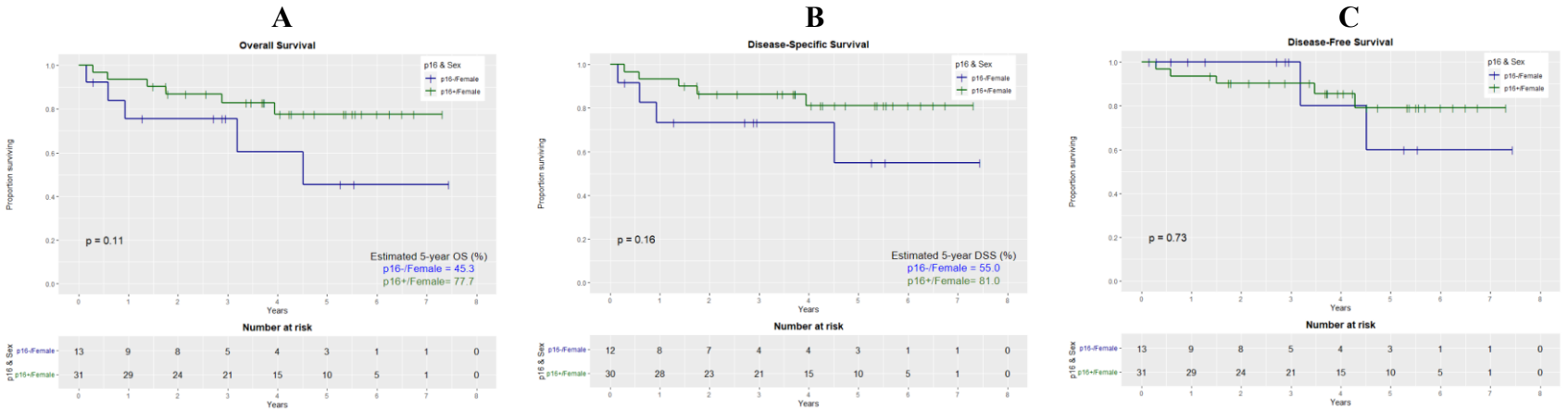


Appx B Figure 9: Survival Analysis of p16/HPV and Age 70+: A) OS B) DSS C) DFS

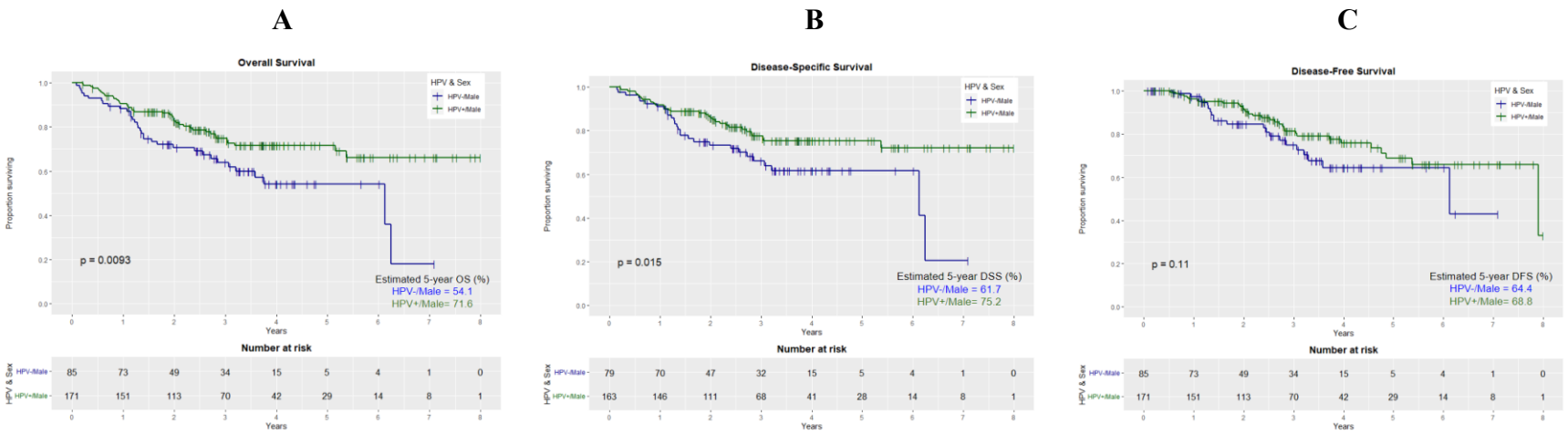
Appendix C: Multivariate Survival Analysis Stratified by Sex



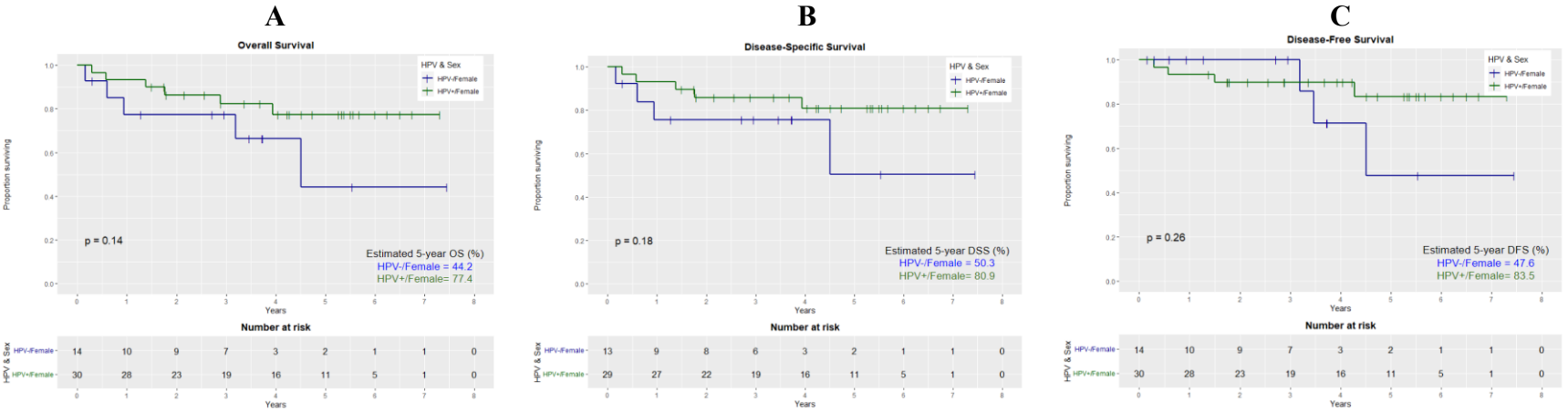
Appx C Figure 1: Survival Analysis of p16+ vs p16- Male: A) OS B) DSS C) DFS



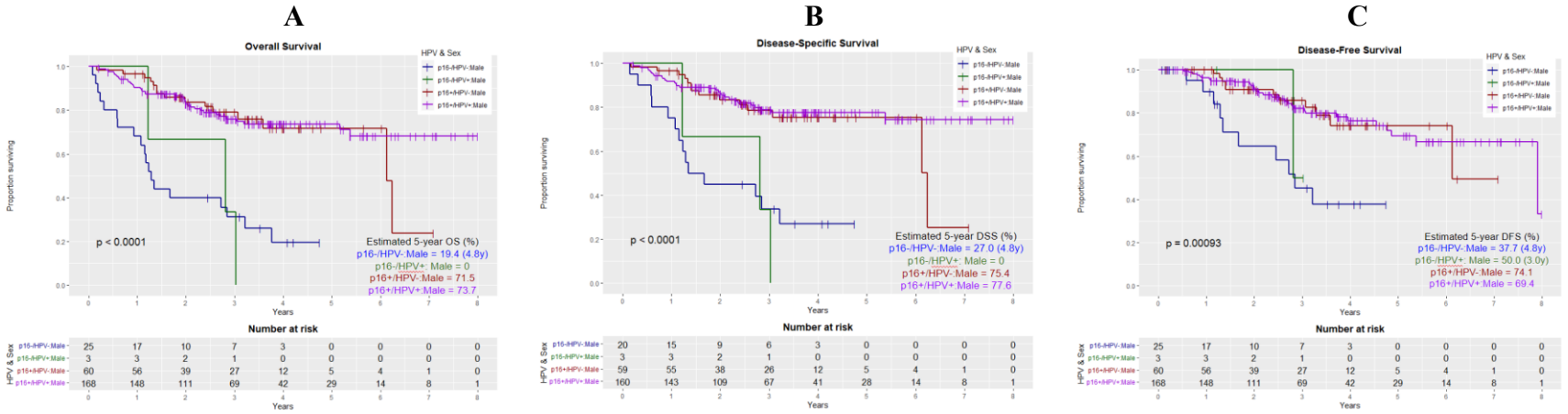
Appx C Figure 2: Survival Analysis of p16+ vs p16- Female: A) OS B) DSS C) DFS



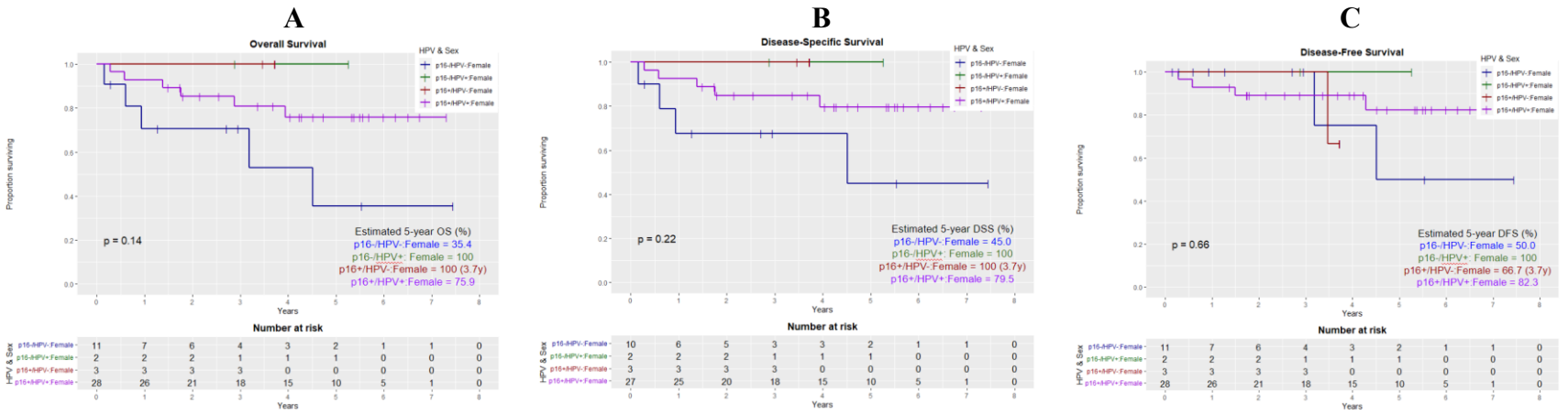
Appx C Figure 3: Survival Analysis of HPV+ vs HPV- Male: A) OS B) DSS C) DFS



Appx C Figure 4: Survival Analysis of HPV+ vs HPV- Female: A) OS B) DSS C) DFS

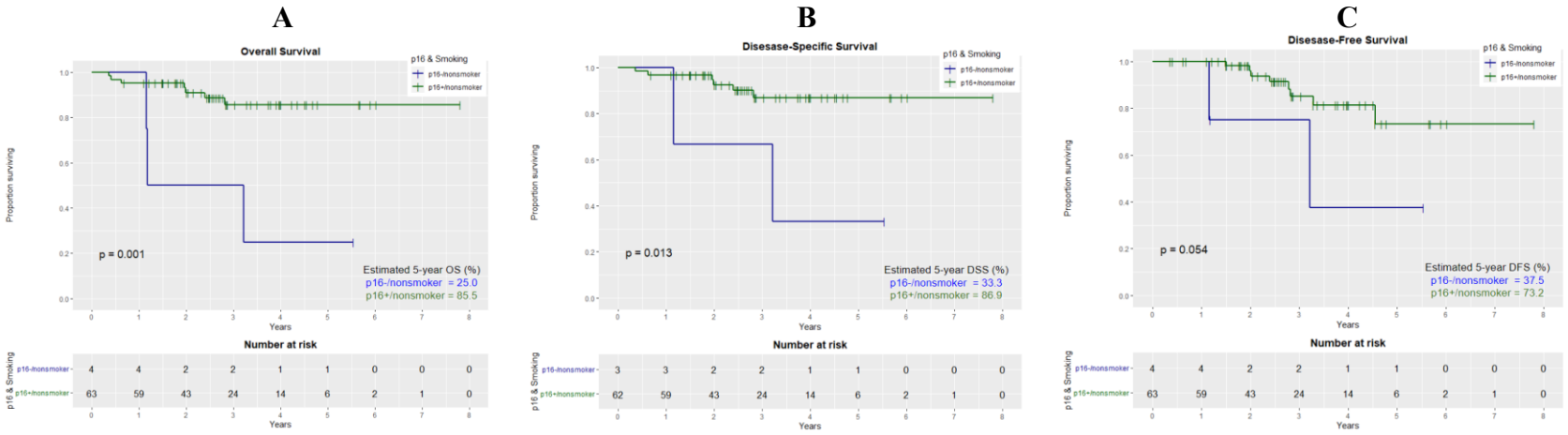


Appx C Figure 5: Survival Analysis of p16/HPV and Male: A) OS B) DSS C) DFS

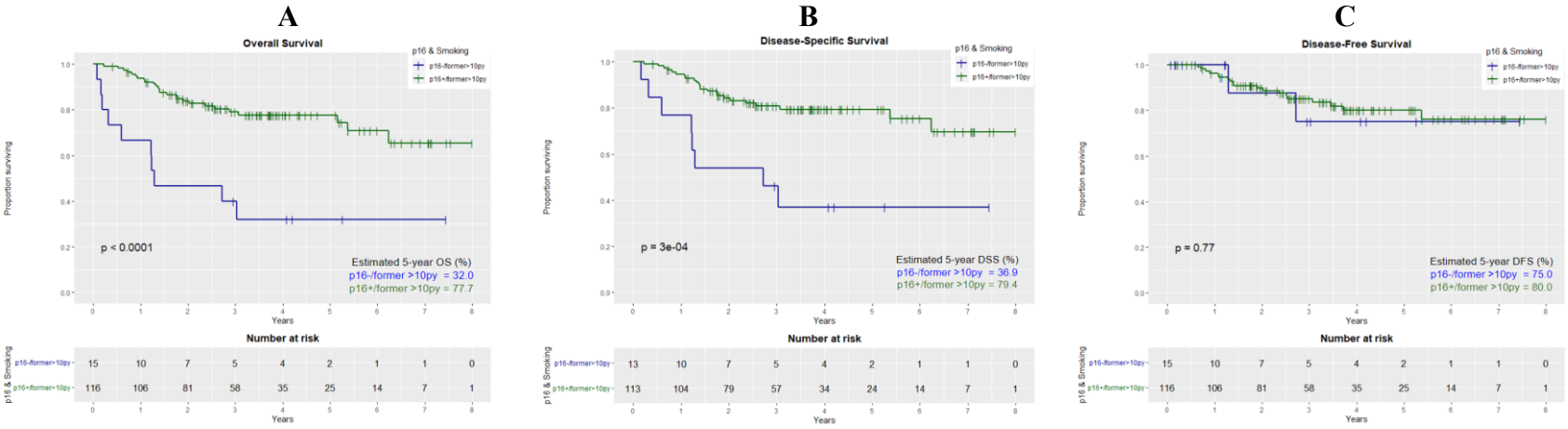


Appx C Figure 6: Survival Analysis of p16/HPV and Female: A) OS B) DSS C) DFS

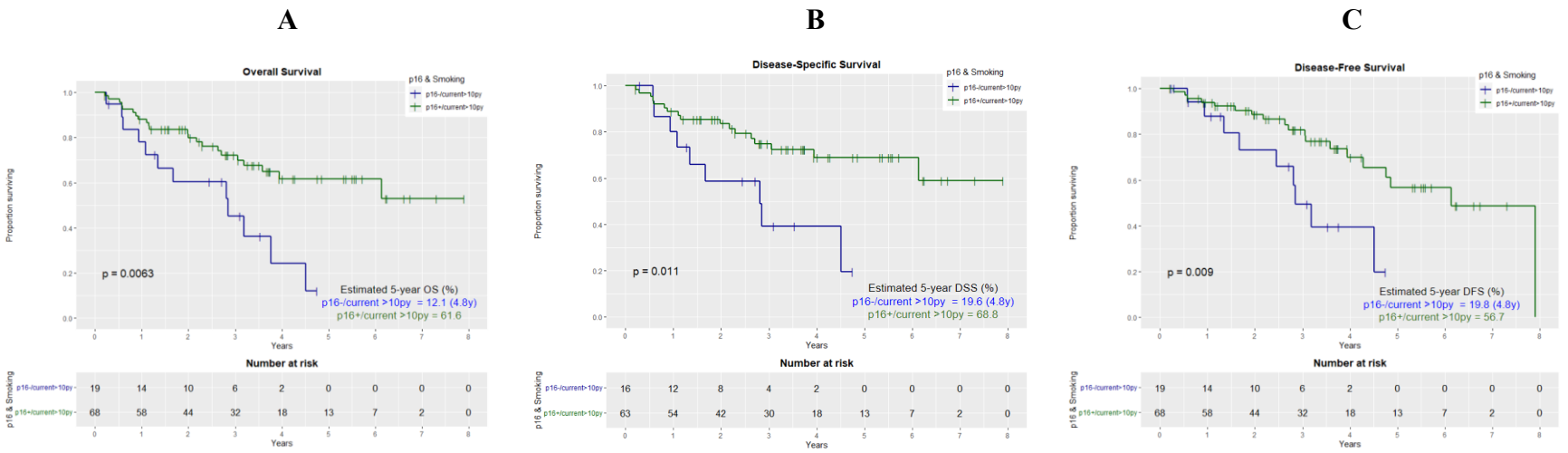
Appendix D: Multivariate Survival Analysis Stratified by Smoking



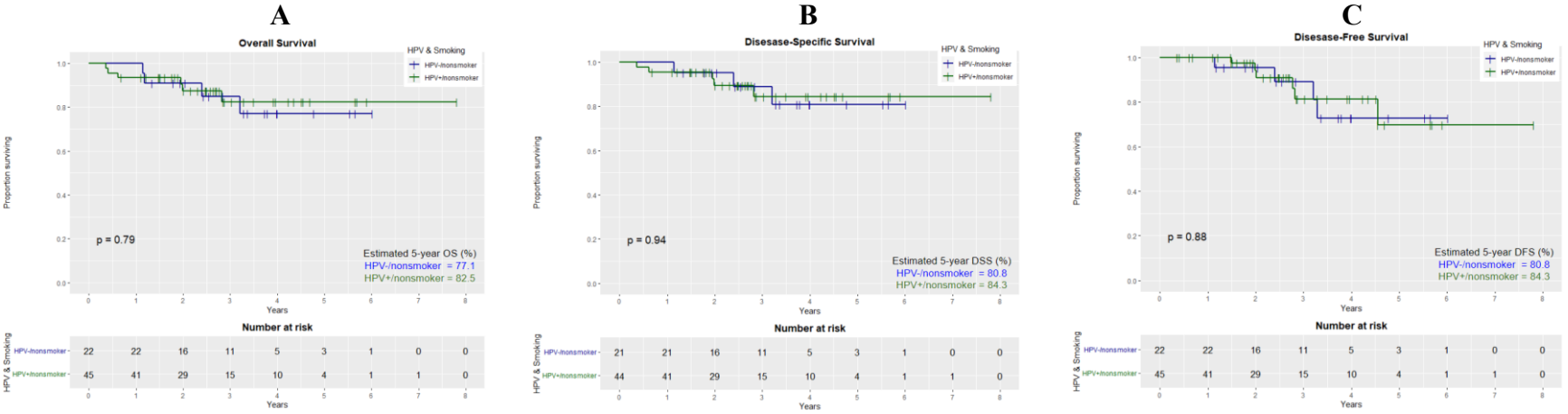
Appx D Figure 1: Survival Analysis of p16+ vs p16- Nonsmoker: A) OS B) DSS C) DFS



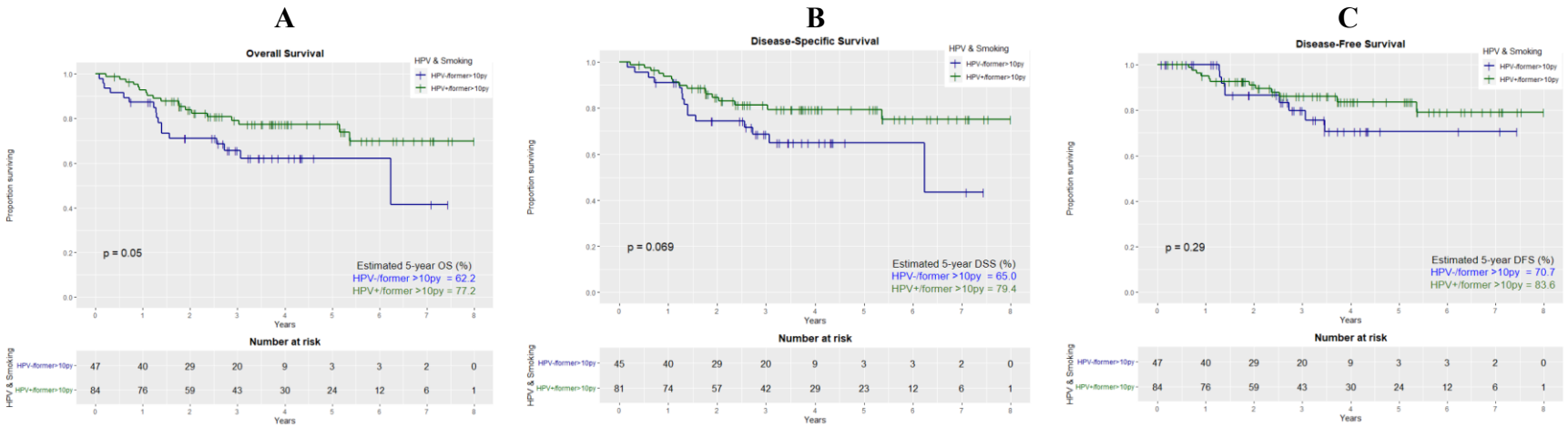
Appx D Figure 2: Survival Analysis of p16+ vs p16- Former smoker >10py: A) OS B) DSS C) DFS



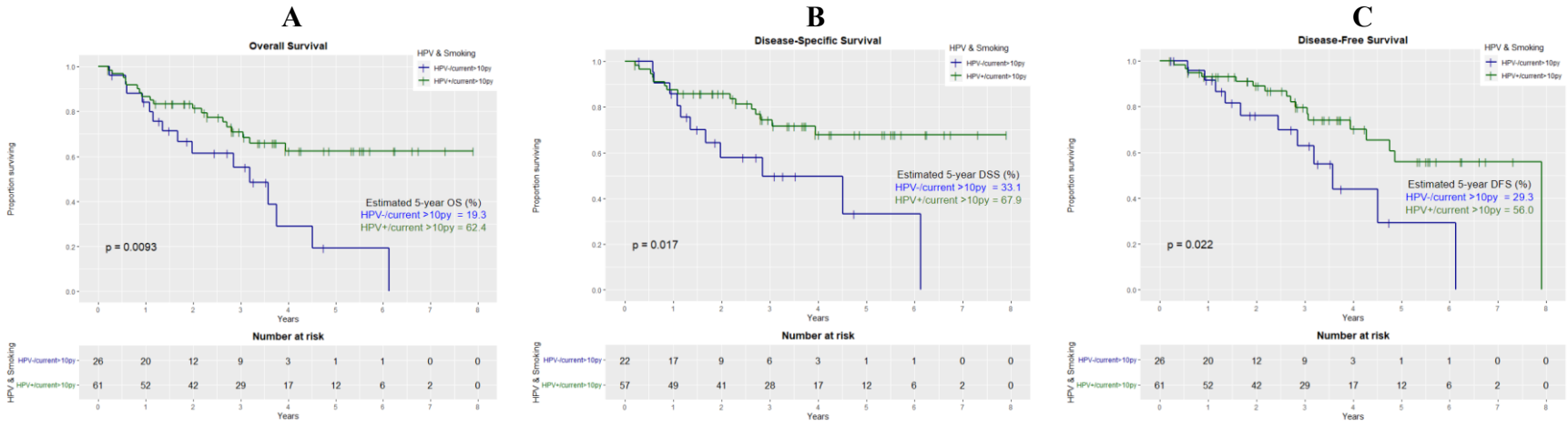
Appx D Figure 3: Survival Analysis of p16+ vs p16- Current smoker >10py: A) OS B) DSS C) DFS



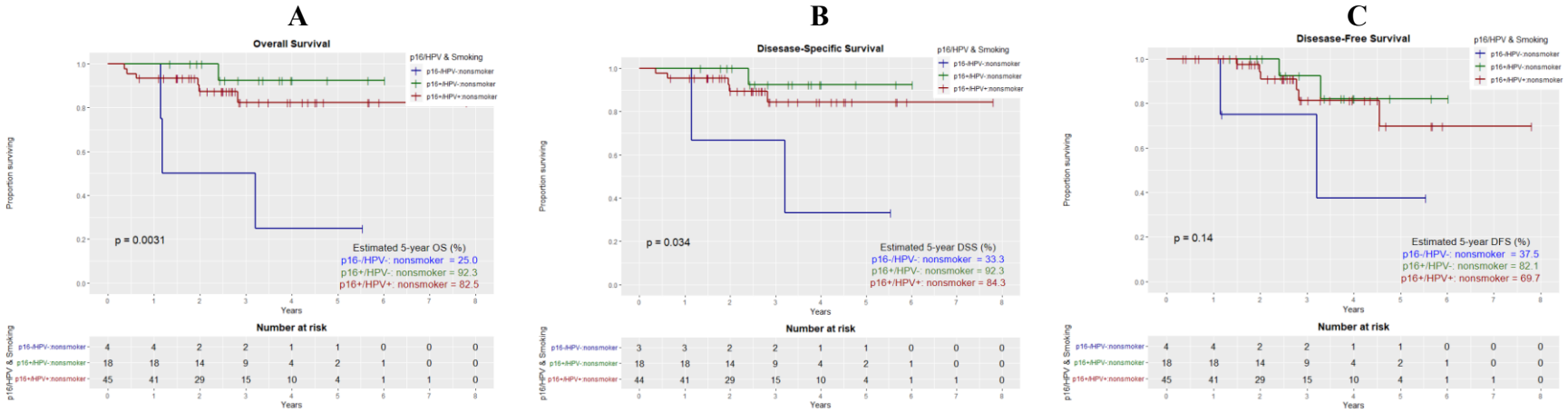
Appx D Figure 4: Survival Analysis of HPV+ vs HPV- Nonsmoker: A) OS B) DSS C) DFS



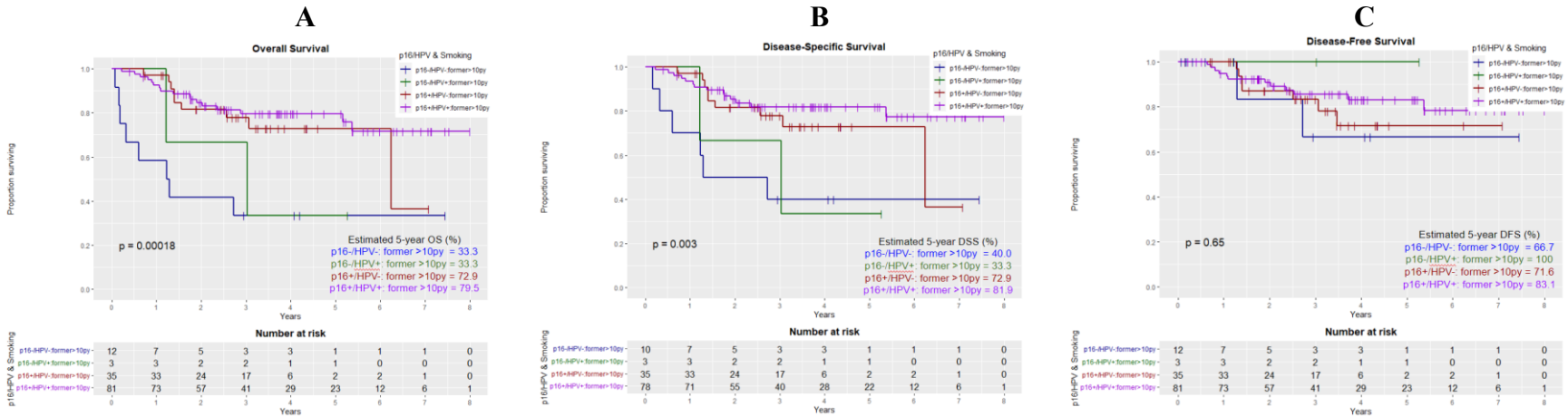
Appx D Figure 5: Survival Analysis of HPV+ vs HPV- Former smoker >10py: A) OS B) DSS C) DFS



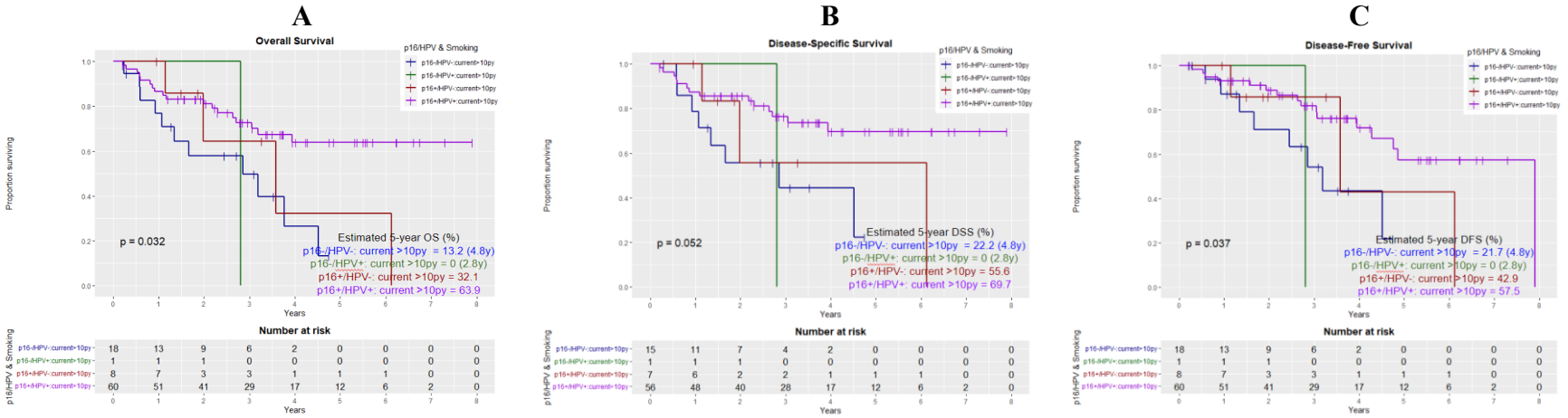
Appx D Figure 6: Survival Analysis of HPV+ vs HPV- Current smoker >10py: A) OS B) DSS C) DFS



Appx D Figure 7: Survival Analysis of p16/HPV and Nonsmoker: A) OS B) DSS C) DFS

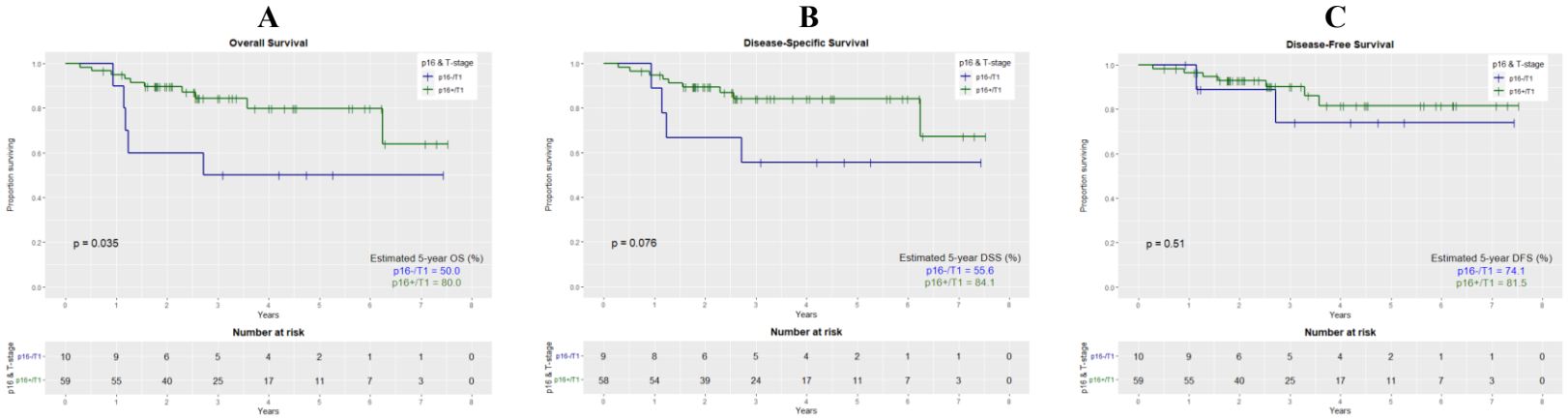


Appx D Figure 8: Survival Analysis of p16/HPV and Former smoker >10py: A) OS B) DSS C) DFS

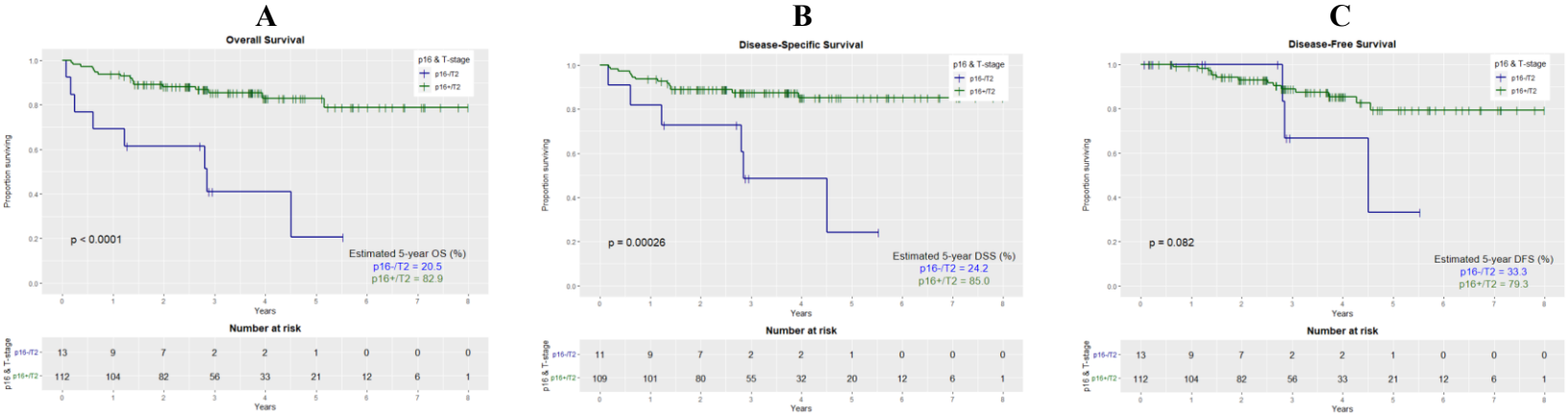


Appx D Figure 9: Survival Analysis of p16/HPV and Current smoker >10py: A) OS B) DSS C) DFS

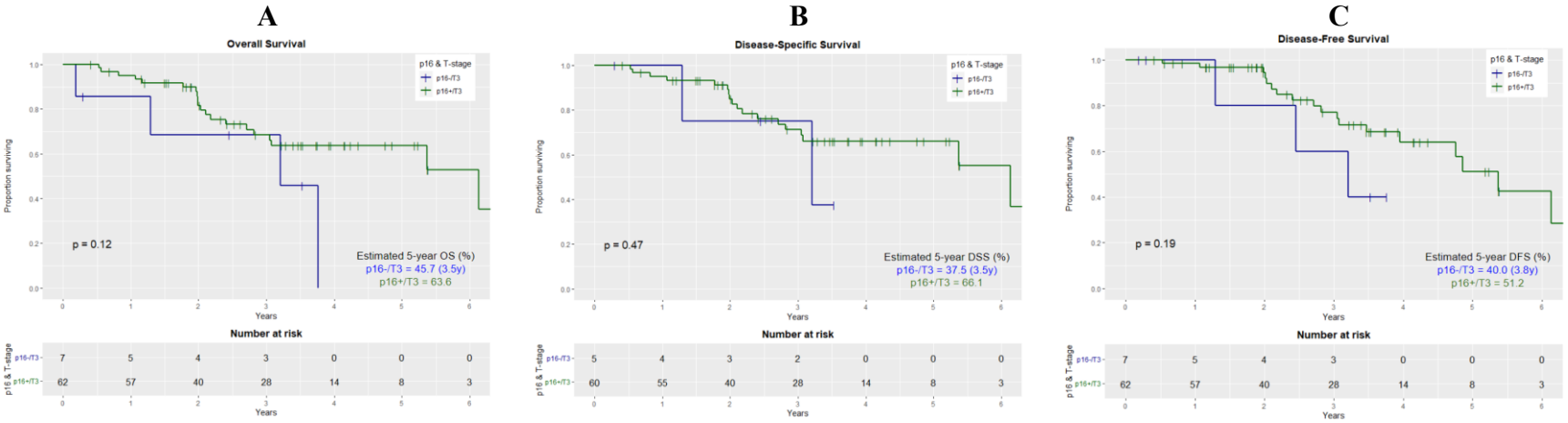
Appendix E: Multivariate Survival Analysis Stratified by Primary Tumor Stage



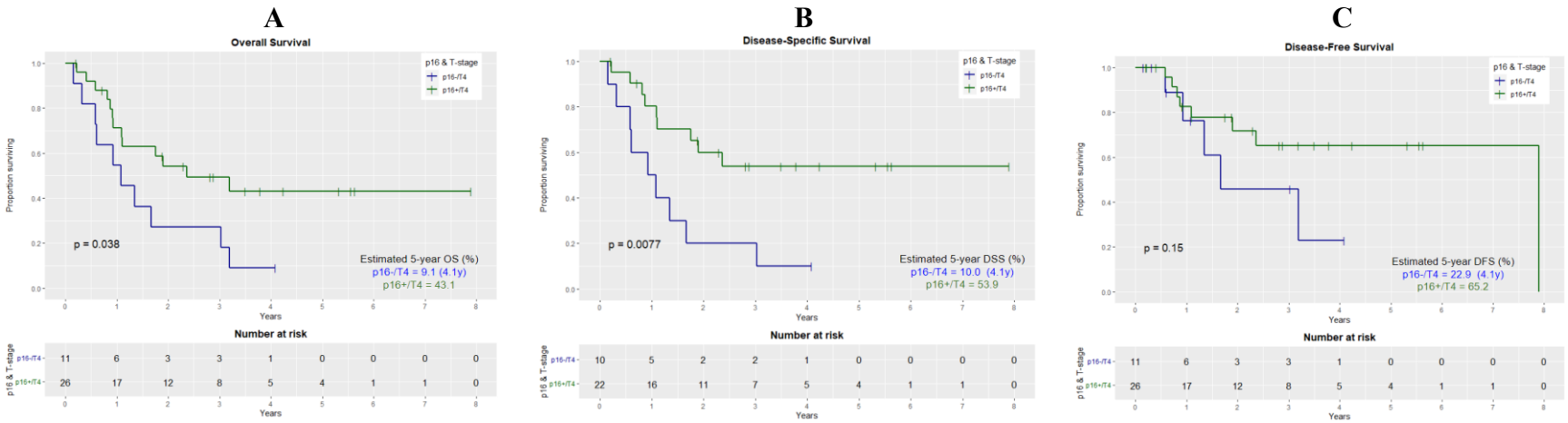
Appx E Figure 1: Survival Analysis of p16+ vs p16- T1: A) OS B) DSS C) DFS



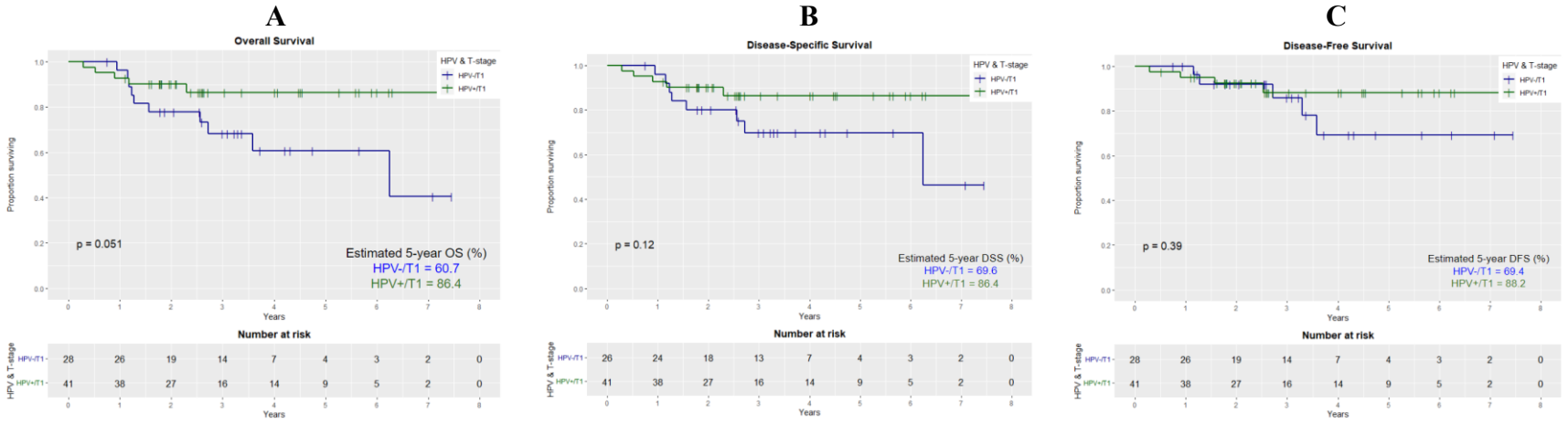
Appx E Figure 2: Survival Analysis of p16+ vs p16- T2: A) OS B) DSS C) DFS



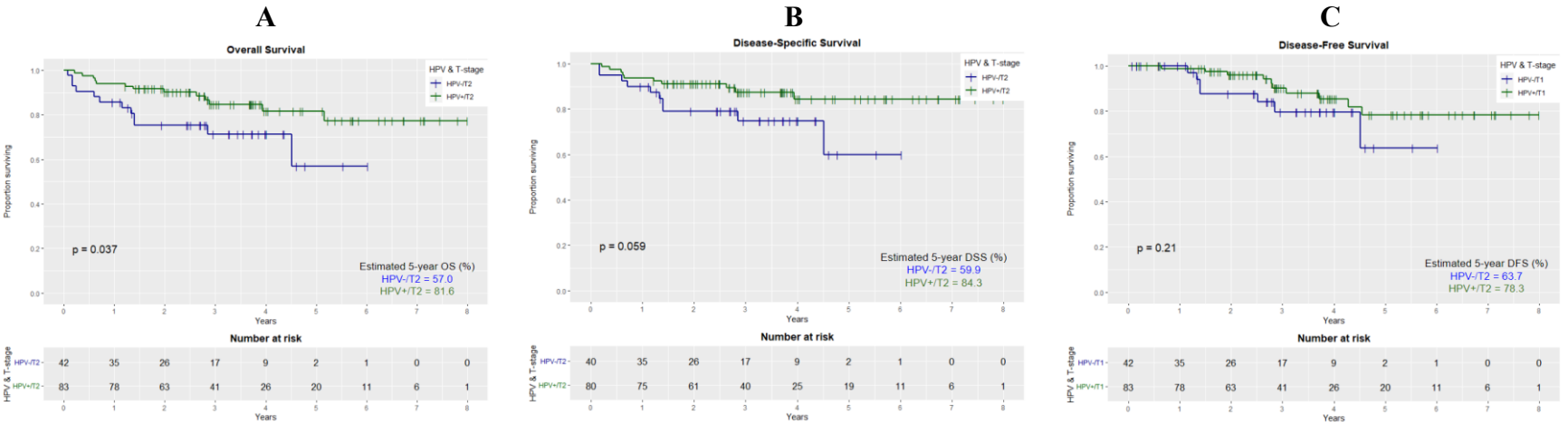
Appx E Figure 3: Survival Analysis of p16+ vs p16- T3: A) OS B) DSS C) DFS



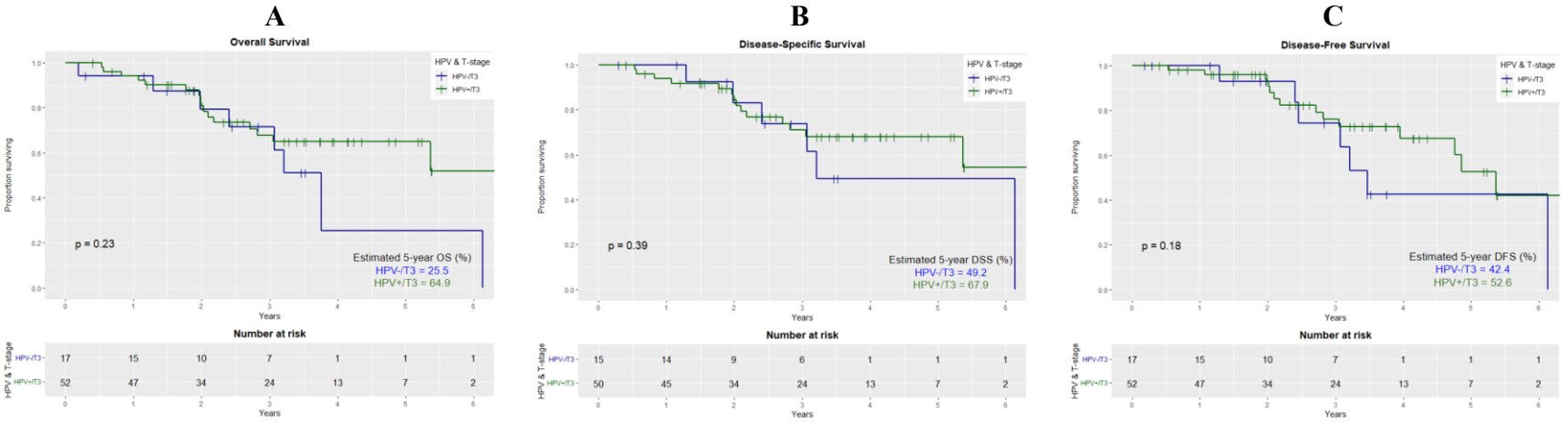
Appx E Figure 4: Survival Analysis of p16+ vs p16- T4: A) OS B) DSS C) DFS



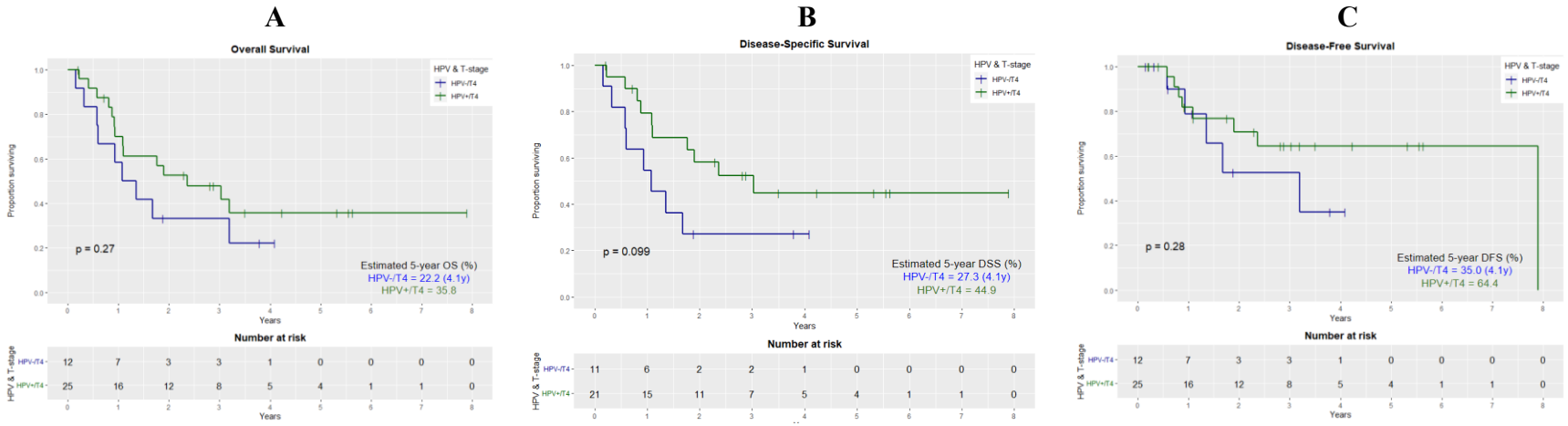
Appx E Figure 5: Survival Analysis of HPV+ vs HPV- T1: A) OS B) DSS C) DFS



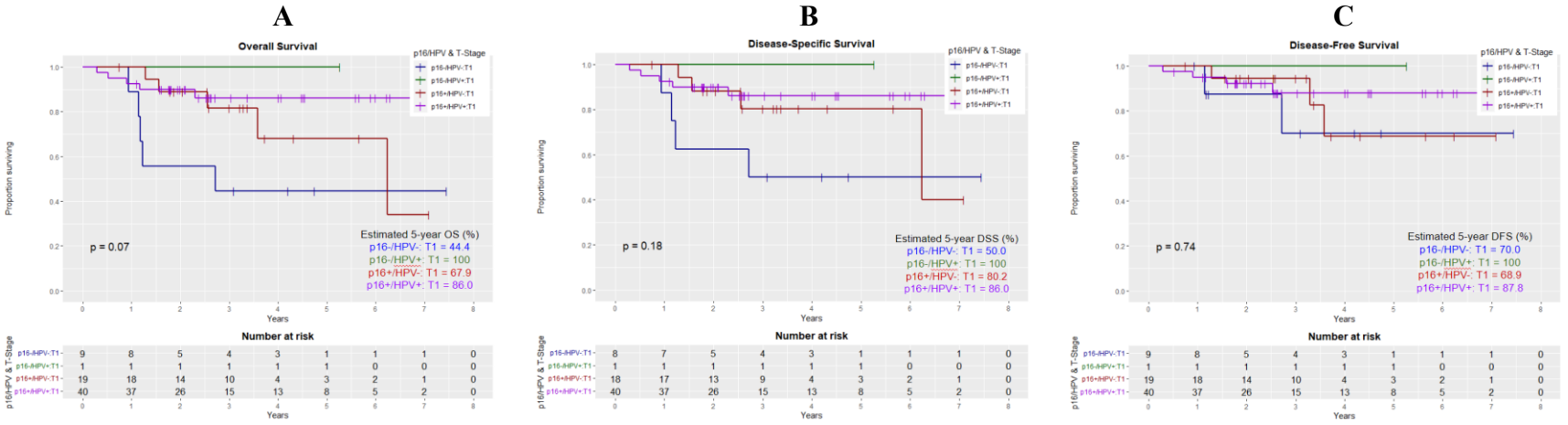
Appx E Figure 6: Survival Analysis of HPV+ vs HPV- T2: A) OS B) DSS C) DFS



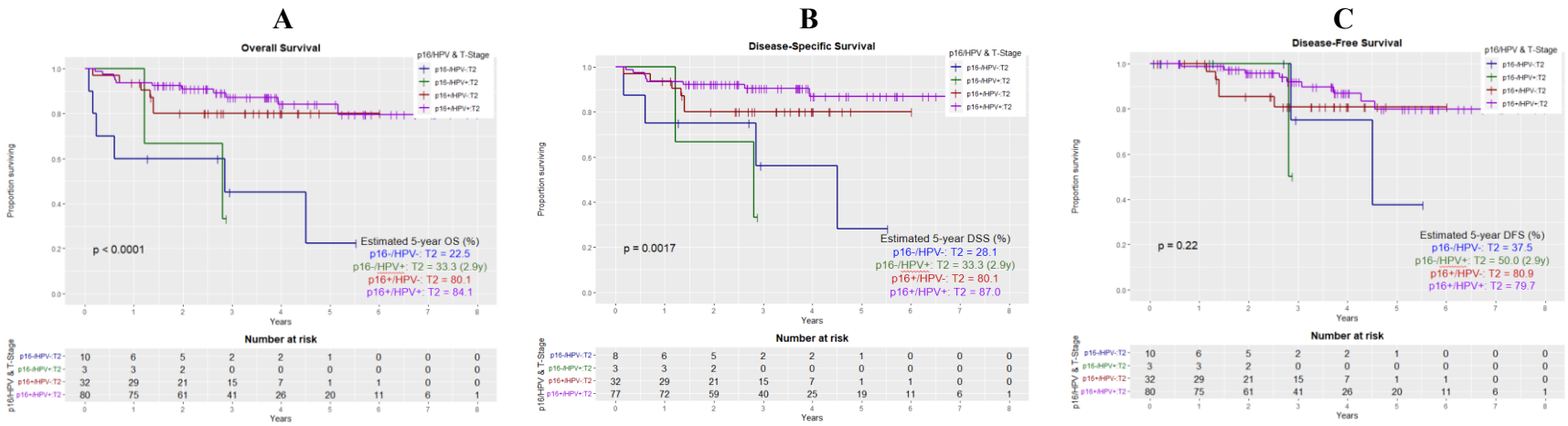
Appx E Figure 7: Survival Analysis of HPV+ vs HPV- T3: A) OS B) DSS C) DFS



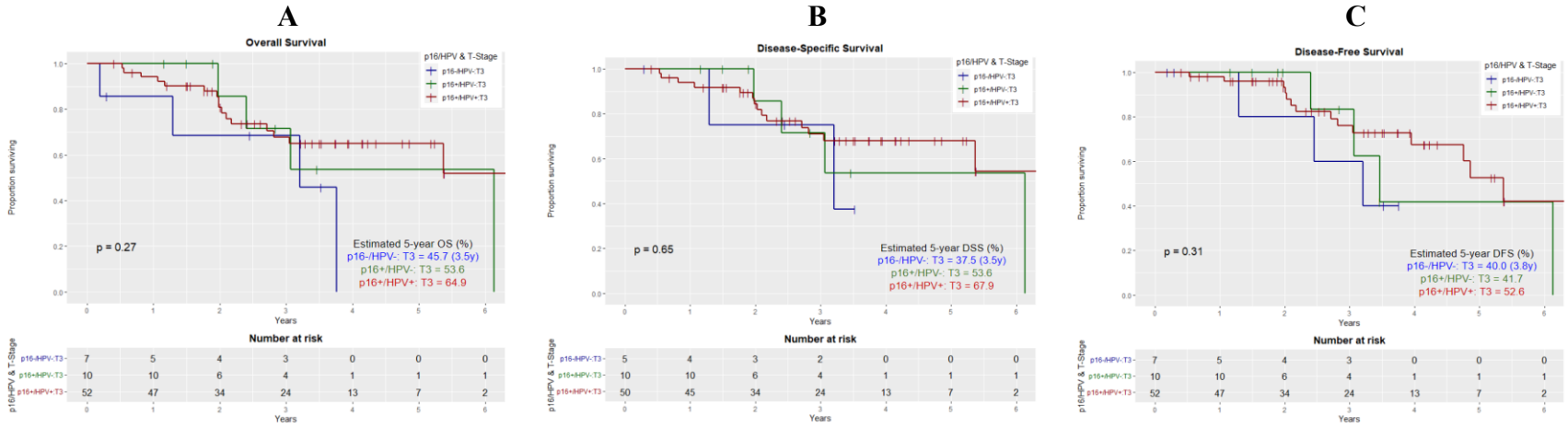
Appx E Figure 8: Survival Analysis of HPV+ vs HPV- T4: A) OS B) DSS C) DFS



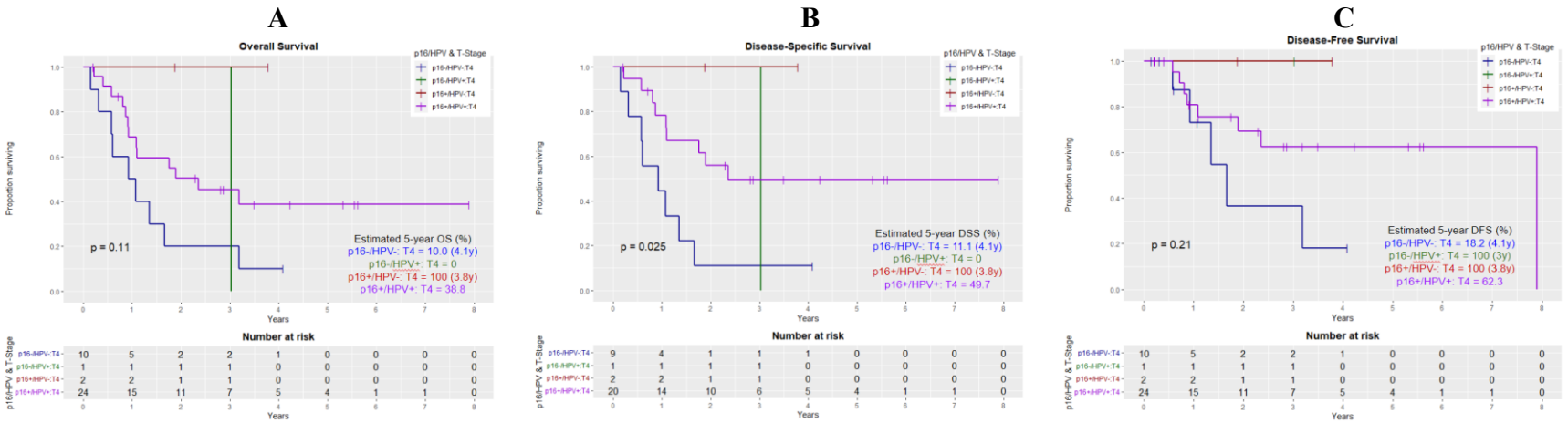
Appx E Figure 9: Survival Analysis of p16/HPV and T1: A) OS B) DSS C) DFS



Appx E Figure 10: Survival Analysis of p16/HPV and T2: A) OS B) DSS C) DFS

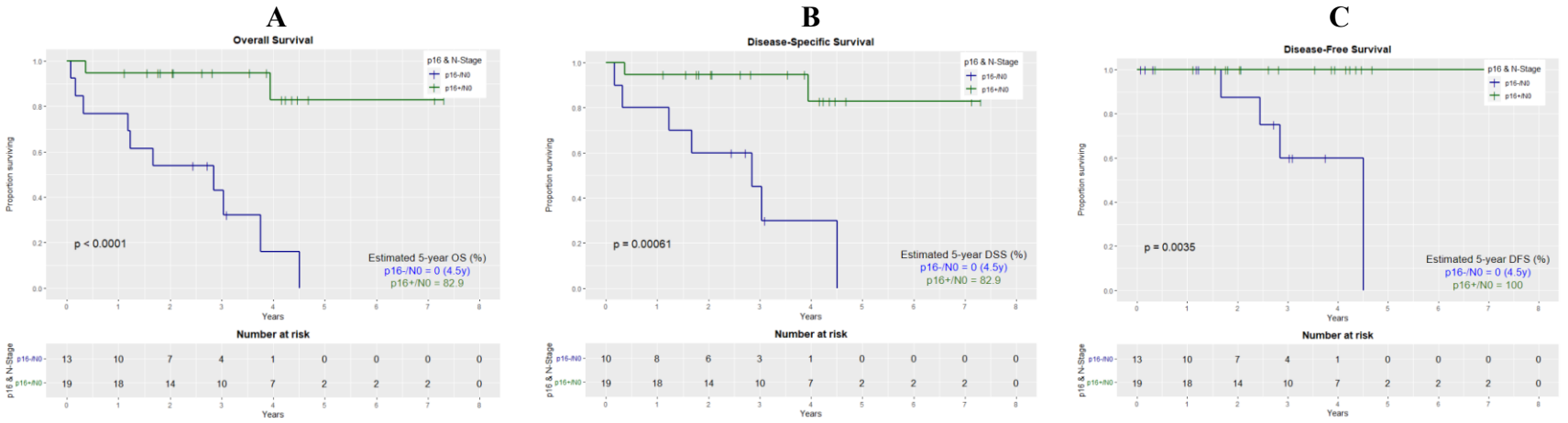


Appx E Figure 11: Survival Analysis of p16/HPV and T3: A) OS B) DSS C) DFS

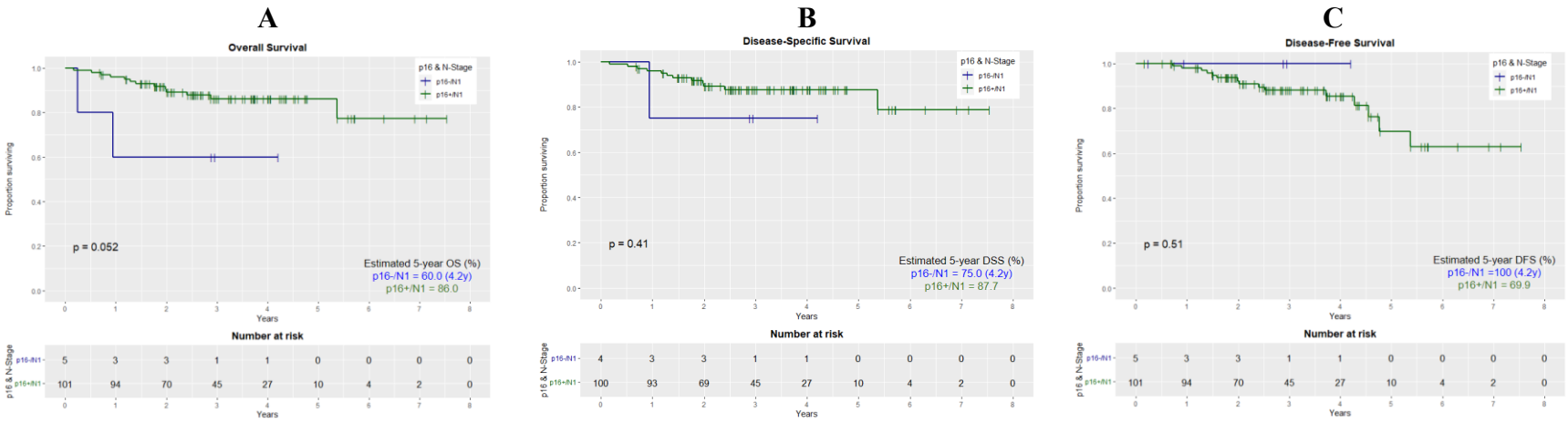


Appx E Figure 12: Survival Analysis of p16/HPV and T4: A) OS B) DSS C) DFS

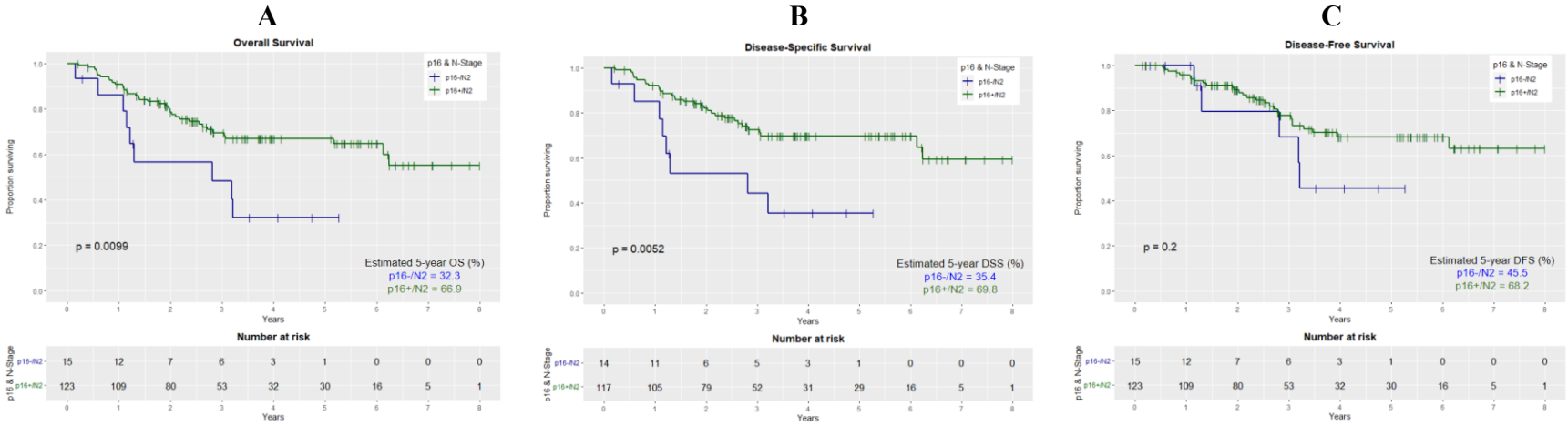
Appendix F: Multivariate Survival Analysis Stratified by Nodal Stage



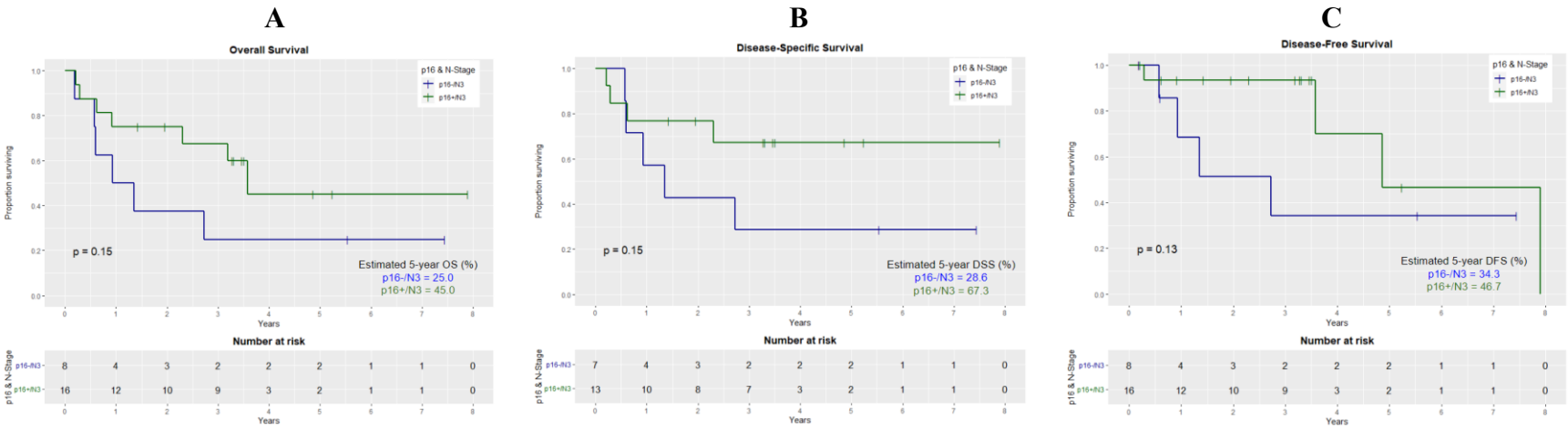
Appx F Figure 1: Survival Analysis of p16+ vs p16- N0: A) OS B) DSS C) DFS



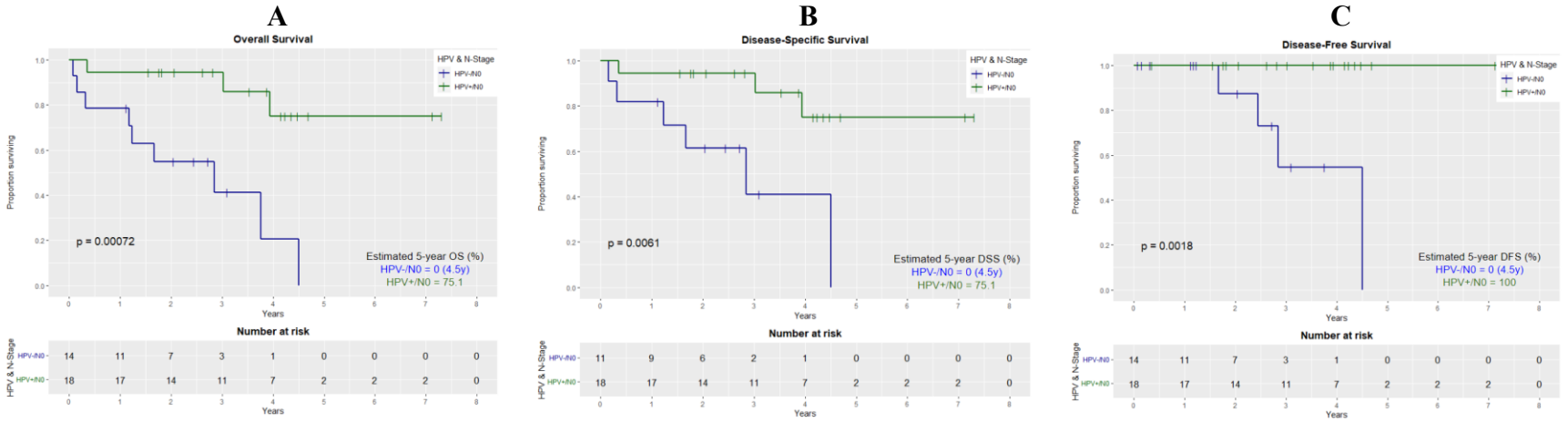
Appx F Figure 2: Survival Analysis of p16+ vs p16- N1: A) OS B) DSS C) DFS



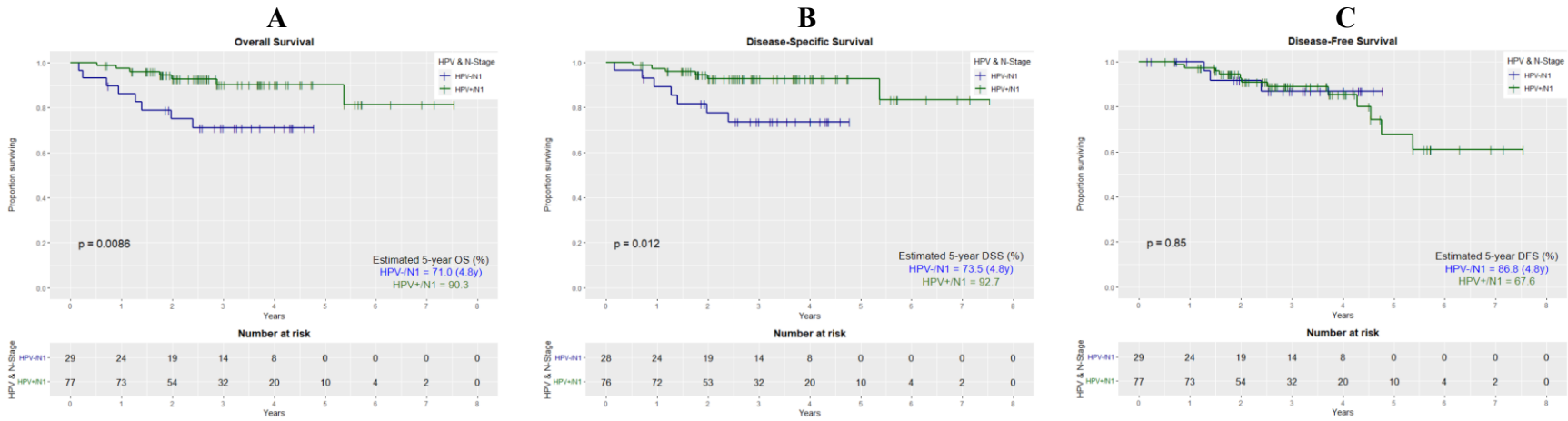
Appx F Figure 3: Survival Analysis of p16+ vs p16- N2: A) OS B) DSS C) DFS



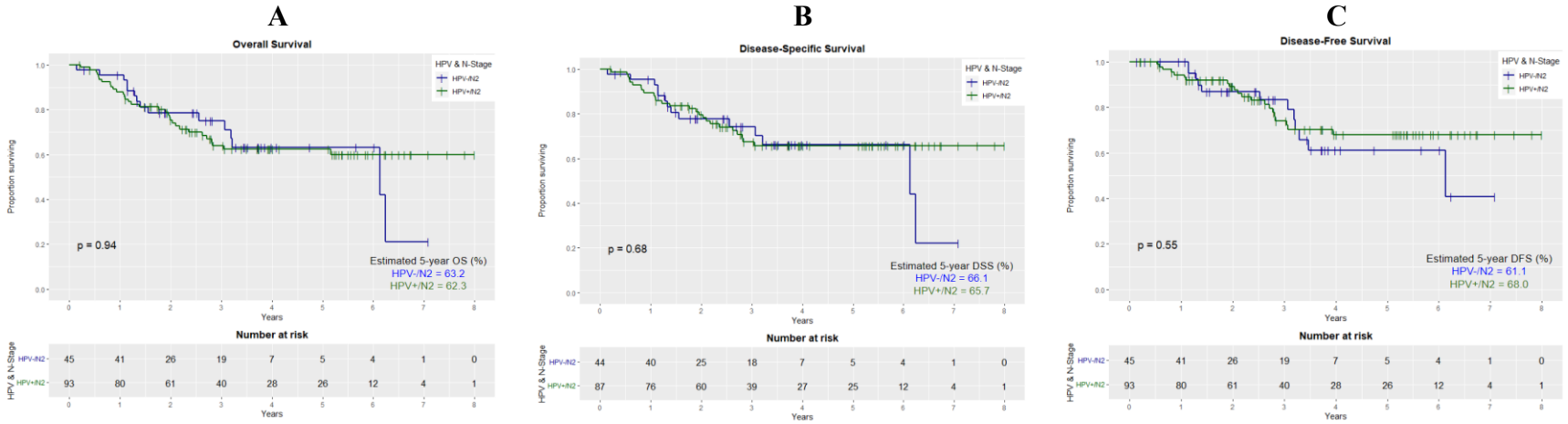
Appx F Figure 4: Survival Analysis of p16+ vs p16- N3: A) OS B) DSS C) DFS



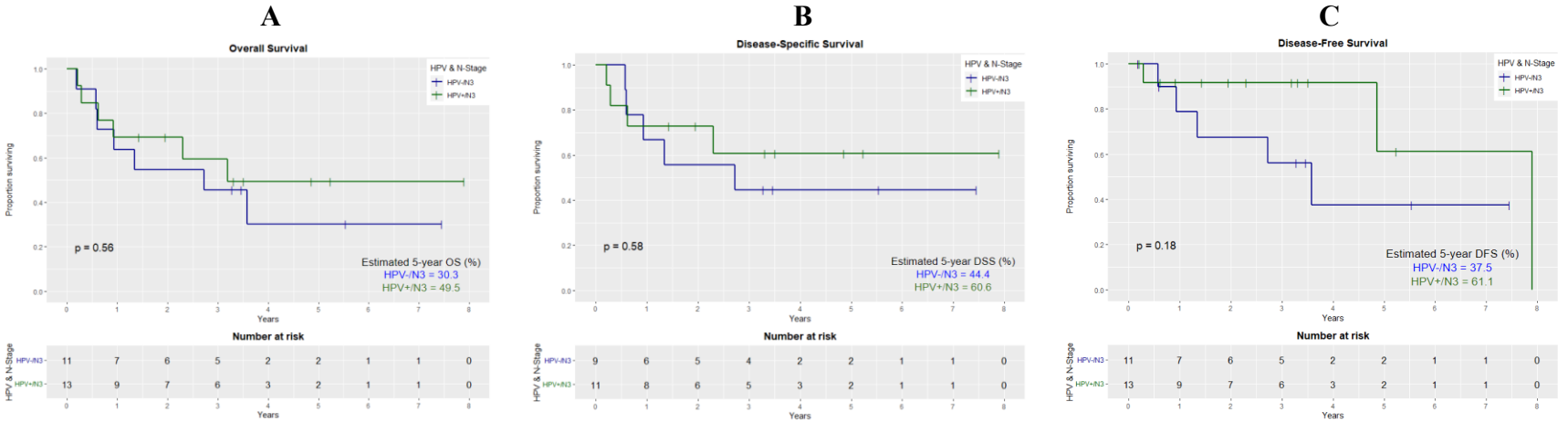
Appx F Figure 5: Survival Analysis of HPV+ vs HPV- N0: A) OS B) DSS C) DFS



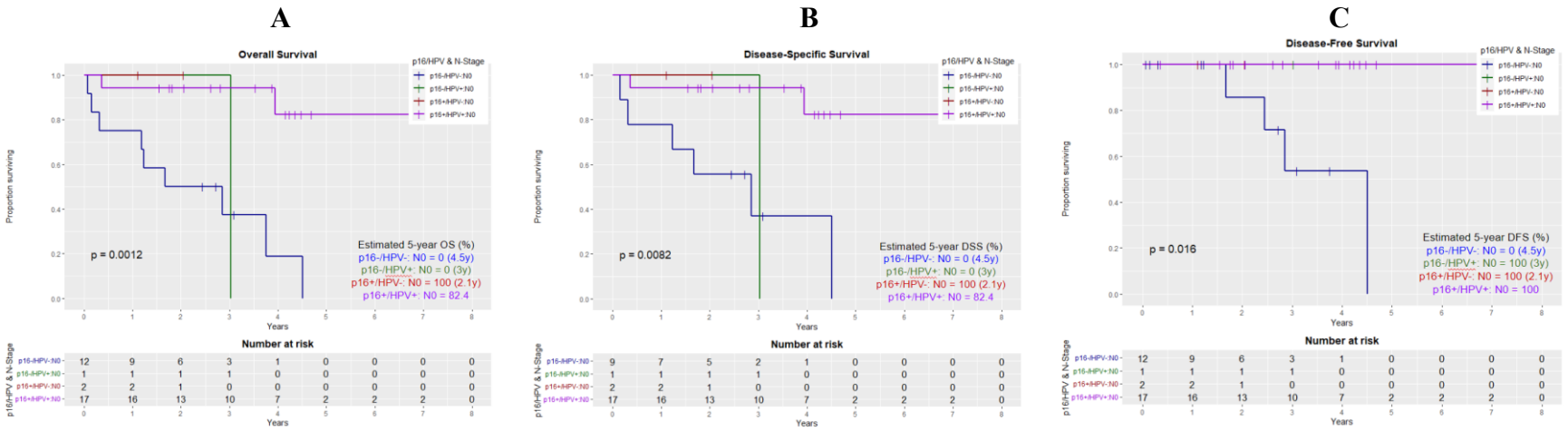
Appx F Figure 6: Survival Analysis of HPV+ vs HPV- N1: A) OS B) DSS C) DFS



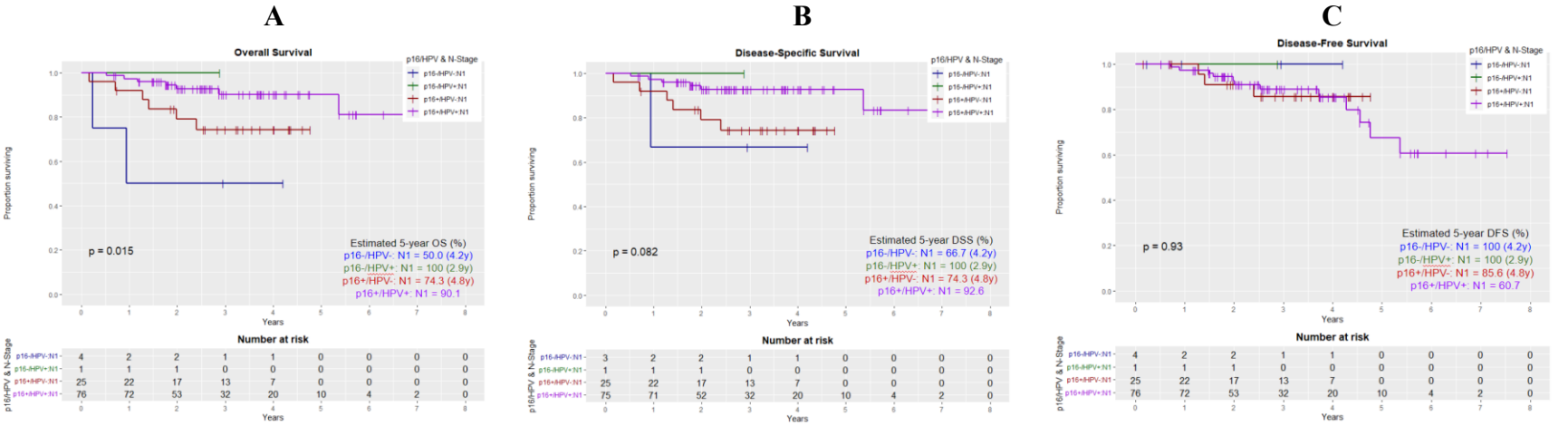
Appx F Figure 7: Survival Analysis of HPV+ vs HPV- N2: A) OS B) DSS C) DFS



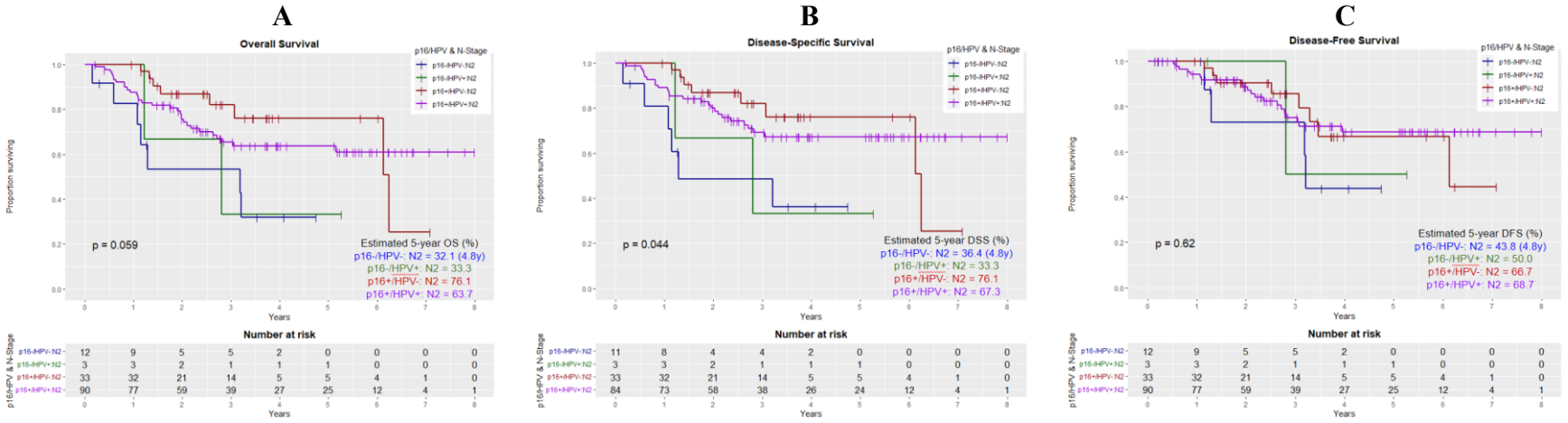
Appx F Figure 8: Survival Analysis of HPV+ vs HPV- N3: A) OS B) DSS C) DFS



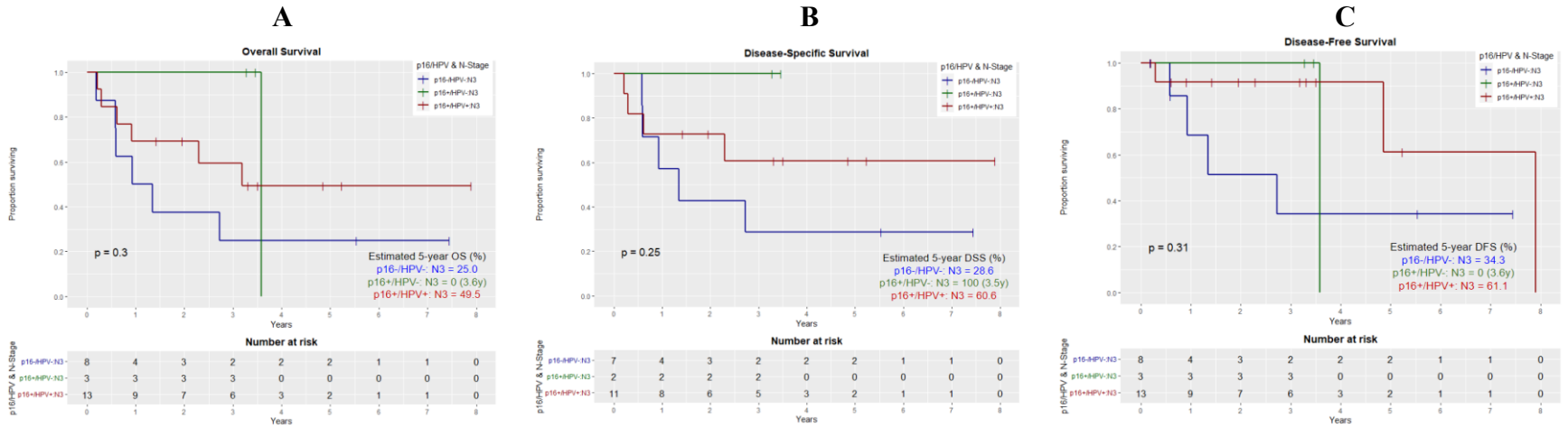
Appx F Figure 9: Survival Analysis of p16/HPV and N0: A) OS B) DSS C) DFS



Appx F Figure 10: Survival Analysis of p16/HPV and N1: A) OS B) DSS C) DFS

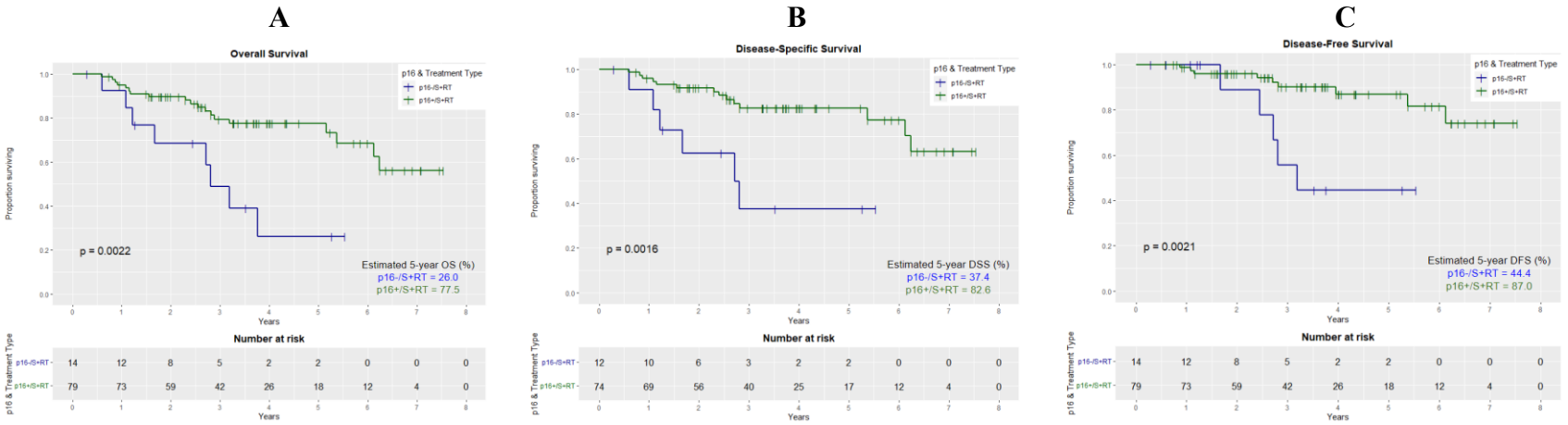


Appx F Figure 11: Survival Analysis of p16/HPV and N2: A) OS B) DSS C) DFS

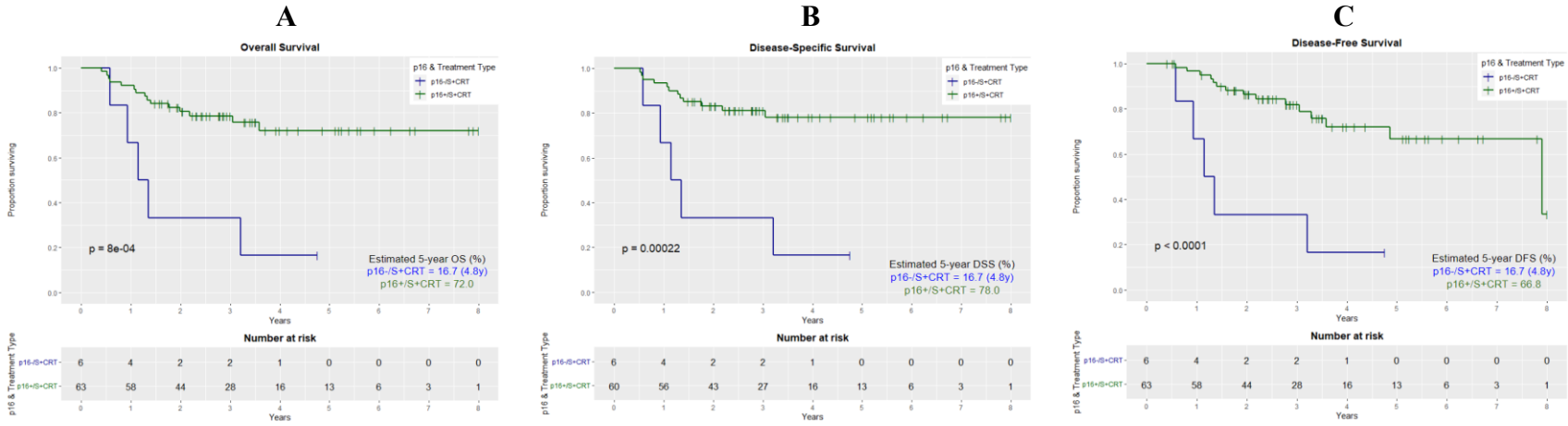


Appx F Figure 12: Survival Analysis of p16/HPV and N3: A) OS B) DSS C) DFS

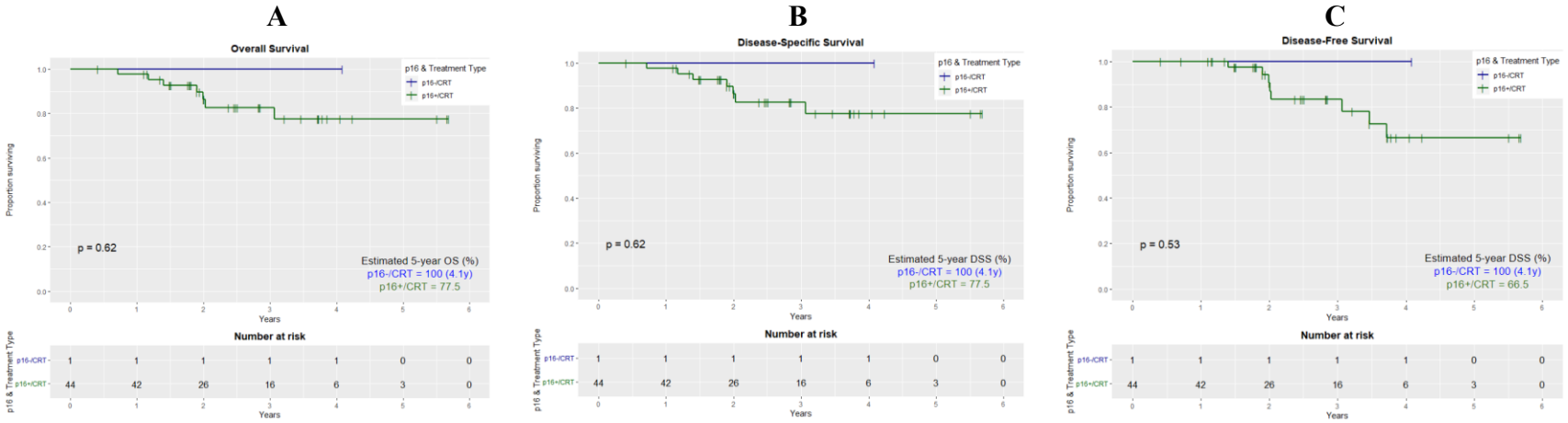
Appendix G: Multivariate Survival Analysis Stratified by Treatment Type



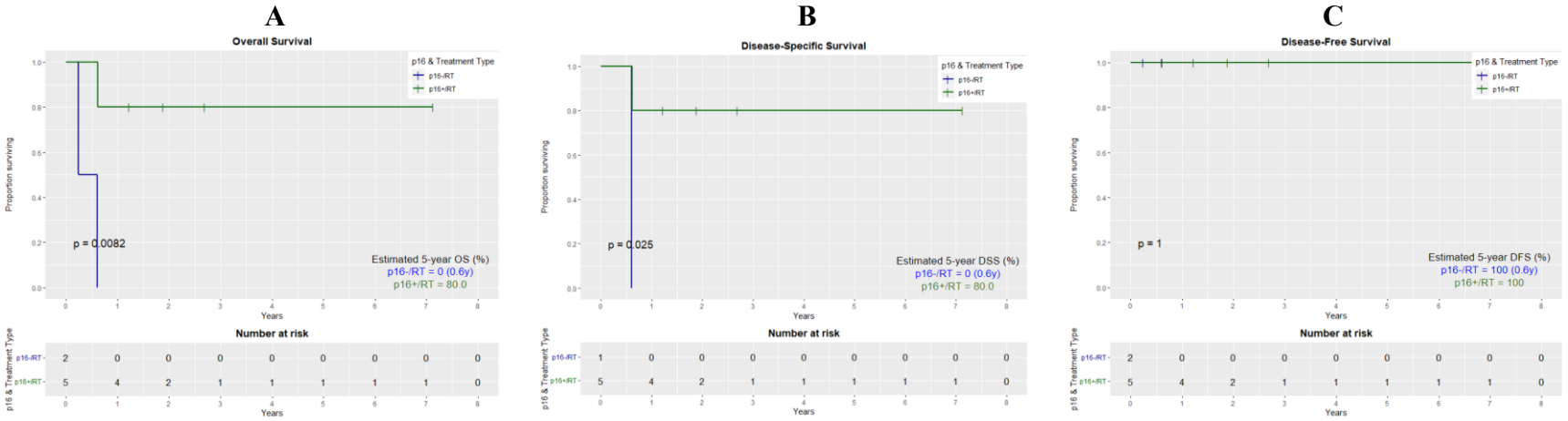
Appx G Figure 1: Survival Analysis of p16+ vs p16- S+RT: A) OS B) DSS C) DFS



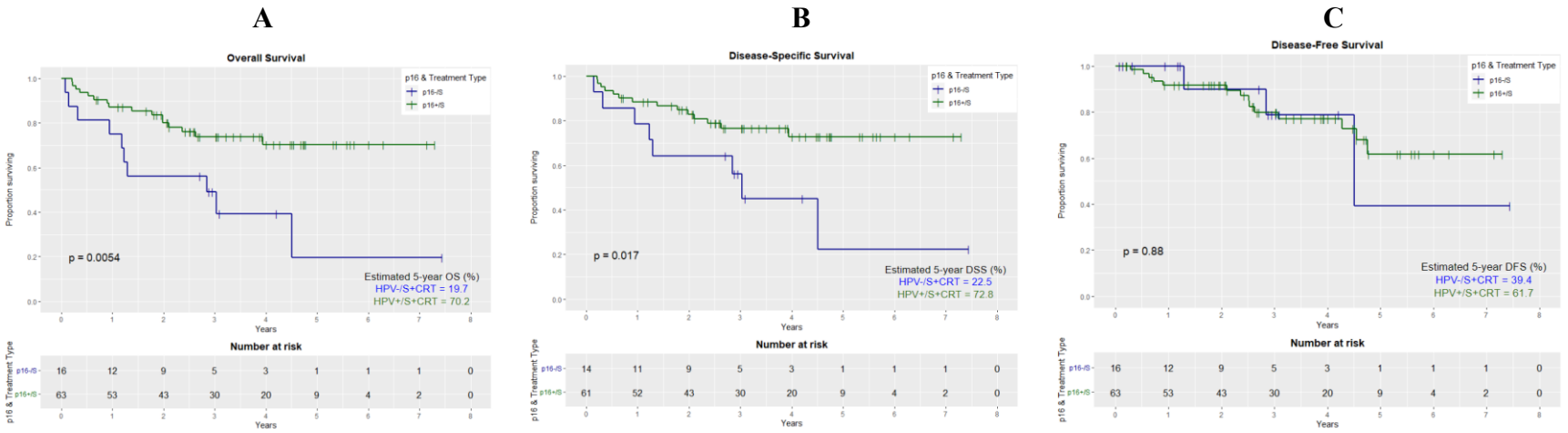
Appx G Figure 2: Survival Analysis of p16+ vs p16- S+CRT: A) OS B) DSS C) DFS



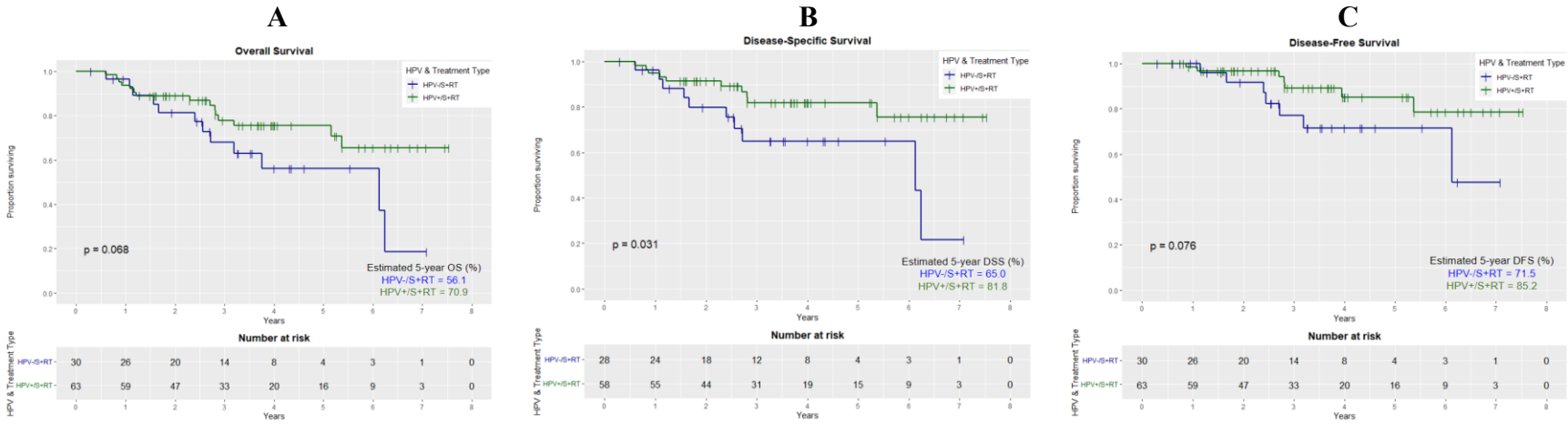
Appx G Figure 3: Survival Analysis of p16+ vs p16- CRT: A) OS B) DSS C) DFS



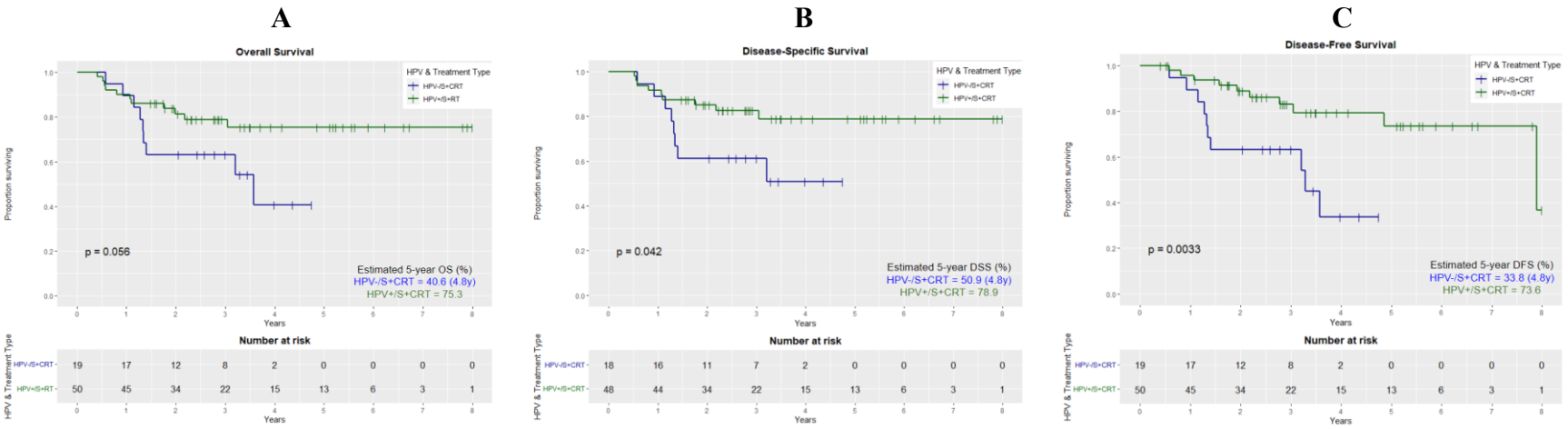
Appx G Figure 4: Survival Analysis of p16+ vs p16- RT: A) OS B) DSS C) DFS



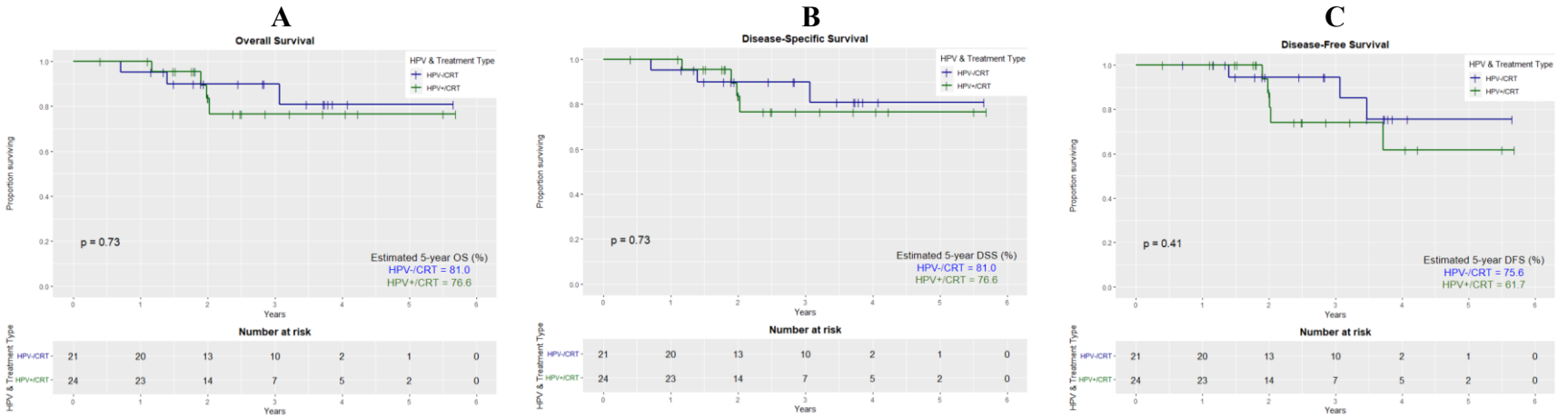
Appx G Figure 5: Survival Analysis of p16+ vs p16- S: A) OS B) DSS C) DFS



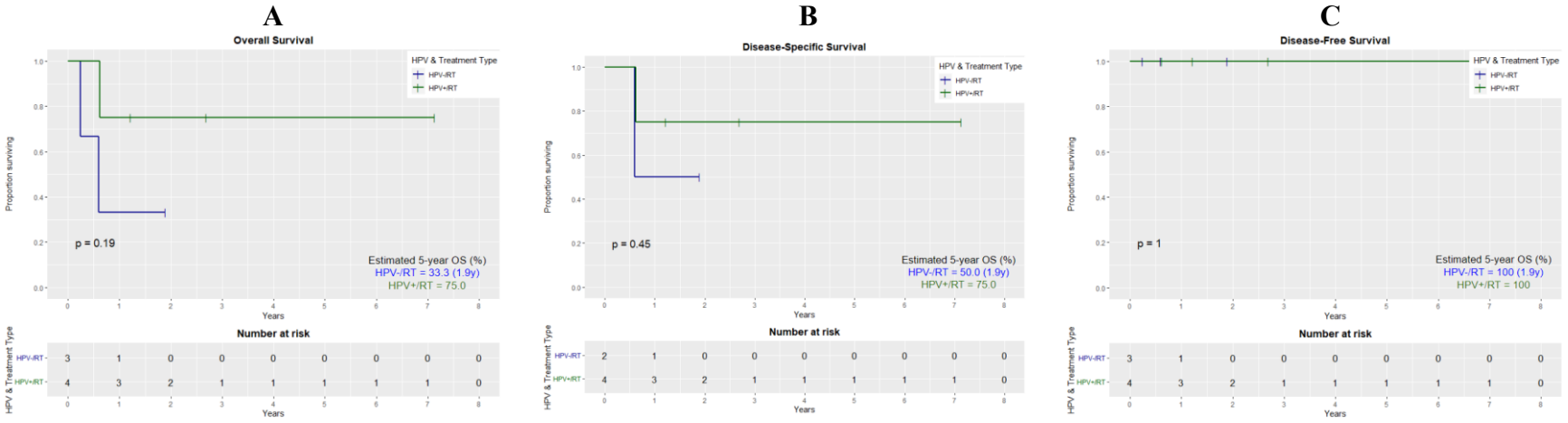
Appx G Figure 6: Survival Analysis of HPV+ vs HPV- S+RT: A) OS B) DSS C) DFS



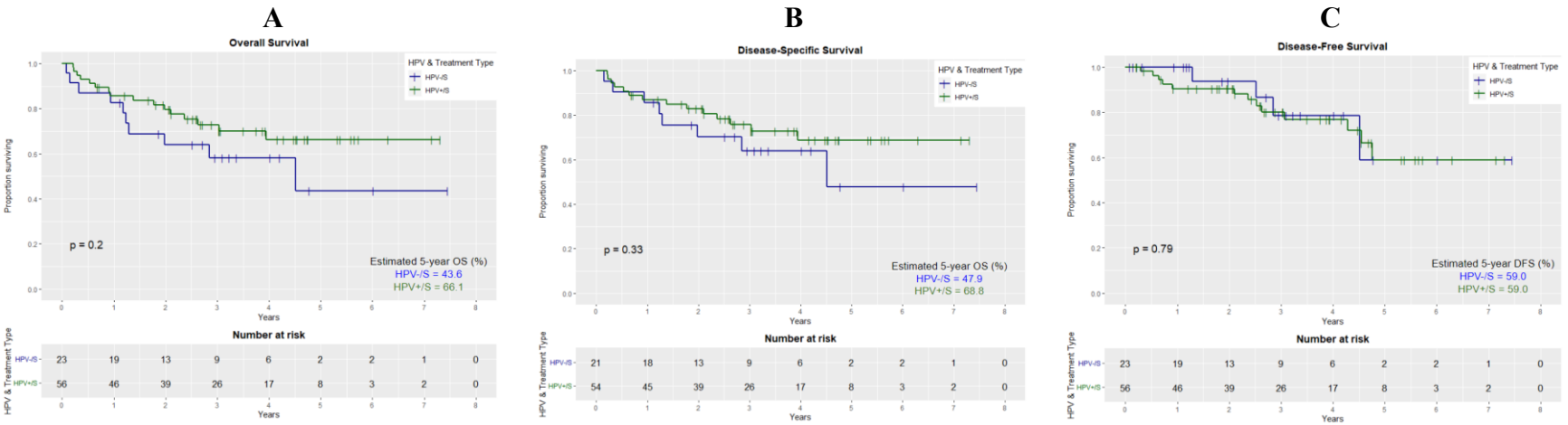
Appx G Figure 7: Survival Analysis of HPV+ vs HPV- S+CRT: A) OS B) DSS C) DFS



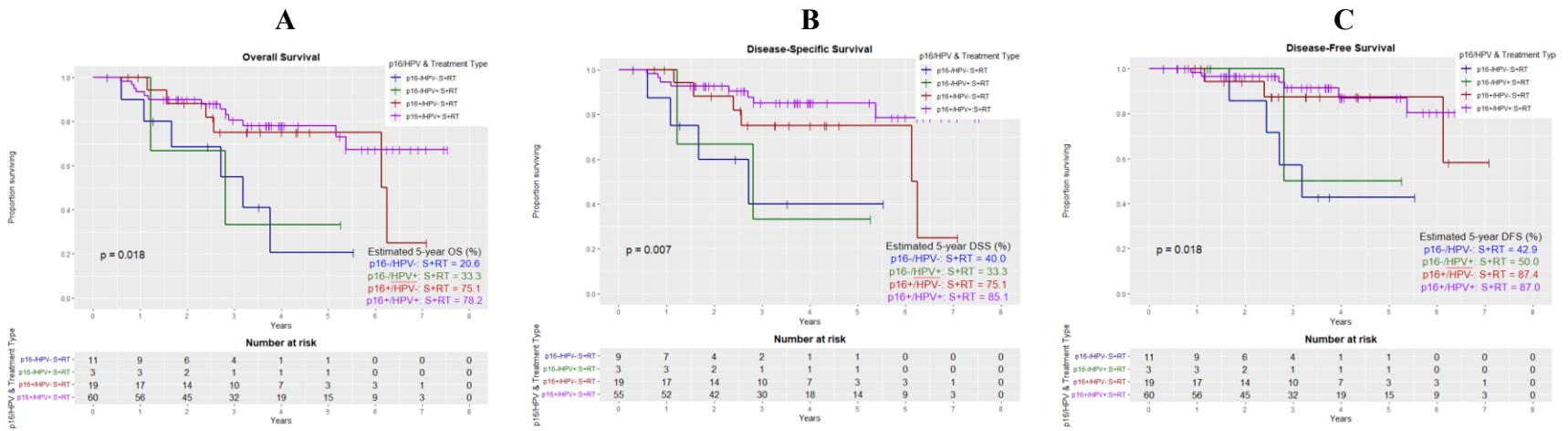
Appx G Figure 8: Survival Analysis of HPV+ vs HPV- CRT: A) OS B) DSS C) DFS



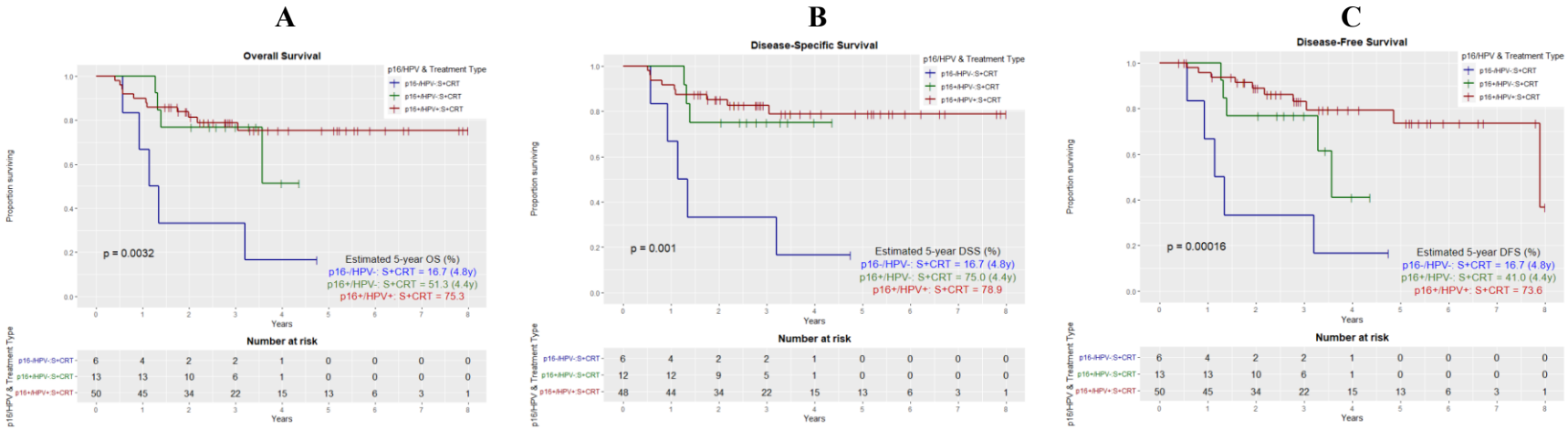
Appx G Figure 9: Survival Analysis of HPV+ vs HPV- RT: A) OS B) DSS C) DFS



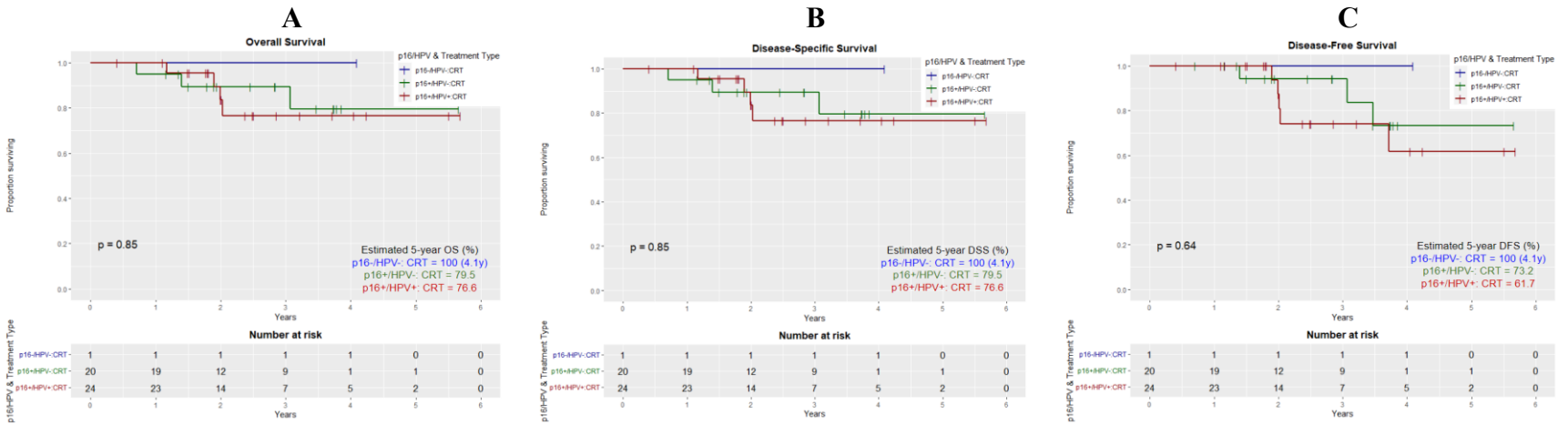
Appx G Figure 10: Survival Analysis of HPV+ vs HPV- S: A) OS B) DSS C) DFS



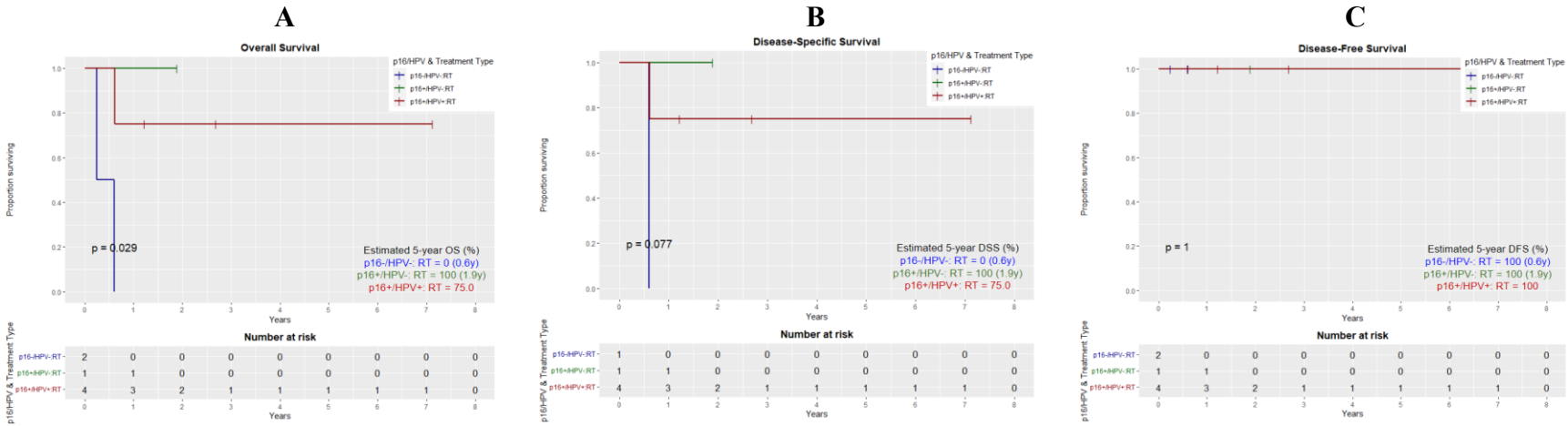
Appx G Figure 11: Survival Analysis of p16/HPV and S+RT: A) OS B) DSS C) DFS



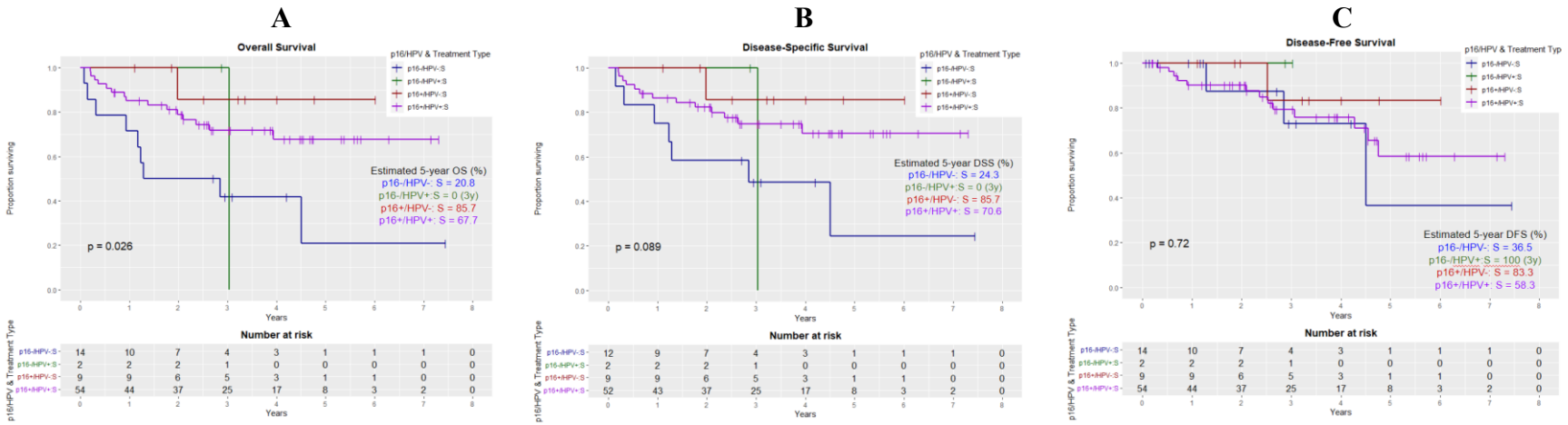
Appx G Figure 12: Survival Analysis of p16/HPV and S+CRT: A) OS B) DSS C) DFS



Appx G Figure 13: Survival Analysis of p16/HPV and CRT: A) OS B) DSS C) DFS



Appx G Figure 14: Survival Analysis of p16/HPV and RT: A) OS B) DSS C) DFS



Appx G Figure 15: Survival Analysis of p16/HPV and S: A) OS B) DSS C) DFS