Advances in the treatment and diagnosis of human papillomavirus-related oropharyngeal cancer: the advantages of de-escalated therapeutic strategies and the need for standardized viral testing

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Abstract

Cases of head and neck cancer (HNC), particularly of oropharyngeal cancer (OPC), have risen in incidence due to human papillomavirus (HPV) infections. Recently, HPV-positive OPC has been recognized as a distinct HNC subtype due to its unique etiology, marked molecular characteristics, and superior patient prognoses and treatment responses. However, treatment modalities remain the same regardless of HPV status, consisting of surgery, radiation, and chemotherapy combinations, which often result in severe acute or chronic toxicities. With favorable responses in HPV-positive OPC, de-escalated therapeutic strategies have been explored in these patients to minimize treatment-related adverse events while maintaining anti-cancer efficacy. Despite promising responses to these treatments, de-escalated strategies have yet to be incorporated into the standard of care for HPV-positive OPC patients. The objective of this thesis is to address the major barriers hindering the widespread implementation of de-escalated therapies in the treatment of HPV-positive OPC, notably the limited evidence supporting their efficacy, and the lack of a standardized HPV testing regimen in OPC.

First, recent advances in de-escalated therapies for HPV-positive HNC were assessed in a narrative literature review, providing an updated summary of results from clinical trials evaluating their efficacy. Recent studies have reported lower toxicities and favorable efficacy with reduced-dose radiotherapy, neoadjuvant docetaxel or paclitaxel chemotherapy, and adjuvant, neoadjuvant or induction nivolumab immunotherapy. However, established targeted therapies like cetuximab have been cautioned in HPV-positive patients due to unfavorable outcomes. Nevertheless, findings from recent clinical trials highlight the benefits of novel de-escalated treatments for HPV-related OPC, warranting a re-evaluation of their status in the standard of care.

Next, to address the lack of standardized HPV testing in OPC, the differences between the most common testing methods used in OPC, namely p16 immunohistochemistry (IHC) and direct molecular HPV detection, were examined in an original research article. In this study, molecular HPV detection was assessed in 124 HPV-positive OPC formalin-fixed paraffin-embedded (FFPE) tissue specimens using the Anyplex™ II HPV28 Detection (Anyplex II) genotyping assay and compared against detection rates of three p16 IHC antibodies. The p16 antibody E6H4 had the best HPV detection of the IHC clones tested. Molecular HPV detection significantly outperformed IHC at p16 positivity thresholds of 50% and 70%, supporting the addition of direct HPV detection to

validate p16 IHC in OPC. However, molecular HPV detection rates differ based on the assay used. Results from the Anyplex II genotyping were compared against an *in-house* real-time polymerase chain reaction (qPCR) assay, finding superior HPV detection with the commercial test. Conversely, molecular genotyping was validated using the INNO-LiPA® HPV Genotyping Extra II assay in a subset of samples, finding significantly higher HPV detection than the Anyplex II assay, though these results should be interpreted with caution due to a small sample size. Overall, these results support the use of molecular HPV testing in addition to p16 IHC in OPC, though further investigations may be needed to determine the optimal molecular assay.

The studies presented in this thesis highlight the advantages of de-escalated therapies for the treatment of HPV-related OPC and the importance of establishing a standardized HPV testing regimen for OPC that combines p16 IHC and direct molecular testing. Together, they may help improve the treatment and diagnosis of HPV-related OPC.

Résumé

L'incidence des cancers otorhinolaryngées (ORLs), particulièrement des cancers de l'oropharynx, a augmenté en raison des infections du virus du papillome humain (VPH). Récemment, le cancer de l'oropharynx lié au VPH a été différencié des autres cancers ORL en raison de son étiologie et de ses caractéristiques moléculaires uniques, ainsi que les prognostiques favorables des patients. Cependant, les choix de traitement pour le cancer de l'oropharynx demeurent les mêmes indépendamment des infections du VPH. Ces derniers comprennent des combinaisons de chirurgie, de radiothérapie et de chimiothérapie qui entraînent souvent des toxicités sévères. En raison des prognostiques favorables des patients atteints du cancer de l'oropharynx lié au VPH, des stratégies thérapeutiques moins intenses sont en développement afin de minimiser les effets indésirables des traitements tout en maintenant leurs efficacités anticancéreuses. Malgré leur succès initial, ces stratégies ne sont pas actuellement inclues dans les normes de traitement pour les patients atteints du cancer de l'oropharynx lié au VPH. Ainsi, l'objectif de cette thèse est d'adresser les facteurs principaux qui limitent l'adoption des traitements moins intenses pour le cancer de l'oropharynx lié au VPH, soit la nécessité de recherches plus approfondies sur leur efficacité et le manque d'un test standard de dépistage du VPH pour l'oropharynx.

Premièrement, les avancements dans le développement et la validation des thérapies moins intenses pour le cancer de l'oropharynx lié au VPH ont été résumés dans une revue de littérature afin d'offrir un bilan des résultats des essais cliniques récents. Notamment, plusieurs études ont rapporté des effets secondaires réduits et des réponses antitumorales favorables avec l'utilisation de la radiothérapie à dose réduite, de la chimiothérapie docetaxel ou paclitaxel en tant que traitements néoadjuvants et du nivolumab comme immunothérapie néoadjuvante, adjuvante ou d'induction. Toutefois, d'autres études ont rapporté que la thérapie ciblée cetuximab, qui est fréquemment utilisée contre le cancer de l'oropharynx, n'est pas recommandée pour les patients atteints des infections du VPH en raison d'une mauvaise survie comparé aux traitements standards. Néanmoins, les résultats des études cliniques récentes soulignent les avantages des traitements moins intenses pour le cancer de l'oropharynx lié au VPH, justifiant une réévaluation de leur statut dans les normes de traitement pour ce cancer.

Deuxièmement, plusieurs méthodes de détection du VPH ont été comparés dans une étude de recherche originale afin d'adresser le manque d'un test standard de dépistage du VPH pour

l'oropharynx. Particulièrement, l'immunohistochimie (IHC) de la protéine p16 et les méthodes moléculaires de détection du VPH, dont le génotypage du virus et un test interne d'amplification en chaîne par polymérase (ACP), ont été comparés. Dans cette étude, le génotypage du VPH a été effectué avec le test commercial Anyplex™ II HPV28 Detection (Anyplex II) dans 124 échantillons de tissus fixés au formol et inclus en paraffine provenant des patients atteints du cancer de l'oropharynx. Le taux de détection du VPH avec le génotypage a ensuite été comparé à celui de l'IHC réalisé avec trois anticorps ciblant la protéine p16 et trois seuils pour attribuer un statut de VPH positif selon l'expression de p16. L'anticorps E6H4 a permis la meilleure détection du VPH parmi ceux testés, mais le génotypage avait considérablement plus de détection du VPH que l'IHC à des seuils de 50% et 70% d'expression de p16. Concernant les méthodes de détection moléculaires, les taux de détection du VPH variaient en fonction du test de dépistage utilisé. Les résultats du génotypage Anyplex II ont été comparés à ceux obtenus avec le test interne d'ACP, ayant une plus grande détection du VPH que ce-dernier. Le génotypage Anyplex II a aussi été validé avec un autre test commercial, soit le INNO-LiPA® HPV Genotyping Extra II, dans un sous-échantillon de tissus, mais avait une détection inférieure du VPH à celui-ci. Pourtant, ces résultats doivent être interprétés avec prudence en raison de la petite taille du sous-échantillon. Ensemble, ces résultats supportent l'utilisation des méthodes de détection moléculaire en plus de l'IHC pour améliorer la détection du VPH dans l'oropharynx, mais des recherches additionnelles seront nécessaires afin de déterminer la méthode moléculaire optimale.

En bref, les études présentées dans cette thèse soulignent les avantages des traitements moins intenses pour le cancer de l'oropharynx lié au VPH, ainsi que l'importance d'établir un test standard de dépistage du VPH pour l'oropharynx qui utilise à la fois l'IHC de p16 et la détection moléculaire. Ensemble, elles pourraient contribuer à l'amélioration du diagnostic et du traitement du cancer de l'oropharynx lié au VPH.

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Contribution of Authors

Jenna Bouassaly performed the writing of this thesis in its entirety. The source articles from which Figures 1, 4, and 5 were entirely or partially reproduced are cited in each figure legend, along with the appropriate license where applicable.

For Manuscript 1 titled "Rethinking Treatment Paradigms: Neoadjuvant Therapy and De-Escalation Strategies in HPV-Positive Head and Neck Cancer", Jenna Bouassaly performed the literature search, data retrieval, synthesis, and curation, writing of the first draft, and creation of the figures and tables. All authors (Jenna Bouassaly, Naser Karimi, Luiz Paulo Kowalski, Khalil Sultanem, Moulay Alaoui-Jamali, Alex Mlynarek, Marco Mascarella, Michael Hier, Nader Sadeghi, and Sabrina Daniela da Silva) were involved in the revision and editing of the manuscript. Sabrina Daniela da Silva provided resources and supervision throughout the creation of this manuscript. This article was published in the journal *Critical Reviews in Oncology/Hematology* in April 2024 (https://doi.org/10.1016/j.critrevonc.2024.104326). All authors previously read and approved of the published version, which has been included in its entirety in this thesis.

For Manuscript 2 titled "Comparative Analysis of Histological and Molecular Methods for HPV Detection in Oropharyngeal Cancer", Jenna Bouassaly reviewed and established the experimental methodologies, and performed the data analyses, writing of the first draft, and creation of the figures and tables. Jenna Bouassaly, Megan Araujo, Naser Karimi, and Sabrina Daniela da Silva were involved in the data collection, notably by performing the DNA extraction and molecular HPV detection and genotyping tests. Jenna Bouassaly, Lilianny Querino Rocha de Oliveira, and Sabrina Daniela da Silva performed the data collection from p16 IHC results. Jenna Bouassaly performed all statistical analyses. All authors (Jenna Bouassaly, Lilianny Querino Rocha de Oliveira, Megan Araujo, Naser Karimi, Michael Hier, Alex Mlynarek, Marco Mascarella, and Sabrina Daniela da Silva) were involved in reviewing and editing the manuscript. Sabrina Daniela da Silva provided resources and supervision. Though not authors, François Coutlée and Julie Guenoun provided the facilities, equipment, and expertise needed to complete the Anyplex™ II HPV28 Detection (Seegene Inc., South Korea) assay. François Coutlée and Naciba Benlimame were also involved in a complete and partial review of the article, respectively, prior to submission. This article was submitted to the International Journal of Oral Sciences in October 2024, after being read and approved by all authors.

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List of Abbreviations

ACRIN	American College of Radiology Imaging Network
ACP	Amplification en chaîne par polymérase
AJCC	American Joint Committee on Cancer
Anyplex II	Anyplex™ II HPV28 Detection kit
ASCO	American Society of Clinical Oncology
ASR	Age-standardized rates
cCR	Clinical complete response
CDK(s)	Cyclin-dependent kinase(s)
CRT	Chemoradiotherapy
DC	Distant control
DCR	Disease control rate
DFS	Disease-free survival
DM	Distant metastasis
DMFS	Distant metastasis-free survival
dsDNA	Double-stranded DNA
DNA	Deoxyribonucleic acid
E6AP	E6-associated protein
ECOG	Eastern Cooperative Oncology Group
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FFPE	Formalin-fixed paraffin-embedded
HB-EGF	Heparin-binding EGF-like growth factor
HNC(s)	Head and neck cancer(s)
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HPV+	Human papillomavirus positive
HPV-	Human papillomavirus negative
IARC	International Agency for Research on Cancer
IFN(s)	Interferon(s)

IHC	Immunohistochemistry, immunohistochimie (français)
IL-1β	Interleukin-1 beta
IMRT	Intensity-modulated radiotherapy
INNO-LiPA	INNO-LiPA® HPV Genotyping Extra II kit
ISH	*In-situ* hybridization
LA	Locally advanced
LA HNSCC	Locally advanced head and neck squamous cell carcinoma
LCR	Long control region
LRC	Locoregional control
LRF	Locoregional failure
LRP	Locoregional progression
MAPK	Mitogen-activated protein kinase
MHC I	Major histocompatibility complex I
mPR	Major pathological response
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NA	Not applicable
NF-κB	Nuclear factor-kappa B
OPC(s)	Oropharyngeal cancer(s)
OPSCC	Oropharyngeal squamous cell carcinoma
ORLs	Otorhinolaryngées
ORR	Objective response rate
OS	Overall survival
p16	p16 INK4A protein
p16+	p16 positive
PI3K	Phosphatidylinositol-3-kinase
pCR	Pathological complete response
PCR	Polymerase chain reaction
PD-1	Programmed cell death receptor 1
PDE5	Phosphodiesterase-5
PD-L1	Programmed cell death ligand 1

PEG	Percutaneous endoscopic gastrotomy
PFS	Progression-free survival
pPR	Pathological partial response
pRb	Retinoblastoma protein
qPCR	Real-time PCR
RB1	Retinoblastoma 1 gene
RFS	Recurrence-free survival
RNA	Ribonucleic acid
RT	Radiotherapy
RT-PCR	Reverse-transcription PCR
SBRT	Stereotactic body radiation therapy
TGF-α	Transforming growth factor alpha
TIL(s)	Tumor-infiltrating lymphocyte(s)
TIM	Tumor immune microenvironment
TLM	Transoral laser microsurgery
TLR(s)	Toll-like receptor(s)
TOS	Transoral surgery
TORS	Transoral robotic surgery
UK	United Kingdom
USA	United States of America
VPH	Virus du papillome humain
WHO	World Health Organization

Preface

This thesis was written in conformance to the 2024 guidelines for a manuscript-based thesis established by McGill University's Graduate and Postdoctoral Studies, to fulfill the requirements for completion of the Master of Science degree in Experimental Medicine. Following an introduction of the research topic that will be explored in this thesis, as well as an in-depth review of the literature, the projects undertaken to address any gaps in the field will be presented in the form of two manuscripts. The first constitutes a narrative literature review assessing the current status of treatment advances for HPV-positive HNC patients. The second consists of original research which evaluates the efficacy of various HPV detection methods, to aid in the implementation and standardization of HPV testing in the diagnosis of HNC. Between both manuscripts, a bridging section will link the topics to better situate this work in the overarching field of HNC research. This will be followed by a comprehensive discussion of all works presented in this thesis, as well as a final summary and an exploration of future research directions.

These manuscripts and the research efforts on which they are based were made possible by an interdisciplinary team of basic science researchers and clinicians from various cancer research domains. The individual contribution of each author to the manuscripts has been previously stated on page ix. The supplemental materials of each manuscript can be found in Chapter 9: Appendices. The supplemental material for Manuscript 1 (Chapter 3) is located in section 9.1 Appendix A, while the supplemental material for Manuscript 2 (Chapter 5) is found in section 9.2 Appendix B.

Chapter 1: Introduction

1.1 Rationale

Head and neck cancer (HNC) is the 6th most common malignancy, affecting over 946 000 individuals globally and accounting for over 482 000 cancer-related deaths[1]. HNCs, which comprise cancers of the oral cavity, pharynx, larynx, nasal cavity, and salivary glands, among other subtypes, are traditionally caused by an overconsumption of tobacco and alcohol products[2–4]. However, rapid increases in HNC rates, particularly in oropharyngeal cancer (OPC) rates, have been observed in recent times due to human papillomavirus (HPV) infections[2–4]. HPV is the most common sexually transmitted virus worldwide, with concerning increases in younger populations[5], and a rise in HPV-related HNC rates across all age groups[2–4]. With over 25% of all HNC cases in Canada[6], and over 50% and 70% of OPC cases in the United Kingdom (UK) and United States of America, (USA) respectively[4], being attributed to HPV infections, the virus has emerged as a major risk factor for the disease[2–4].

HPV-related OPC has recently been recognized as a distinct HNC subtype, characterized by a reduced mutational burden and superior patient prognoses[3,4]. Despite these differences, the standard of care for HPV-related HNC remains combinations of surgery, radiotherapy (RT), and platinum chemotherapy[4,7–9], which often induce severe acute and chronic toxicities, like dysphagia, dysarthria or dysphonia, necrosis, feeding tube dependency, and more[10,11]. For this reason, research efforts have aimed to develop de-escalated treatments for HPV-positive patients that would minimize adverse effects while maintaining therapeutic efficacy[3,4,7,12].

Though de-escalated treatment strategies have shown promise, their widespread acceptance has been limited by a lack of evidence on their efficacy[9]. While early phase I and II trials showcasing the promise of de-escalated strategies have been previously reviewed[7,9,12,13], recent large-scale studies on reduced-dose RT[14–16], neoadjuvant chemotherapy[17–19], and immunotherapy[20–22] have shown much success, expanding the available literature on these therapies and warranting an up-to-date review of these advancements.

Conversely, a lack of standardized HPV testing in HNC is also preventing the implementation of these therapeutic strategies[23,24]. As de-escalated treatments would benefit HPV-positive HNC patients due to their favorable prognoses, accurate and reliable detection of the virus

is essential for appropriate treatment allocation. The most common HPV testing method for HNC used in clinical settings is $p16^{INK4A}$ (p16) immunohistochemistry (IHC), which detects an accumulation of the protein p16 following HPV infections[23,24]. Though this method is rapid, sensitive, and cost-effective[23,24], its reliability as a surrogate marker for HPV has been debated, leading clinicians and researchers to turn to molecular-based tests that directly detect the presence of viral genomic material[23–27]. Thus, the differences between indirect histological testing and direct molecular testing must be investigated further for HPV to be used as a modality for treatment allocation.

1.2 Objectives

Considering the challenges hindering the implementation of de-escalated therapies, the objectives of this thesis are to (1) explore and summarize the recent advancements in de-escalated treatment strategies for HPV-positive HNC patients, and (2) compare various HPV-testing methods to aid in the standardization of HPV-positive HNC diagnosis and treatment allocation.

Chapter 2: A Comprehensive Review of the Literature

2.1 Head and Neck Cancer Development and Risk Factors

HNC remains one of the most common cancer types worldwide, accounting for 4.7% of new cancer cases and nearly 5% of cancer-related mortalities in 2022[1]. HNC encompasses cancers of the oral and nasal cavities, pharynx, oropharynx, hypopharynx, salivary glands, and larynx[2–4,28,29] (**Figure 1**). Like other malignancies, HNCs arise from an accumulation of genomic alterations resulting in the downregulation of critical tumor suppressors genes, the overexpression of oncogenes, and the stimulation of pro-proliferative pathways, allowing for affected cells to divide uncontrollably[28,29]. Notably, many HNCs are marked by inactivating mutations in the tumor suppressor genes *TP53* and *PTEN*[28,29], among others, and the upregulation of receptors like epidermal growth factor receptor (EGFR), resulting in the activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways[28]. Together, these events can induce continued pro-proliferative signaling and cell survival, which are key hallmarks of cancer[30]. The upregulation of these pathways also promotes other processes that enable tumor progression, including immune evasion, angiogenesis, invasion, and metastasis[28,30]. In HNCs, these processes are associated with malignant transformation of the mucosal epithelia, leading to the formation of a head and neck squamous cell carcinoma (HNSCC), which represent nearly all head and neck tumors[28,29].

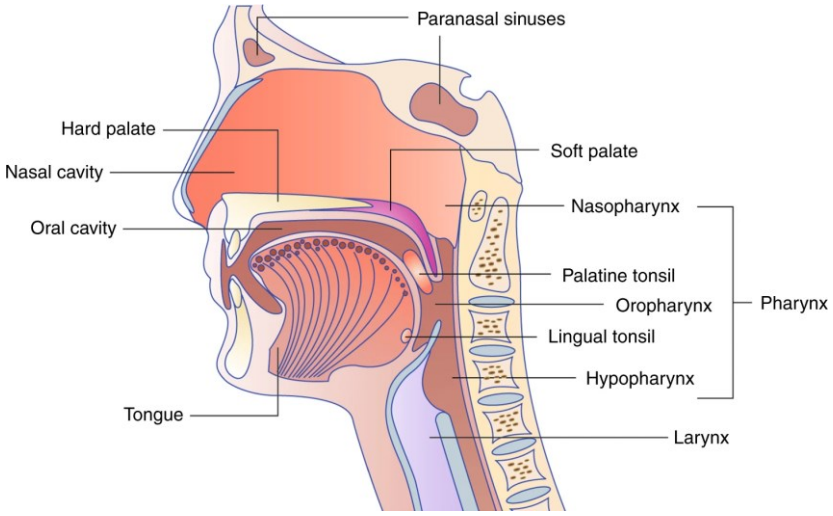

Figure 1. Anatomical Regions in which HNCs Occur. HNCs primarily arise in the epithelial tissues of the upper aerodigestive tract, including several key anatomical regions. The oral cavity

includes the lips, the front two-thirds of the tongue, the gingiva, the floor of the mouth, the hard palate, and the inside lining of the cheeks[29]. The pharynx is divided into three areas: the nasopharynx, which is the upper part of the throat behind the nose, the oropharynx, which includes the palate, base of the tongue, and tonsils, and the hypopharynx, which is situated in the lower part of the throat, just above the esophagus and windpipe[29]. The larynx is located just below the pharynx and contains the vocal cords[29]. The paranasal sinuses and nasal cavity are air-filled spaces in the bones around the nose[3]. This figure was reproduced without modifications from Sabatini & Chiocca, *British Journal of Cancer* (2020), under the Creative Commons Attribution (CC BY) 4.0 International License (http://creativecommons.org/licenses/by/4.0/)[3]. Abbreviations: HNCs, head and neck cancers.

The development of HNCs is highly influenced by lifestyle factors, with tobacco and alcohol consumption constituting the main risk factors for the disease[2,28,29]. Indeed, in Western populations, up to 75% of HNC cases may be related to smoking[2,28], with alcohol increasing the risk of developing a malignancy when combined with tobacco[2,28,29] or when consumed in excess by non-smokers[2,28]. Both tobacco smoke and alcohol products contain carcinogenic aldehydes, while the former also contains polycyclic aromatic hydrocarbons and nitrosamines, which are known to cause cancer[2,29]. In addition to these factors, a low socioeconomic status, poor oral hygiene, and dietary habits can also increase the risk of developing HNCs[2,29].

With cultural changes in smoking habits, particularly a decline in Western countries, HPV infections have recently emerged as a leading risk factor for HNC[3,4], with over 25% of all HNCs in Canada being attributed to HPV[6]. HPV is the most common sexually transmitted disease, affecting over 11% of individuals worldwide[5]. The virus is oncogenic, inducing cancer development through the production of oncoproteins that dysregulate cell growth pathways, leading to unrestricted proliferation and tumor formation[3–5,31,32]. However, not all HPV infections lead to cancer. With over 200 strains, infections with high-risk strains like HPV16 or 18 are most associated with oncogenesis, while low-risk strains like HPV6 or 11 lead to the formation of warts[3–5,31,32]. Furthermore, as transient infections can be cleared by the immune system[5], HPV oncogenesis requires persistent infections and the production of oncogenic proteins, which in turn often necessitates the integration of viral deoxyribonucleic acid (DNA) into the human genome[3–5,33,34]. Though it remains a risk factor for all HNC subtypes, HPV infections are primarily

associated with OPC due to the microenvironment of the tonsillar crypts[3]. These sites are highly populated by foreign microorganisms, leading to high rates of immune cell infiltration[3,35]. Consequently, this also results in elevated programmed cell death ligand 1 (PD-L1) expression on tonsillar epithelia[3,35], which binds to the programmed cell death 1 (PD-1) receptor expressed on T cells, preventing their activation to reduce autoimmune responses[36]. However, the suppression of T cell activity in the tonsillar crypts by the PD-1/PD-L1 axis allows for HPV infections to persist and induce malignant transformation, thus enabling infected cells to escape immune surveillance and form neoplasms[3]. Since HPV oncogenesis is facilitated in the oropharynx, most OPCs are attributable to the virus, as seen with over 50% and 70% of OPCs being HPV-related in UK and USA, respectively[4].

2.2 HPV Oncogenesis in Head and Neck Cancer

2.2.1 The Molecular Processes Governing HPV Oncogenesis

Papillomaviruses have a double-stranded episomal DNA (dsDNA) genome, with most containing six early genes (E1, E2, E4, E5, E6, E7), two late genes (L1 and L2), and a long control region (LCR) involved in the regulation of viral replication **(Figure 2A)**[31,32]. In the case of HPV16, the most common oncogenic HPV type[3–5], the early genes include E1 and E2, which encode DNA helicases and transcription factors, respectively, that help initiate viral DNA replication and transcription[31]. The early genes of HPV16 also comprise E4, which is poorly elucidated but may be implicated in viral replication[37], E5, which supports the proliferation, transformation, and invasion of infected cells through interactions with the MAPK and PI3K pathways, among others[32,34,38]. Early genes E6 and E7 are the main drivers of oncogenesis[3,4,31,32,34]. HPV infects basal epithelial cells of mucosal surfaces[4,32,34], stimulating their proliferation with E5[32,34,38], and allowing for the propagation of viral DNA as they expand and differentiate **(Figure 2B)**[32,34]. In differentiated keratinocytes, the late genes L1 and L2 encode proteins that encapsulate the viral genome, allowing it to be released and transmitted to another host **(Figure 2B)**[32,34,39].

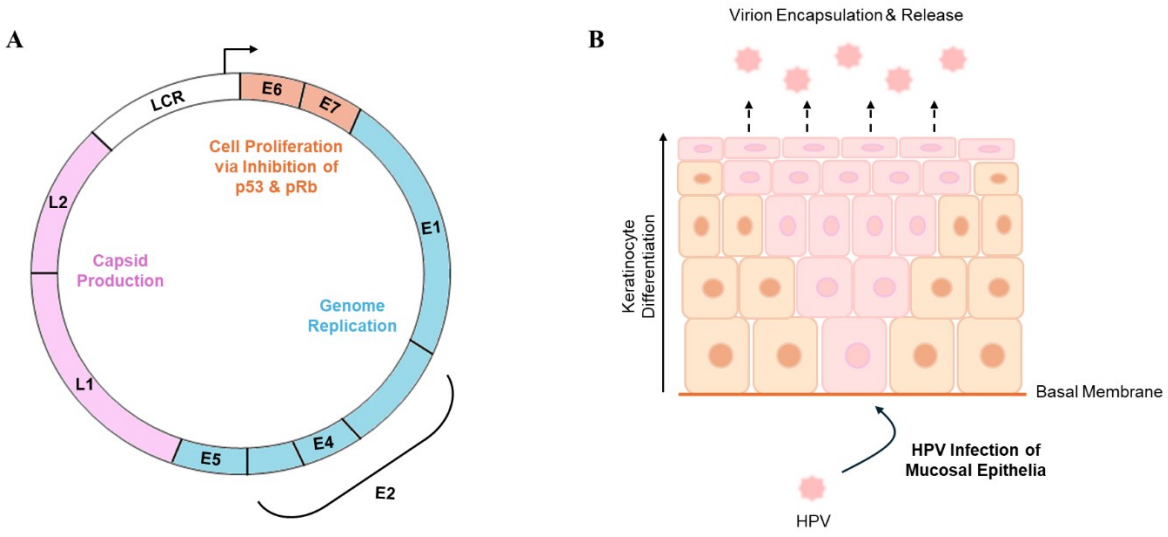

Figure 2. Genomic Composition and Infection Process of HPV. (**A**) The composition of the dsDNA genome of HPV16, containing the early genes (E1, E2, E4, E5, E6, E7), the late genes (L1, L2), and the long control region (LCR)[31,32]. The open reading frame of E4 is located within that of E2, and oncogenes E6 and E7, which encode the oncogenic proteins of the same name, are indicated in red. This figure was adapted from Münger *et al*., *Journal of Virology* (2004)[31]. (**B**) Schematic representation of the HPV infection process. In HNC, basal cells of the mucosal epithelia are infected by HPV[4,32,34]. Infected cells replicate, expand laterally, and differentiate into keratinocytes, propagating the viral genome[32,34]. The viral genome is encapsulated in terminally differentiated keratinocytes and released[32,34,39]. This figure was adapted from zur Hausen, *Nature Reviews Cancer* (2002)[32]. Abbreviations: dsDNA, double-stranded DNA; E, early; HNC, head and neck cancer; HPV, human papillomavirus; L, late; LCR, long control region.

As mentioned, in HPV-related HNCs, oncogenesis often occurs following viral integration, where the circular episomal genome is linearized and incorporated into the host's genetic material[32–34]. Though the mechanisms underlying HPV integration remain poorly understood, the activity of early genes E1 and E2 are disrupted during genomic linearization by cleavage or epigenetic silencing, resulting in the upregulation of E6 and E7 which were previously repressed[33]. These genes encode the HPV E6 and E7 proteins, which induce oncogenesis by repressing key cell cycle checkpoints p53 and retinoblastoma (pRb)[3,4,32,34]. Indeed, the HPV E6 protein complexes with E6-associated protein (E6AP) and targets p53 for proteasomal degradation by ubiquitination, preventing apoptosis (**Figure 3A**)[32,34]. Conversely, the HPV E7 protein binds and inhibits pRb,

sequestering it from the transcription factor E2F which can activate downstream cyclins E and A, inducing cell cycle progression (**Figure 3B**)[32,34]. The inhibition of pRb by E7 also results in the upregulation of *CDKN2A*, which encodes the tumor suppressor p16 (**Figure 3B**)[4,32,34]. p16 maintains pRb activity by inhibiting cyclin-dependent kinases (CDKs) 4 and 6 and preventing the progression of the cell cycle[4,32,34,40], though the direct inhibition of pRb by E7 negates this function[32,40]. As such, the combined effects of the HPV E6 and E7 proteins results in unrestricted cell proliferation and entry in S phase, promoting genomic instability and malignant transformation[31,32,34].

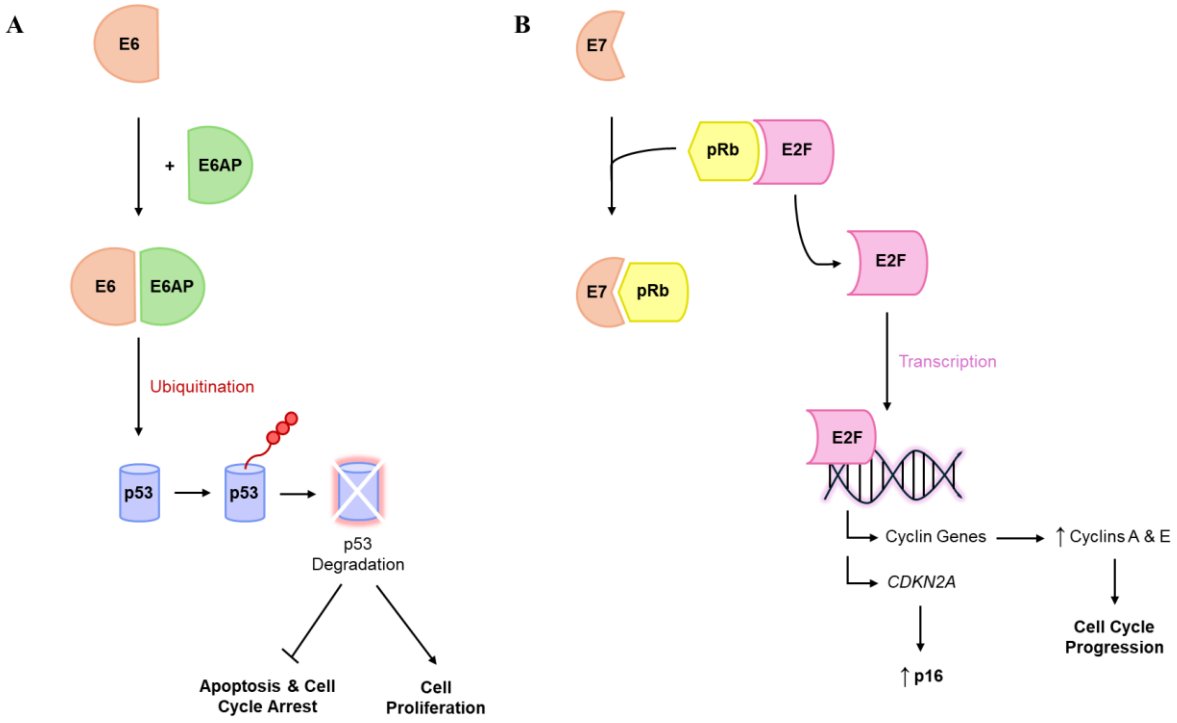

Figure 3. Oncogenic Mechanisms of the HPV E6 and E7 Proteins. (**A**) Mechanism of action of the HPV E6 protein. E6 binds to E6AP, forming a complex that ubiquitinates the tumor suppressor p53, triggering its degradation[32,34]. Loss of p53 due to E6 results in the inhibition of the mechanisms governing apoptosis and consequently, uncontrolled cell proliferation[32,34]. This figure was adapted from Pal & Kundu, *Frontiers in Microbiology* (2020)[34]. (**B**) Mechanism of action of the HPV E7 protein. E7 binds and sequesters pRb, allowing the transcription factor E2F, which was previously inhibited, to activate the expression of cyclins A and E[32,34]. These promote the progression of the cell cycle and proliferation[32,34]. E2F also stimulates the expression of *CDKN2A*,

which encodes the protein p16, a prominent biomarker in HPV-related HNCs[4,32,34]. This figure was adapted from Pal & Kundu, *Frontiers in Microbiology* (2020)[34]. Abbreviations: E6AP, E6-associated protein; HNC, head and neck cancer; HPV, human papillomavirus; pRb, retinoblastoma protein.

2.2.2 The Role of HPV in Promoting Immune Evasion and Inflammation

In addition to directly inducing oncogenesis, viral HPV proteins may also promote cancer development by facilitating immune evasion and persistent infections. Indeed, the oncogenic E5, E6, and E7 proteins have been found to modulate the expression of receptors involved in anti-cancer immune processes[41]. Notably, the HPV proteins E6 and E7 have been found to affect innate immunity by influencing the expression of toll-like receptors (TLRs), which are responsible for initiating anti-viral immune and inflammatory responses[42]. During viral infections, TLRs activate of phagocytic immune cells, as well as the transcription factor nuclear factor-kappa B (NF-κB), which in turn, promotes the production of interferons (IFNs) and other pro-inflammatory cytokines[42]. Both HPV E6 and E7 have been shown to impair the expression of TLR9[43], with E7 inducing epigenetic changes to downregulate transcription[44]. Though these findings suggest that HPV proteins modulate innate immunity through TLRs, further validation in HNC may still be warranted, as much of these studies examined these effects in cervical cancer, the most common HPV-related cancer[5,45,46], and mixed results have been reported on the expression of TLRs, notably TLR9, in OPC[47,48].

Similarly, the E5 and E7 proteins of multiple HPV genotypes have also been found to be implicated in immune evasion through the downregulation of the major histocompatibility complex I (MHC I)[38,49–52]. As MHC I aids in the activation of cytotoxic T cells, this suggests that oncogenic HPV proteins may prevent infected cells from being eliminated by the immune system[38], allowing them to persist and become malignant. In HNC, the HPV E5 protein has also been shown to inhibit the immunoproteasome, which reduces anti-viral IFN production and indirectly limits HPV-related antigen presentation, thus facilitating immune escape[53].

Furthermore, persistent HPV infections have been shown to enable immune evasion through the PD-1/PD-L1 axis. As mentioned, PD-1/PD-L1 interactions facilitate HPV infections in the oropharynx, and in turn the development of OPC, by repressing T cell activity[3]. Likewise, the PD-1/PD-L1 axis is exploited to help maintain HPV-related malignancies, with the HPV E7

protein promoting the upregulation of PD-L1[54], and elevated PD-L1 expression being characteristic of HPV-related OPC[55–57].

Finally, oncogenic HPV proteins produced during lasting infections can also induce chronic inflammation, particularly through the actions of E6 and E7, which stimulate the pro-inflammatory interleukin-1 beta (IL-1β)[58], while E6 also activates NF-κB[59]. Though this transcription factor is usually involved in anti-viral IFN cascades[42], continuous inflammatory responses can lead to DNA damage and exacerbate oncogenesis[58]. Despite the interaction between HPV-related inflammation and tumorigenesis being heavily studied in cervical cancer[58], increased inflammation has also been associated with positive HPV status in HNC[60].

2.3 HPV-Related Oropharyngeal Cancer as a Distinct Cancer Subtype

Due to its unique etiology, HPV-related OPC has been increasingly considered as a distinct HNC subtype from HPV-unrelated disease, due to striking differences in prognoses and treatment responses between HPV-positive and -negative patients. Indeed, a landmark study by Fakhry *et al*. reported a superior response rate of 84% to chemoradiotherapy (CRT) in patients with HPV-related HNC compared to 57% in their HPV-negative counterparts, as well as increased overall survival (OS), and reduced disease progression and death risk[61]. These findings are supported by multiple studies also showing higher OS, progression-free survival (PFS), and a lower death risk in HPV-positive patients[62–66], ultimately establishing a clear discrepancy in prognostic outcomes based on HPV status in HNC. This distinction has led to a separate classification of OPC based on HPV status by the American Joint Committee on Cancer (AJCC)[67] and the World Health Organization (WHO)/International Agency for Research on Cancer (IARC)[68], recognizing HPV-related OPC as a HNC subtype. This change has also led to new staging guidelines for HPV-positive OPC, incorporating the increased nodal involvement observed in these tumors[67]. In light of these changes, much research has been devoted to the development of de-escalated treatment strategies for HPV-positive patients with superior prognoses, to mitigate the severe toxicities associated with the standard of care for HNC[69].

2.4 Epidemiological Trends of HPV-Related Head and Neck Cancers

Globally, HPV is the leading viral cause of cancer[46], with HPV16 being responsible for most HPV-related OPCs[3–5,70]. However, the prevalence of HPV strains among OPC patients have been found to vary between populations. For instance, despite HPV16 being the most common strain found in

OPC patients, it is most prevalent among White OPC patients, while HPV18 is highest among Black patients[3,71]. In the USA, the incidence of HPV-related OPC is highest in White populations compared to Black or Hispanic populations[72]. Further, the combined expression of p16 with integrated HPV DNA is predominantly observed in White OPC populations, while this status has been found to be less apparent in Black and Asian populations[3,71].

Similarly, large discrepancies in HPV-positive OPC incidence are seen based on sex, with the disease disproportionately affecting males globally[4–6,45,70,72]. In the USA, oral HPV infections are higher in males at 11.5% compared to 3.3% in females[72], while the incidence of HPV-related OPC is over four times higher in males than females in both Canada[6] and the USA[70]. Sex-based differences in rates of HPV-positive OPC may be explained by a larger number of different sexual partners in males[3,73], as well as reduced vaccination among this group[4,6]. Most vaccination programs were previously directed towards females due to the association between HPV infections and cervical cancer[4,6,74], while vaccination has only recently begun to be recommended for males with the emergence of the virus as a prominent risk factor for HNC[4,6,74].

Furthermore, striking geographical differences are seen in the incidence of HPV-related OPC, with cases being much higher in high income countries (**Figure 4**)[3–5,45,45,72]. Indeed, North American and Western European countries have the highest rates of HPV-related OPC cases[4,5,45,75], with OPC becoming the predominant HPV-related malignancy in the USA and UK[4]. These disparities may be attributable to cultural differences in sexual habits[3,70,72]. Finally, rates of HPV-related OPC vary greatly by age, with many studies previously reporting a high incidence in younger individuals[3,4,70,72]. However, rising diagnoses among those over the age of 65 have shifted this trend[3,4,72,76], though this forecast is likely due to aging individuals representing a large fraction of the total population[4,76], and the effects of HPV vaccination among young cohorts[72].

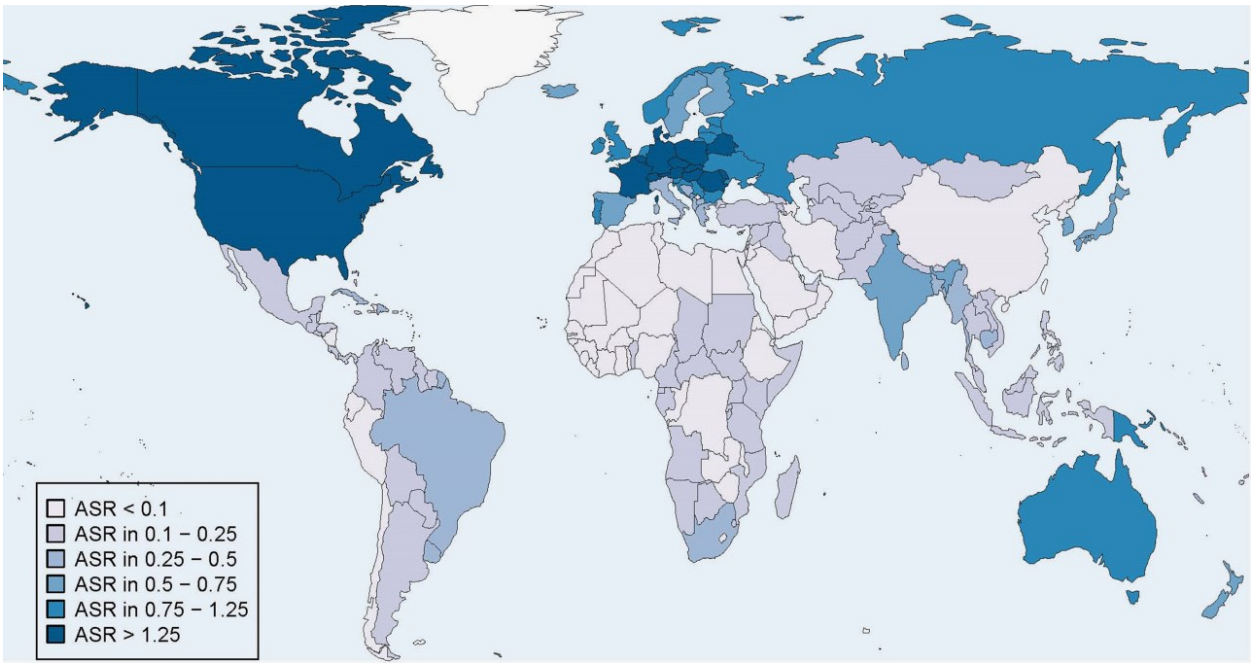

Figure 4. HPV-Related HNC Incidence According to Geographic Location. The age-standardized rates (ASR) of global HNC cases related to HPV infections[45]. The incidence of HPV-related HNC per 100 000 individuals is depicted including males and females[45]. Rates of HPV-related HNC are elevated among Western and developed countries, with the highest number of cases in North America and Europe, while African and East-Asian countries have lower incidences[45]. This figure was reproduced from de Martel *et al.*, *International Journal of Cancer* (2017) under the Creative Commons IGO license (CC BY-NC 3.0 IGO https://creativecommons.org/licenses/by-nc/3.0/igo/legalcode) without modifications[45]. Abbreviations: ASR, age-standardized rates; HNC, head and neck cancer; HPV, human papillomavirus.

2.5 Diagnostic Measures and HPV Detection in Head and Neck Cancer

2.5.1 Head and Neck Cancer Diagnosis

Most HNCs are diagnosed following the identification of a mass, ulcer, or lesion by patients who often experience pain and difficulty swallowing, eating, or speaking, among other symptoms[29]. These symptoms are often present in more aggressive or advanced HPV-negative oropharyngeal tumors, while HPV-positive OPC often fails to cause symptoms and instead presents with nodal spread[29]. Though novel measures like brush biopsies, salivary liquid biopsies, and tissue stains are

being assessed for the early detection of other HNCs[77], radiologic imaging, excisional or incisional tissue biopsy, fine needle aspiration, and histopathological examination are primarily used in the diagnosis of OPC[29,78]. Once OPC is diagnosed, there are currently no secondary diagnostic measures, and both HPV-positive and -negative patients receive the same standard of care[3,4,9,12,29]. However, with the development of de-escalated treatment strategies for HPV-positive patients, the implementation of a screening program to detect HPV-positive tumors would facilitate patient stratification for the allocation of less intense treatment regimens[4,23,24]. Despite the lack of a standardized HPV testing regimen for OPC, clinical trials often employ indirect or direct methods to detect and stratify HPV-positive patients.

2.5.2 Indirect HPV Detection

Despite the lack of a standardized testing regimen, p16 IHC is the most used method to detect HPV in OPC[4,23–25]. This method uses the detection of p16 as a marker for HPV infections, due to its overexpression induced by the HPV E7 protein[23,24]. Briefly, p16 antibodies tagged with reporter molecules are applied to biopsied tissues, allowing cells with protein expression to be identified[79]. The substrate to the reporter molecule is then added, inducing a chromogenic change and allowing for positive tissue sections to be visualized by light microscopy[79]. While this method is discouraged in other HNC types due to high rates of non-viral p16 upregulation[80], it has a high sensitivity in OPC[23,81], and is often preferred by clinicians due to its simplicity, low cost, and quick completion[23–25]. However, the accuracy of p16 IHC in predicting HPV status remains limited by non-viral factors that may influence p16 expression, which could lead to false positive or negative diagnoses[25,82]. Particularly, variability in the p16 antibody clones used[82] and positivity thresholds that attribute HPV status[23,82–85] can dramatically impact the reliability of the technique, while gene mutations in *TP53*[86] and increased inflammation have been associated with aberrant p16 expression[23]. Multiple antibody clones are available for p16 detection, with many differing in staining intensity, negative predictive potential, and sensitivity[82]. The p16 positivity threshold used to define HPV-positive tumors can also impact the accuracy of detection rates, with increasing disagreement on the optimal threshold[23,83–85]. The p16 positivity threshold can be defined as the proportion of p16-positive cells needed to classify the tumor as HPV-positive. Currently, a p16 positivity threshold of 70% is most recommended[23,87], though it has been found to exclude OPCs with integrated HPV DNA but low p16 expression[83], and lead to false-negative HPV classifications

when used with different antibodies[82] or when applied to fine-needle aspiration biopsies[84,85]. Instead, lower thresholds between 10% and 50% have been proposed to better represent HPV positivity[83–85]. Conversely, non-viral p16 upregulation can lead to false-positive HPV diagnoses[25], with patients without integrated HPV DNA being classified as HPV-positive due to high p16 expression. Indeed, false-positive HPV detection rates with p16 IHC differ geographically, ranging from 5.6% in the Netherlands to 26.3% in Chile[25]. Since patients with discordant p16 and HPV statuses have been found to have worse prognoses[4,23,26,27,88–90], additional direct molecular testing has been recommended to ensure proper patient diagnoses[4,25–27,81,89,90].

2.5.3 Direct HPV Detection

While direct molecular HPV testing has been recommended in addition to p16 IHC, many different molecular assays are currently being used, and a consensus on the best molecular method has yet to be reached. Molecular HPV testing targets viral genetic material, allowing for direct detection of the virus.

Some of the most common molecular testing methods include polymerase chain reaction (PCR)-based HPV genotyping, which has shown high specificity and sensitivity in OPC, especially when combined with p16 IHC[23,91]. Many of these assays detect the presence of integrated HPV DNA through the amplification of conserved sequences in the L1, E6, or E7 genes[23,92]. The first iteration of this method consisted of the Gp5/Gp6 primers, which were designed to detect 11 different HPV genotypes, including the high-risk HPV16 and 18[93]. These primers have since been expanded to increase overall and strain-specific HPV detection, detecting at least 22 HPV strains as the Gp5+/Gp6+ primers[94]. These primers have been shown to be accurate and reliable in the detection of HPV in OPC, when used in combination with p16 IHC[95,96]. Many commercial HPV genotyping assays have been developed based on this method, using quantitative real-time PCR (qPCR) to detect regions of HPV L1, like the Cobas 4800/6800/8800 HPV Tests, Anyplex II HPV HR Detection, and INNO-LiPA HPV Genotyping Extra II Assay, among others[92]. Conversely, PCR-based assays have also been developed to target the HPV E6 and E7 genes, including the BD Onclarity HPV Assay, Xpert HPV assay, and Cervista HPV HR Test[92]. Although many of these are commonly used in cervical cancer[92], a commercial HPV detection test has yet to be approved for use in OPC[23], despite showing favorable detection rates, sensitivity, and concordance with p16 IHC in this cancer type[91,97–101]. Their acceptance and implementation in

OPC may be limited by the common storage of biopsied specimens as formalin-fixed paraffin-embedded (FFPE) tissues that often have low viral load and poor DNA quality, which can impair the sensitivity of PCR-based tests[23].

Similarly, reverse-transcription PCR (RT-PCR) assays have been used to detect the presence of HPV E6 and E7 genetic transcripts, confirming that tumors originated due to the effects of the E6 and E7 proteins[23]. Often deemed the gold-standard for molecular HPV detection, HPV E6 and E7 messenger ribonucleic acid (mRNA) detection has shown high reliability in OPC[102], though it remains limited by a lower availability of genetic material in FFPE tissues[23]. Nevertheless, commercial HPV mRNA detection tests have been developed, with the APTIMA HPV Assay approved for cervical cancer[92] showing high sensitivity and comparability with p16 IHC in OPC[103–105].

Finally, molecular methods like DNA and mRNA *in-situ* hybridization (ISH) are also used to directly detect the presence of HPV DNA and mRNA, respectively, in tissue specimens[23]. Briefly, nucleic acid probes are designed to hybridize to specific sequences within the viral genome, emitting a signal that can be visualized using light microscopy and identifying HPV-infected cells[23]. Although mRNA ISH has a high sensitivity and specificity in OPC[23], HPV detection by DNA ISH has been shown to be unreliable, with high rates of false-negative diagnoses due to variations in probe efficacy, availability of genetic material, and consistency in experimental manipulations[23].

2.5.4 The Rising Need for Standardized HPV Testing in Oropharyngeal Cancer

As mentioned, p16 IHC remains the most used HPV detection method in OPC[4,23–25]. However, it is limited by non-viral factors that may result in false-negative and false-positive diagnoses. For the proper implementation of de-escalated treatment strategies, the appropriate diagnosis of HPV-positive patients is crucial. Patients that are p16-positive but negative for HPV DNA integration have been found to have worse prognoses than those with both p16 positivity and integrated HPV DNA[4,23,26,27,88–90], meaning a de-escalated treatment may not be aggressive enough if allocated. Considering studies have reported between 5% and 25% of OPC patients having discrepant p16 and HPV statuses[25], the use of p16 IHC as the sole method of HPV detection has been debated and the addition of molecular HPV testing has been recommended[4,25–27,81,88–90]. Without a standardized clinically approved HPV testing regimen for OPC, proper diagnosis and treatment allocation, as

well as the approval of de-escalated treatments, will remain hindered by variability in HPV testing methods[23–25].

2.6 Treatment Strategies for HPV-Related Head and Neck Cancers

2.6.1 Current Therapies for Head and Neck Cancers

Despite differences in etiology, staging, and patient prognoses, HPV-related and -unrelated HNCs have the same standard of care, consisting of surgery, radiation, and chemotherapy combinations depending on tumor stage and location (**Figure 5**)[3,4,8,9,12,29]. For recurrent, metastatic, or refractory disease, CRT with concomitant targeted therapy, single-agent immunotherapy has also been approved (**Figure 5**)[4,8,29].

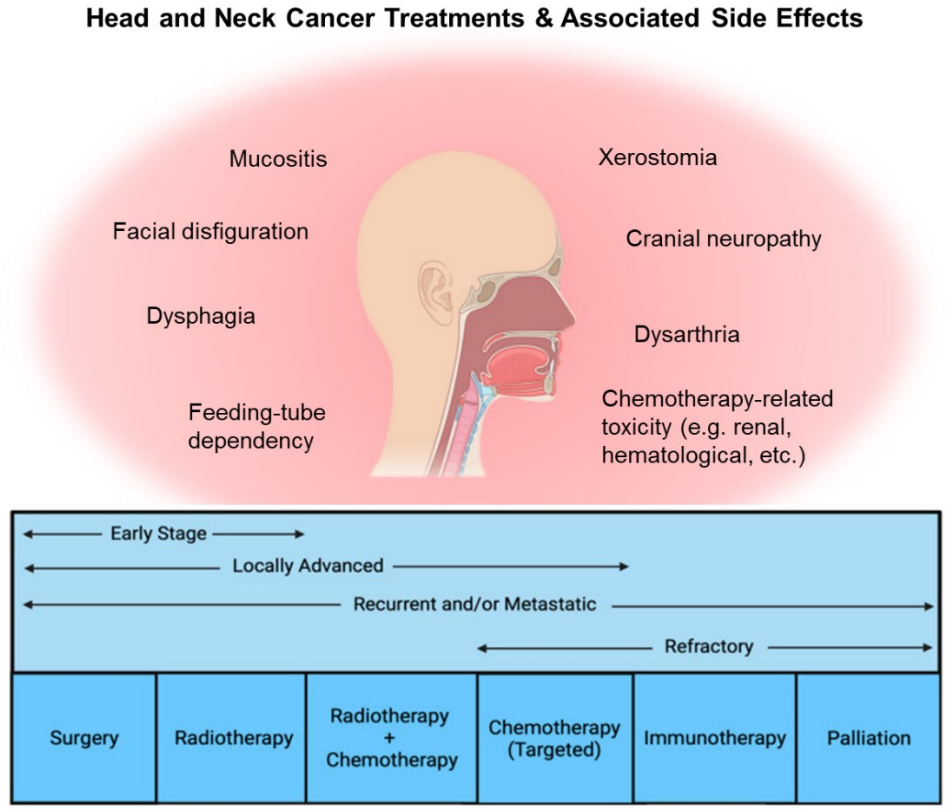

Figure 5. Current Standard of Care for HNC and Common Treatment-Related Morbidities. Currently, the standard of care for HNC remains the same regardless of HPV status, and often induces severe side effects[4,7–9]. First-line treatments for early-stage tumors consist of surgery and RT, while systemic or targeted chemotherapy is added for locally advanced tumors[8]. Refractory HNCs are treated with targeted therapy, immunotherapy, or palliative care, while all treatment

modalities are considered for recurrent or metastatic cases[8]. Both surgery and RT can result in functional morbidities like feeding-tube dependency, dysphagia, and dysarthria[4,9,10,106–109], while RT may also induce xerostomia, mucositis, and cranial neuropathy, among others[10,11,109]. Chemotherapy-related effects like renal toxicity and hematological deficiencies are also common[8,110,111]. This figure was partially reproduced from Muniz *et al*., *International Journal of Molecular Sciences* (2024) with modifications under the Creative Commons Attribution (CC BY) 4.0 International license (https://creativecommons.org/licenses/by/4.0/)[8]. Abbreviations: HNC, head and neck cancer; HPV, human papillomavirus; RT, radiotherapy.

In the case of OPC, the main surgical intervention was previously radical open surgery and neck dissection, as tumors in this location are often difficult to access[4,9,106,107,112]. However, this often resulted in severe functional morbidities, as well as facial disfiguration, which in turn greatly impaired the physical and psychological quality of life of patients (**Figure 5**)[4,9,106–108]. For this reason, minimally invasive surgical techniques like transoral laser microsurgery (TLM) and transoral robotic surgery (TORS) have been developed and implemented for the management of HNCs, particularly for OPC[4,9,106,107,112]. These techniques allow for better targeting and visualization of the tumor with minimal functional morbidities and are often followed by standard RT or CRT regimens to maximize anti-tumor responses[4,9,106,107,112]. Although these advances have reduced the sequelae of surgical treatments, their widespread use remains limited by the need for costly equipment, specialized training, and hard-to-reach or contraindicated tumors[4,106,107,112], leading to the incorporation of RT and CRT as first-line treatments[4,9,29].

Regardless of its administration as a primary therapy or postoperative adjuvant, the standard of care for OPC often comprises high-dose radiation[4,12,113], which can result in high-grade toxicities like dysphagia, dysarthria, dysphonia, cranial neuropathy, and feeding tube dependency (**Figure 5**)[10,11,109]. As such, advancements in RT instruments have led to the development of targeted modalities like intensity-modulated radiotherapy (IMRT) which allows radiation beams to target the tumor more precisely while minimizing the impact on healthy surrounding tissues[12,114]. Though technique has been shown to reduce the incidence of xerostomia[114–118], acute and late RT-related epidermal and mucosal toxicities are still prevalent (**Figure 5**)[115,118].

Chemotherapy is often offered as an adjuvant to radiation (**Figure 5**), with systemic drugs that target DNA repair mechanisms, such as cisplatin and 5-fluorouracil, being the most used[8,110].

Similarly, the incorporation of microtubule-targeting taxanes like docetaxel and paclitaxel into chemotherapy regimens has been explored due to improved survival outcomes[8,111,119,120], leading to their approval for use in HNCs and alongside targeted therapies[8,121]. However, due to their systemic nature, these chemotherapeutic agents often result in severe adverse events like nausea, mucositis, renal toxicity, hematological deficiencies, and hearing loss, among others (**Figure 5**)[8,110,111]. For this reason, targeted therapies have been explored, with the EGFR inhibitor cetuximab showing mixed success in improving anti-cancer efficacy in late-stage, recurrent or refractory HNCs[122]. Cetuximab has shown superior survival and disease control when combined with RT[123] or systemic therapies[121,124,125], gaining approval for use in HNC treatments. Despite its initial success among HPV-negative HNC patients, recent studies have reported worse survival outcomes with cetuximab compared to platinum chemotherapies[126–128], particularly among HPV-positive cohorts[127,129–131]. For this reason, its use warrants more investigation in HPV-positive patients, highlighting the importance of distinguishing HPV-related OPC as a HNC subtype and developing separate treatment guidelines based on HPV status.

Finally, immunotherapy has been approved in the frontline setting for recurrent and metastatic HNC, as well as platinum-refractory tumors (**Figure 5**)[8,29]. Immunotherapy acts as an effective antineoplastic agent by influencing the complex tumor environment, with immune checkpoint inhibitors (ICIs) being of much interest due to their ability to inhibit immunosuppressive pathways, like the PD-1/PD-L1 axis, among others, resulting in greater immune activation[35]. Particularly, PD-1 inhibitors nivolumab and pembrolizumab have shown improved survival outcomes and favorable rates of treatment-related toxicities as a primary treatment[132–135] and as an adjuvant to combination chemotherapy[132,135] in both HPV-positive and -negative HNC. While these ICIs are already approved for use in late-stage HNCs, their benefit and success when combined with surgery or radiation in HPV-positive HNC patients has been reported in recent clinical trials, making them promising candidates for treatment de-escalation[69].

2.6.2 De-Escalated Treatment Strategies for HPV-Related Head and Neck Cancers

Considering the severe side effects resulting from standard treatments, de-escalated strategies have been proposed as less intense alternatives for HPV-positive OPC patients who historically have better prognoses. Though these treatments have yet to be widely accepted, many investigations have reported promising treatment responses and reductions in toxicities, warranting a

reconsideration of their status in the standard of care[69]. Novel de-escalated treatments for HPV-related OPC will be introduced in this section and will be explored in greater depth as the topic of focus in Manuscript 1 (Chapter 3).

The most researched de-escalated therapies for HP-related OPC include reduced-dose RT, neoadjuvant chemotherapy, and neoadjuvant or adjuvant immunotherapy[69]. Indeed, reduced-dose RT has been explored to minimize treatment-related toxicities, with multiple studies showing that the modality resulted in promising response rates and toxicity outcomes in HPV-positive OPC patients[136–138]. When administered as a definitive treatment, reduced radiation doses below 66 Gy have been shown to result in a high OS comparable to that of standard doses[136], while pathological complete response (pCR) rates above 80%, low disease progression, and favorable toxicity rates were achieved when combined with platinum or taxane chemotherapy[137,138].

Similarly, taxane chemotherapies docetaxel and paclitaxel have shown much success as neoadjuvant or induction agents to shrink tumors prior to surgery or RT, respectively, facilitating their management. Most notably, studies by Sadeghi *et al.* showed high pCR[18], as well as exceptional disease-free survival (DFS) and low feeding-tube dependency compared to standard CRT[139] with neoadjuvant docetaxel before TORS in HPV-positive OPC patients. HPV-positive OPC patients receiving low-dose RT following induction paclitaxel have also been found to have high OS and PFS, as well as significantly lower rates of mucosal toxicities and feeding tube dependencies compared to those with standard CRT after induction[140].

Finally, neoadjuvant and adjuvant immunotherapies, particularly PD-1 inhibitors, have opened a promising avenue for de-escalated treatments, taking advantage of the high PD-L1 expression and increased immune cell infiltration observed in HPV-positive HNC[141] to improve tumor responses. Nivolumab has shown moderate tumor responses with acceptable adverse effects when administered alone as a neoadjuvant to surgery[21], while its use as an adjuvant to reduced-dose RT has resulted in high OS and PFS, as well as tolerable toxicity rates[142]. Furthermore, the addition of taxane and platinum chemotherapies to induction nivolumab resulted in an overall response rate of 89% and higher OS and PFS in patients receiving de-escalated CRT following induction[143].

The studies presented above show the promise of de-escalated strategies in the treatment of HPV-positive HNC, with many more recent clinical trials supporting their implementation[69].

These studies and other recent advances in de-escalated therapies for HPV-related HNC will be explored thoroughly in Manuscript 1 presented in Chapter 3.

2.6.3 Challenges in the Implementation of De-Escalated Treatments

Despite their promising success, novel de-escalated therapies have yet to be clinically approved for HPV-positive HNC. Although they have shown favorable outcomes and safety, hesitancy surrounding their acceptance still stems from a need for further evidence supporting their ability to induce strong anti-tumor responses while minimizing toxicities[9]. The possibility of implementing de-escalated treatment strategies in the standard of care for HPV-positive HNC was last evaluated by the American Society of Clinical Oncology (ASCO) in 2019[9]. Since then, many clinical trials assessing de-escalated modalities in HPV-positive HNC patients have been published, warranting a re-evaluation of their status. The narrative literature review in Chapter 3 summarizes the results from these studies in hopes of reopening the discussion on the use of de-escalated treatments for HPV-related HNC management. However, as previously mentioned, the implementation of de-escalated therapies is also hindered by a lack of standardized HPV testing for OPC[23,24]. With differences in patient prognoses and the accuracy of testing methods, a critical assessment and comparison of HPV detection methods is needed to ensure appropriate patient diagnosis and treatment allocation. Manuscript 2 presented in Chapter 5 sheds light on the discrepancies in HPV detection between histological and molecular methods, in hopes of aiding the standardization of HPV testing in OPC.

Chapter 3: Manuscript 1

Rethinking Treatment Paradigms: Neoadjuvant Therapy and De-Escalation Strategies in HPV-Positive Head and Neck Cancer

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3.1 Highlights

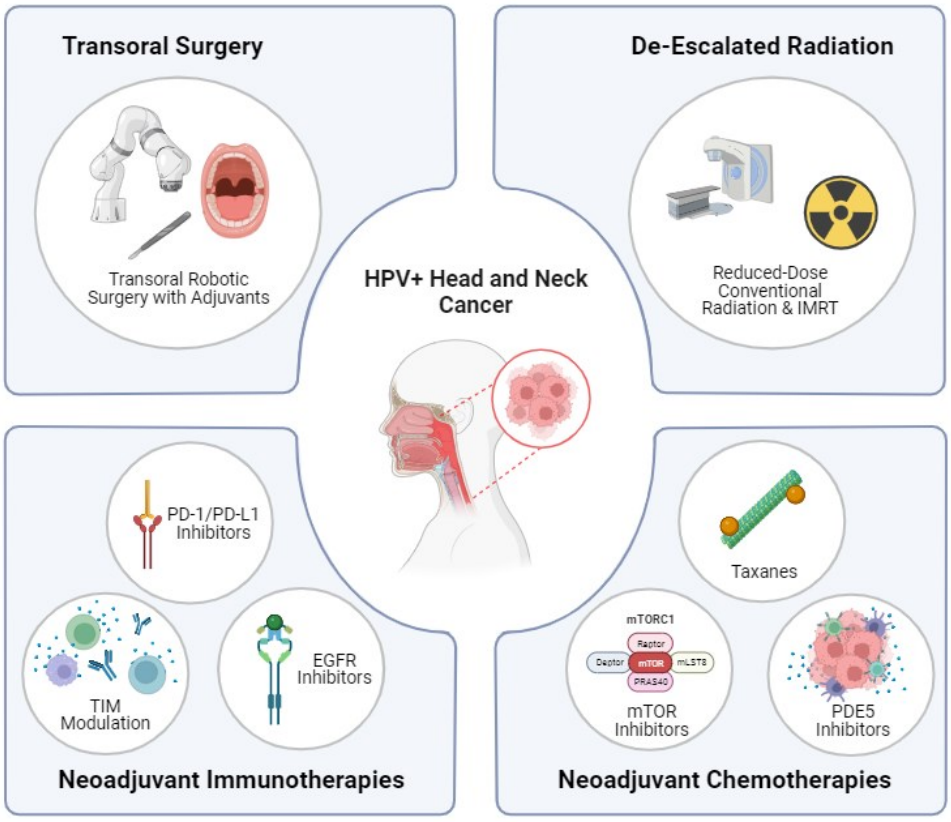
- HPV+HNC is rising in incidence, but patients have better prognoses, warranting the investigation of de-escalation strategies to mitigate toxicities.
- Reduced-dose radiotherapy and transoral surgery have been shown to limit adverse effects without compromising survival.
- Taxane chemotherapies and immunomodulatory agents show promising anti-tumor activity in HPV+HNC when used in adjuvant and neoadjuvant settings.

3.2 Abstract

Head and neck cancer (HNC) is the 6th most common cancer across the world, with a particular increase in HNC associated with human papilloma virus (HPV) among younger populations. Historically, the standard treatment for this disease consisted of combined surgery and

radiotherapy or curative platinum-based concurrent chemoradiotherapy, with associated long term and late toxicities. However, HPV-positive HNC is recognized as a unique cancer subtype, typically with improved clinical outcomes. As such, treatment de-escalation strategies have been widely researched to mitigate the adverse effects associated with the current standard of care without compromising efficacy. These strategies include treatment de-escalation, such as novel surgical techniques, alternative radiation technologies, radiation dose and volume reduction, as well as neoadjuvant chemotherapies, immunotherapies, and combined therapies. Although these therapies show great promise, many of them are still under investigation due to hesitation surrounding their widespread implementation. The objective of this review is to summarize the most recent progress in de-escalation strategies and neoadjuvant therapies designed for HPV-positive HNC. While specific treatments may require additional research before being widely adopted, encouraging results from recent studies have highlighted the advantages of neoadjuvant chemotherapy and immunotherapy, as well as radiation and surgical de-escalation approaches in managing HPV-positive HNC.

Fig. 6. Graphical Abstract



Summaryof the treatment de-escalation approaches recently investigated in the literature. Recent advances in de-escalated treatment strategies include transoral robotic surgery coupled with adjuvant therapies and reduced-dose radiation using conventional RT or IMRT. Emerging adjuvants, including taxane chemotherapies, mTOR inhibitors, and PDE5 inhibitors, as well as immunotherapies like PD-1/PD-L1 and EGFR inhibitors, along with agents that modulate the tumor immune microenvironment, have also been investigated. Overall, these de-escalation strategies have demonstrated diverse outcomes, each accompanied by their own advantages and limitations. Abbreviations: EGFR, epidermal growth factor receptor; HPV+, human papillomavirus positive; IMRT, intensity-modulated radiotherapy; mTOR, mammalian target of rapamycin; PDE5, phosphodiesterase-5; PD-1, programmed cell death receptor 1; PD-L1, programmed cell death ligand 1; TIM, tumor immune microenvironment. Created with BioRender.com.

Keywords

Head and neck cancer (HNC); Human papilloma virus (HPV); Treatment de-escalation strategies; Neoadjuvant therapies; HPV-positive HNC; Clinical outcomes.

3.3 Introduction

Head and neck cancer (HNC) is the 6th most common cancer, accounting for over half a million new cancer cases each year and rising in incidence globally[1,2]. HNCs include cancers of the oral cavity, oropharynx, larynx, and nasal cavity, among others[1,3]. Alcohol and tobacco consumption comprise the major risk factors for HNC, along with poor oral hygiene and socio-economic disparities[1,4]. However, a recent rise in human papilloma virus (HPV)-associated oropharyngeal cancer (OPC), particularly in Western nations, has established HPV infections as substantial risk factors for HNC[1,4]. HPV-positive cancers are distinct from HPV-negative cancers, possessing low genetic heterogeneity and undergoing malignant transformation due to the viral E6 and E7 proteins, which inhibit the tumor suppressors genes *TP53* and *RB1* (retinoblastoma)[4]. While HPV-positive OPCs generally exhibit superior response rates to standard cancer treatments such as chemo-radiation and surgery[1,4], the increased incidence of these cancers in younger populations remains concerning[5].

Currently, the recommended treatment standards for HNC involve surgical resection followed by radiotherapy or definitive radiotherapy concurrently with cisplatin chemotherapy, regardless of HPV status[3,5,6]. While the efficacy of these interventions in the treatment of HNC vary based on tumor characteristics, like tumor site and stage at presentation, the treatments frequently lead to pronounced physical and psychological consequences, coupled with acute, chronic, and late toxicities[5,7]. Depending on the progression and location of the disease, surgical procedures can lead to functional impairments and facial disfigurements, often contributing to psychological distress and depression[8]. Conversely, radiation therapy can significantly impact quality of life, giving rise to challenges such as dysphagia, cranial neuropathy, dysphonia or dysarthria, xerostomia, and more[7,9,10].

Due to the excellent treatment response of HPV-positive OPC, as well as recent advances in both surgical and radiation techniques, treatment de-escalation strategies have garnered much interest to alleviate adverse effects. Transoral robotic surgery (TORS) and radiation dose de-escalation have shown promising reductions in adverse events without compromising patient survival[5]. Neoadjuvant therapies, such as systemic or targeted chemotherapies and immunotherapies, have also emerged as innovative approaches for treatment de-escalation in HPV-positive HNC, as they have the potential to enhance treatment outcomes by minimizing tumor burden before primary treatment interventions[11,12]. These strategies often encompass novel surgical techniques, alternative radiation technologies, radiation dose reduction, or the use of targeted therapies to achieve effective treatment while preserving quality of life and minimizing treatment-related morbidity. In this comprehensive review, we aim to investigate the latest advancements in treatment de-escalation strategies for HPV-positive HNC, placing a particular focus on the therapies that exhibit promising clinical potential, such as the de-escalation of radiotherapy and novel surgical techniques, as well as neoadjuvant chemotherapies and immunotherapies.

3.4 De-escalation strategies and novel surgical approaches

3.4.1 Radiotherapy de-escalation

Given the improved prognoses and clinical responses observed among patients with HPV-positive HNC, de-escalation strategies have been proposed as potential avenues to mitigate the toxicities

associated with radiotherapy (RT), chemoradiotherapy (CRT), and surgery, without compromising treatment effectiveness. Among these strategies, reduced-dose RT and transoral surgery (TOS) have emerged as prevalent approaches for treatment de-escalation, demonstrating promising results (**Table 1**). A recent study conducted by the Eastern Cooperative Oncology Group (ECOG) and the American College of Radiology Imaging Network (ACRIN) Cancer Research Group assessed the impact of TOS followed by various low doses of RT or CRT in HPV-positive OPC[13]. Despite the limitations in assessing statistical disparities, it estimated comparable overall survival (OS) and progression-free survival (PFS) rates between the treatment groups after a two-year period[13]. Notably, lower RT doses were correlated with fewer Grade 3 or higher treatment-related toxicities and an enhanced patient-reported quality of life[13]. Similarly, an observational study confirmed that reduced-dose RT resulted in comparable OS outcomes to those of conventional RT in HPV-positive OPC cases[14], thereby reinforcing the advantages of de-escalated treatments in the context of HPV-positive HNCs.

Table 1. Recent clinical and observational investigations into de-escalated radiotherapy and transoral surgery for HPV-positive HNC*.

Study	Cancer Type & HPV Status	Therapy	Dose	Outcomes
13	HPV+ OPSCC	TOS, then post-operative reduced dose RT vs standard dose RT/CRT	Arm A: TOS only Arm B: 50 Gy Arm C: 60 Gy Arm D: 66 Gy with 40 mg/m2 cisplatin weekly	- 2-year PFS of 96.9% (arm A), 94.9% (arm B), 96.0% (arm C), and 90.7% (arm D) - 2-year OS of 100% (arm A), 99.0% (arm B), 98.1% (arm C), and 96.3% (arm D)
14	HPV+ OPSCC	Reduced dose vs standard dose RT/CRT	Reduced: 50-65 Gy Standard: ≥66 Gy ± chemotherapy in both arms	- No difference in 3-year OS between arms
15	HPV+ OPSCC	Reduced-dose IMRT with chemotherapy	60 Gy with 30 mg/m2 cisplatin weekly	- 86% pCR - Moderate grade 3-4 toxicities, 11% hematological grade 3-4 toxicities
16	HPV+ OPSCC	Reduced-dose IMRT with chemotherapy	60 Gy with 30 mg/m2 cisplatin weekly	- 2-year LRC of 95%, DMFS of 91%, PFS of 86%, OS of 95% - No grade 3+ toxicities

[17]	HPV+ OPSCC	Reduced-dose IMRT with chemotherapy	60 Gy with 30 mg/m2 cisplatin weekly	- 3-year LC, RC, CSS, and DMFS of 100%, OS of 95% - No grade 3+ late toxicities
[18]	HPV+ OPC	Reduced-dose IMRT	43.2 Gy	- 5-year LRC and OS of 100% - 52% grade 3 acute mucositis, 35% grade 3 acute dermatitis
[19]	HPV+ OPSCC	Reduced-dose IMRT ± chemotherapy	60 Gy ± 40 mg/m2 cisplatin weekly	- With chemotherapy: 2-year PFS of 90.5%, OS of 96.7% - IMRT alone: 2-year PFS of 87.6%, OS of 97.3% - Significantly higher acute toxicities with IMRT and chemotherapy
[20]	HPV+ OPSCC	RT/CRT vs TOS & neck dissection	RT/CRT: 60 Gy ± 40 mg/m2 cisplatin weekly	- High grade 2-5 toxicities in the TOS & neck dissection arm

[*] Abbreviations: CRT, chemoradiotherapy; DMFS, distant metastasis-free survival; IMRT, intensity-modulated radiotherapy; HPV+, human papilloma virus positive; HPV-, human papilloma virus negative; LA HNSCC, locally advanced head and neck squamous cell carcinoma; LRP, locoregional progression; pCR, pathological complete response; PFS, progression-free survival; OPC, oropharyngeal cancer; OPSCC, oropharyngeal squamous cell carcinoma; OS, overall survival; RT, radiotherapy; TOS, transoral surgery.

Radiation dose reduction has also been proposed as a de-escalation strategy with intensity-modulated radiotherapy (IMRT), a technique that enhances radiation delivery by dispersing rays across specific curved dosage gradients to minimize radiation exposure to healthy tissues[21]. This radiotherapy modality has demonstrated a capacity to decrease morbidities across various cancer types, particularly in HNC, where IMRT has been associated with decreased xerostomia rates and improved saliva production[21]. Various investigations have shown that the use of de-escalated IMRT with adjuvant platinum-based chemotherapy, taxane chemotherapy, or immunotherapy for HPV-positive OPC cases resulted in excellent clinical outcomes for patients (**Table 1**;[15–18]). In a study investigating reduced-dose IMRT with cisplatin chemotherapy for HPV-positive OPC, the intervention resulted in a pathological complete response (pCR) rate of 86%, as well as moderate rates of grade 3–4 acute toxicities, and low severe hematological toxicities[15]. Updated results from this study showed high survival rates without any high-grade late toxicities after a 3-year follow-up[17]. Another study using the same population demographic and intervention reported similar 2-year survival and toxicity results[16]. Moreover, a study assessing reduced-dose IMRT in HPV-

positive OPC reported 5-year LRC and OS rates of 100%, with moderate and low rates of severe acute and late toxicities, respectively[18]. However, there was a study showing that IMRT combined with adjuvant platinum therapy had higher rates of acute toxicities compared to IMRT alone, though this treatment resulted in a superior PFS and LRC outcomes[19]. Consequently, the promising results from recent trials underscore the need for further research on reduced-dose radiation as a prospective treatment alternative in the context of HPV-positive HNCs.

3.4.2 Novel surgical approaches

Novel surgical approaches for patients with HPV-positive HNC involve innovative techniques aimed at enhancing treatment outcomes while minimizing potential adverse effects. These approaches often focus on preserving organ function and quality of life. For instance, TORS is gaining prominence as a less invasive method, utilizing robotic-assisted tools to access and remove tumors through the mouth, reducing the need for traditional open surgery[5,22]. Similarly, minimally invasive procedures like transoral laser microsurgery (TLM) utilize focused laser energy to precisely target and remove tumors in the oropharynx[22]. These approaches aim to reduce postoperative complications, shorten recovery times, and ultimately improve the patient's experience[5,22]. While novel surgical approaches hold promise for HPV-positive HNC patients, they come with certain drawbacks, including the technical complexity of the procedure demanding specialized training and equipment, the need for careful patient selection based on tumor characteristics, uncertainty regarding long-term outcomes and complications, and patient concerns about unfamiliar procedures and perceived risks[5,22]. Despite these limitations, only a single study revealed that TOS yielded higher instances of severe adverse events in comparison to reduced-dose RT (**Table 1**), which subsequently led to the termination of the trial[20]. Consequently, while TOS exhibits promising potential in treatment de-escalation without compromising efficacy in HPV-positive HNCs, there remains a need for further dedicated investigations. These factors underline the importance of thorough patient discussions and individualized treatment plans when considering these innovative surgical options. By tailoring surgical interventions to individual patients and their specific tumor characteristics, these novel approaches contribute to more effective and patient-centered treatment strategies.

3.5 Neoadjuvant chemotherapy

3.5.1 Taxane-based chemotherapy regimens

An emerging approach for treatment de-escalation involves the use of various chemotherapy drugs as alternatives to the conventional platinum chemoradiotherapy regimen. These chemotherapies are often administered as neoadjuvants before surgeryor radiation, in combination with radiotherapy, or alongside conventional platinum therapies to minimize toxicities while preserving or enhancing treatment effectiveness. Particularly, taxane-based drugs, such as docetaxel or paclitaxel, have been integrated into these strategies. While past reviews have explored taxane use in both HPV-positive and HPV-negative HNC[11,23], recent studies have highlighted their value as neoadjuvants in de-escalation approaches for HPV-positive HNC (**Table 2**). For instance, combining taxanes with platinum-based chemotherapy drugs for induction has yielded promising clinical responses in patients with HPV-positive OPC[24–26]. Sadeghi et al. investigated the use of combined docetaxel and cisplatin as neoadjuvant induction chemotherapy prior to TOS in patients with HPV-positive OPC, achieving high pCR rates (72%) at the primary site and 57% at nodal sites[24]. This strategy also resulted in a significantly higher disease-free survival (DFS) after 5 years compared to a control undergoing standard cisplatin chemoradiation, as well as reduced adverse events[25].

Table 2. Recent clinical investigations on adjuvant chemotherapies in HPV-positive HNC**.

Study	Cancer Type & HPV Status	Primary Therapy & Dose	Neoadjuvant Therapy & Dose	Outcomes
[24]	HPV+ OPC	TOS and selective neck dissection	75 mg/m^2 cisplatin and 75 mg/m^2 docetaxel	- pCR of 72% at primary site, 57% at nodal sites
[25]	HPV+ OPC	TOS and selective neck dissection	75 mg/m^2 cisplatin and 75 mg/m^2 docetaxel	- 5-year DFS of 96.1% - No feeding tube dependency 1 year after treatment - Significantly lower median length of feeding tube use following treatment
[26]	HPV+ OPC	RT/CRT (50 Gy, 45 Gy with chemotherapy, or 75 Gy with chemotherapy)	Induction AUC 6 carboplatin and 100 mg/m^2 *nab*-paclitaxel	- Response rate of 88% to induction - 5-year OS of 90%, PFS of 90%, LRC of 96%, and DC of 96%

[27]	HPV+ OPC	RT/CRT (50 Gy, 45 Gy with chemotherapy, or 75 Gy with chemotherapy)	Induction AUC 6 carboplatin and 100 mg/m^2 *nab*-paclitaxel	- 2-year PFS of 94.5% - Lower rates of grade 3+ mucositis in low-dose RT group vs low- and high-dose CRT groups - No PEG-tube use in low-dose RT group
[28]	HPV+ OPC	Standard IMRT (70 Gy) vs reduced-dose IMRT (56 Gy) with AUC 1.5 carboplatin in both arms	Induction 75 mg/m^2 docetaxel, 100 mg/m^2 cisplatin, and 750 mg/m^2 fluorouracil	- PFS and OS of 87.5% for the standard dose IMRT group vs PFS and OS of 83.3% for the reduced-dose group; no significant differences - pCR or pPR seen in all participants
[29]	HPV+ and HPV- HNSCC	Reduced-dose RT (63.6 Gy) vs standard RT (70.6 Gy)	75 mg/m^2 paclitaxel and 20 mg/m^2 cisplatin, concomitant to reduced-dose RT vs 600 mg/m^2 fluorouracil and 20 mg/m^2 cisplatin, concomitant to standard RT	- No differences in overall 3-year DFS and OS between treatments - No differences in 3-year DFS and OS between treatments in an HPV+ subgroup - Significantly lower grade 3-4 anemia and leukopenia with paclitaxel
[30]	HPV+ OPSCC	Reduced-dose RT (30 Gy or 36 Gy)	15 mg/m^2 docetaxel weekly	- 2-year LRC of 96.2%, PFS of 91.1%, and OS of 98.7% - Low rates of grade 3+ acute and late toxicities
[31]	HPV+ and HPV- HNSCC	Curative surgical or non-surgical therapy within 16 weeks prior to start of adjuvant	10 mg/day everolimus vs placebo	- No significant differences overall in PFS and OS with everolimus - Significantly higher PFS in HPV- participants with everolimus, but no differences in HPV+ participants
[32]	HPV+ and HPV- HNSCC	Definitive surgery	10 mg/day or 20 mg/day tadalafil, vs placebo prior to surgery	- Significant reduction of myeloid-derived suppressor cells and regulatory T cells with tadalafil - Significant increase in tumor-specific CD8+ T cells with tadalafil - No HPV subgroup analyses

[33]	HPV+ and HPV- HNSCC	Unspecified curative therapy external to the trial	20 mg/day tadalafil vs placebo	- Significant increase in ex-vivo T cell expansion and median delayed type hypersensitivity with tadalafil - Significant decrease in myeloid-derived suppressor cells with tadalafil - No differences based on HPV status
[34]	HPV+ and HPV- HNSCC	2 cycles of 240 mg nivolumab prior to surgery	10 mg/day tadalafil vs no adjuvant	- Total response rate of 54% (pPR or pCR), no differences based on HPV status or adjuvant tadalafil - Significant increase in B cells and non-significant increase in T cells with tadalafil, regardless of HPV status - Upregulated B cell and T cell gene expression in HPV+ tumors with tadalafil

** Abbreviations: CRT, chemoradiotherapy; DFS, disease-free survival; DC, distant control; IMRT, intensity-modulated radiotherapy; HNSCC, head and neck squamous cell carcinoma; HPV+, human papilloma virus positive; HPV-, human papilloma virus negative; LRC, locoregional control; pCR, pathological complete response; PEG, percutaneous endoscopic gastrotomy; PFS, progression-free survival; pPR, pathological partial response; OPC, oropharyngeal cancer; OPSCC, oropharyngeal squamous cell carcinoma; OS, overall survival; RT, radiotherapy; TOS, transoral surgery.

Similarly, the OPTIMA trial examined the use of paclitaxel or nab-paclitaxel with carboplatin as induction chemotherapy, followed by RT or CRT at various de-escalated doses[26,27]. Not only did the regimen exhibit promising efficacy in the 2-year follow-up[27], but recently updated findings also highlight a remarkable response rate of 88%[26]. Moreover, the 5-year outcomes demonstrated highly favorable rates of OS, PFS, LRC, and distant control (DC), all exceeding 90%, as well as a decrease in adverse events and the dependency on feeding tubes[26].

In the same manner, the Quarterback trial assessed the effects of induction chemotherapy using docetaxel, cisplatin, and fluorouracil, followed by either standard or reduced-dose CRT in HPV-positive OPC[28]. All patients demonstrated a pCR or a pathological partial response (pPR) following induction chemotherapy[28], underscoring the effectiveness of neoadjuvant docetaxel when used with conventional chemotherapy, as it successfully induces clinical responses prior to radiotherapy and maintains efficacy without compromise.

As adjuvants, taxane drugs have shown promise when administered concurrently with de-escalated radiotherapy in HPV-positive HNC (**Table 2**). The phase III PacCis trial evaluated paclitaxel combined with cisplatin chemotherapy and low-dose RT as an alternative to standard CRT in the treatment of HNC[29]. Despite no differences in 3-year DFS and OS between treatments, subgroup analyses by HPV status revealed that HPV-positive patients receiving the de-escalated regimen had similar DFS and OS to those under the standard treatment[29]. Additionally, MC1273, a phase II clinical trial, demonstrated positive effects of concurrent docetaxel with de-escalated RT as an adjuvant to surgery in HPV-positive OPC[30]. The 2-year OS, PFS, and LRC were all above 90%, with low rates of toxicity after study completion[30].

Though the need for additional large-scale investigations and long-term follow-ups fueled the hesitation surrounding these neoadjuvant chemotherapies[5], these recent studies suggest that integrating taxane drugs as neoadjuvants or adjuvants to de-escalated RT, CRT, or surgery could constitute successful treatment de-escalation strategies in HPV-positive HNC, leading to reduced adverse effects while maintaining clinical efficacy.

3.5.2 Exploring alternative systemic therapies in HPV-positive HNC: beyond taxane chemotherapies

In recent years, researchers have delved into novel systemic therapies as potential treatment options for HPV-positive HNC, supplementing taxane chemotherapies. However, these alternative systemic therapies have shown limited success (**Table 2**). One of these therapies is everolimus, an mTOR inhibitor already established for treating kidney, breast, and neuroendocrine tumors[35]. While previous studies demonstrated everolimus's ability to impede tumor growth in patient-derived xenograft models of various HNCs, its exploration within HPV-positive tumors was limited[35]. A trial examining everolimus as an adjuvant following curative local therapy reported unfavorable outcomes for patients with HPV-positive HNC[31]. Despite lacking significant impacts on OS or PFS, the subgroup analyses revealed that everolimus significantly improved PFS in HPV-negative patients, while no differences were seen in HPV-positive patients[31]. Another avenue under investigation is tadalafil, a phosphodiesterase-5 inhibitor which was previously shown to improve tumor-specific immunity and reshape the tumor immune microenvironment[32–34], though differences in response based on HPV status were either not assessed [32], or not significant[33,34]. Considering these findings, taxanes are the leading neoadjuvant chemotherapies with remarkable

achievements in HPV-positive HNC, underscoring the need for further exploration of alternative novel chemotherapies in this specific cancer context.

3.6 Targeted Therapies in Treatment De-escalation

As alternatives to existing chemotherapies, immunotherapy and targeted therapies have gained attention as potential neoadjuvant treatments that may improve clinical outcomes while minimizing systemic effects. Currently, immunotherapies are being investigated as both neoadjuvant and adjuvant treatments to RT, CRT, and surgery, reflecting a paradigm shift in treatment strategies towards de-escalation. Historically, induction chemotherapies were primarily used to support de-escalation of CRT or RT, aiming to promote more robust tumor responses. However, the effectiveness of these neoadjuvants led to diverse clinical outcomes[12,23]. The use of neoadjuvant immunotherapies has expanded de-escalation strategies, offering additional therapeutic avenues to improve clinical outcomes while reducing related toxicities[12]. This is particularly pertinent in the context of HPV-positive HNCs, which exhibit distinct treatment responses, prognostic profiles, and are increasingly recognized as a unique subgroup within HNC, making them well-suited for alternative treatments strategies and de-escalation approaches[1,4,12].

3.6.1 Therapeutic biomarkers

An increasing number of investigators are conducting multi-omics experiments, which allows a rapid and comprehensive analysis of cancers in individual patients. As the field of targeted therapy rapidly evolves, researchers are identifying and validating biomarkers that can predict treatment outcomes and patient prognosis. By analyzing specific genetic, epigenetic, and protein markers, clinicians could tailor therapy regimens to individual patients, enhancing treatment precision and efficacy while minimizing potential side effects, effectively transforming the landscape of HNC treatment. To direct the development of novel targeted therapies, biomarkers associated with both HPV-positive and negative HNC have been identified (**Fig. 7**). Unfortunately, studies regarding HNC biomarkers are still hampered by several limitations, such as technical parameters such as sample size, suitable analysis strategy, standardized protocols for sample collection and storage, rational study design, detailed methods, as well as the complex nature of the disease itself. HNC is not a uniform disease, but a heterogeneous neoplasm with an array of genetic and epigenetic modifications associated with different risk factors. Though research on biomarkers is still

ongoing, recent breakthroughs on biological targets and their impact in HPV-positive HNC are outlined below.

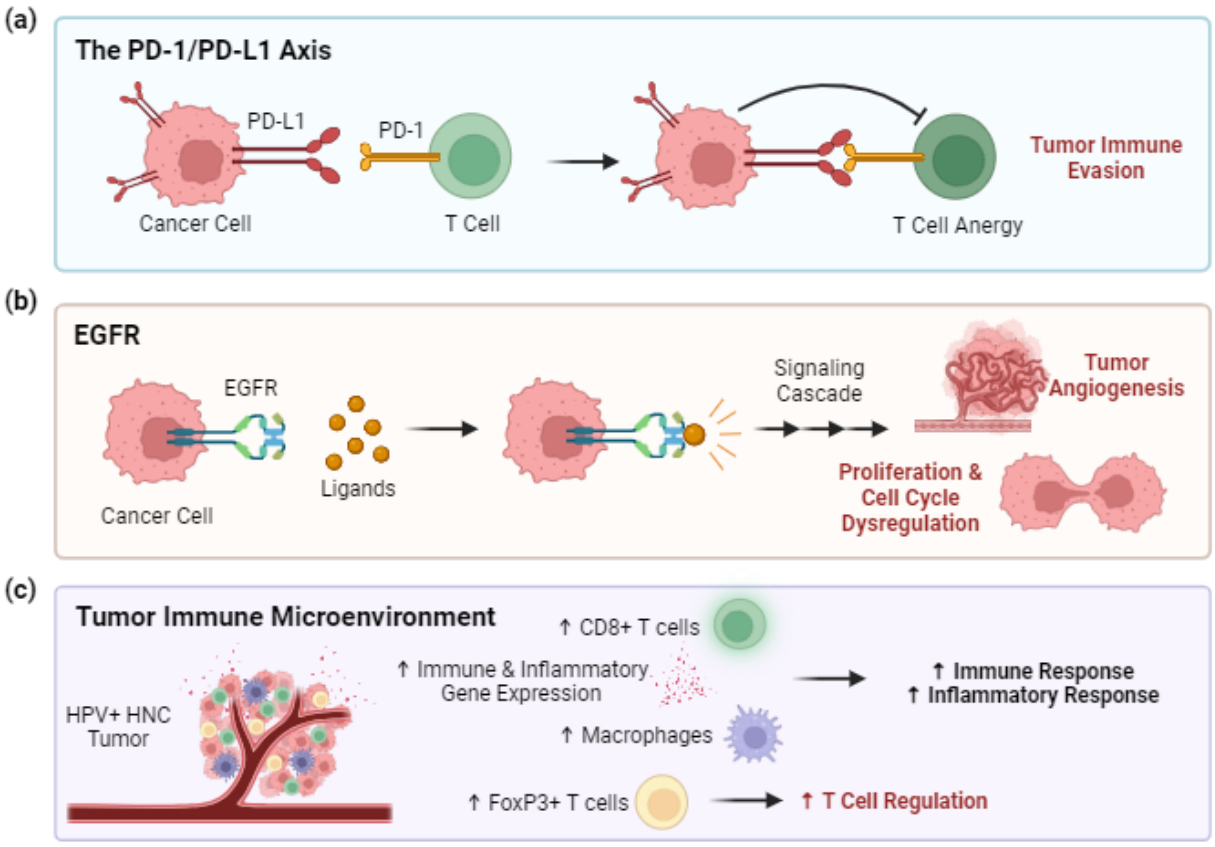


Fig. 7. Biomarkers used in the development of targeted therapies in HPV-positive HNC. (**a**) Interactions between PD-L1 ligands on tumor cells and PD-1 receptors on effector T cells can suppress their activity, leading to T cell inactivation and immune escape. (**c**) Ligands (EGF, TGF-α, HB-EGF, betacellulin, amphiregulin and epiregulin) bind to EGFRs on cancer cells, initiating signaling pathways that promotes angiogenesis, cell proliferation, and cell cycle dysregulation. (**d**) HPV-positive HNCs have distinct tumor immune microenvironments, with increased CD8+ T cells, macrophages, FoxP3+ cells, and immune/inflammatory gene expression. This results in heightened immune and inflammatory responses, as well as T cell regulation. Abbreviations: EGFR, epidermal growth factor receptor; HNC, head and neck cancer; HPV+, human papillomavirus positive; PD-1, programmed cell death receptor 1; PD-L1, programmed cell death ligand 1; EGF: epidermal growth factor; TGF-α - transforming growth factor apha; HB-EGF heparin-binding EGF-like growth factor. Created with BioRender.com.

3.6.1.1 PD-1/PD-L1 Axis

The receptor Programmed Cell Death 1 (PD-1) and its ligand Programmed Cell Death Ligand 1 (PD-L1) form a regulatory axis that plays a pivotal role in both central and peripheral immune tolerance[36,37]. PD-L1, which is expressed by dendritic and epithelial cells, binds to the PD-1 receptor expressed by maturing T cells, inhibiting the activation of these T cells and preventing downstream autoimmune responses[36]. The PD-1/PD-L1 axis is exploited by cancer cells as a mechanism to evade immune surveillance (**Fig. 7a**)[37]. The overexpression of PD-L1 by tumor cells suppresses the activation of effector T cells, consequently promoting proliferation and tumorigenesis[37].

In the context of HPV-positive HNC, the implications of the PD-1/PD-L1 axis become particularly significant. Overexpression of PD-L1 is often observed on the epithelia of tonsillar crypts, a common site of origin for HNCs[38,39]. This is often accompanied by increased PD-1 expression on tumor-infiltrating T cells, ultimately promoting the interaction between PD-1 and PD-L1, and causing these T cells to become anergic and ineffective[38,39]. This anergic state facilitates the persistence of both viral HPV infections and the proliferation of cancer cells[38,39].

Immunotherapies have emerged as promising strategies to counteract the effects of the PD-1/PD-L1 axis in cancer. One notable approach involves the development of checkpoint inhibitors that block the interaction between PD-1 and PD-L1, effectively reversing T cell anergy and enabling the restoration of their functional activity[36].

Given the distinctive immune characteristics of HPV-positive HNC, targeting the PD-1/PD-L1 axis holds immense clinical significance[12,40]. Studies have established the relationship between PD-L1 expression and HPV status, with HPV-positive tumor cells often having higher levels of PD-L1 expression[41–44]. The PD-1/PD-L1 axis offers the potential to restore antitumor immune responses and enhance treatment efficacy in HPV-positive HNC, as explored below in recent clinical trials (**Fig. 8a**; **Table S1**;[45–51]).

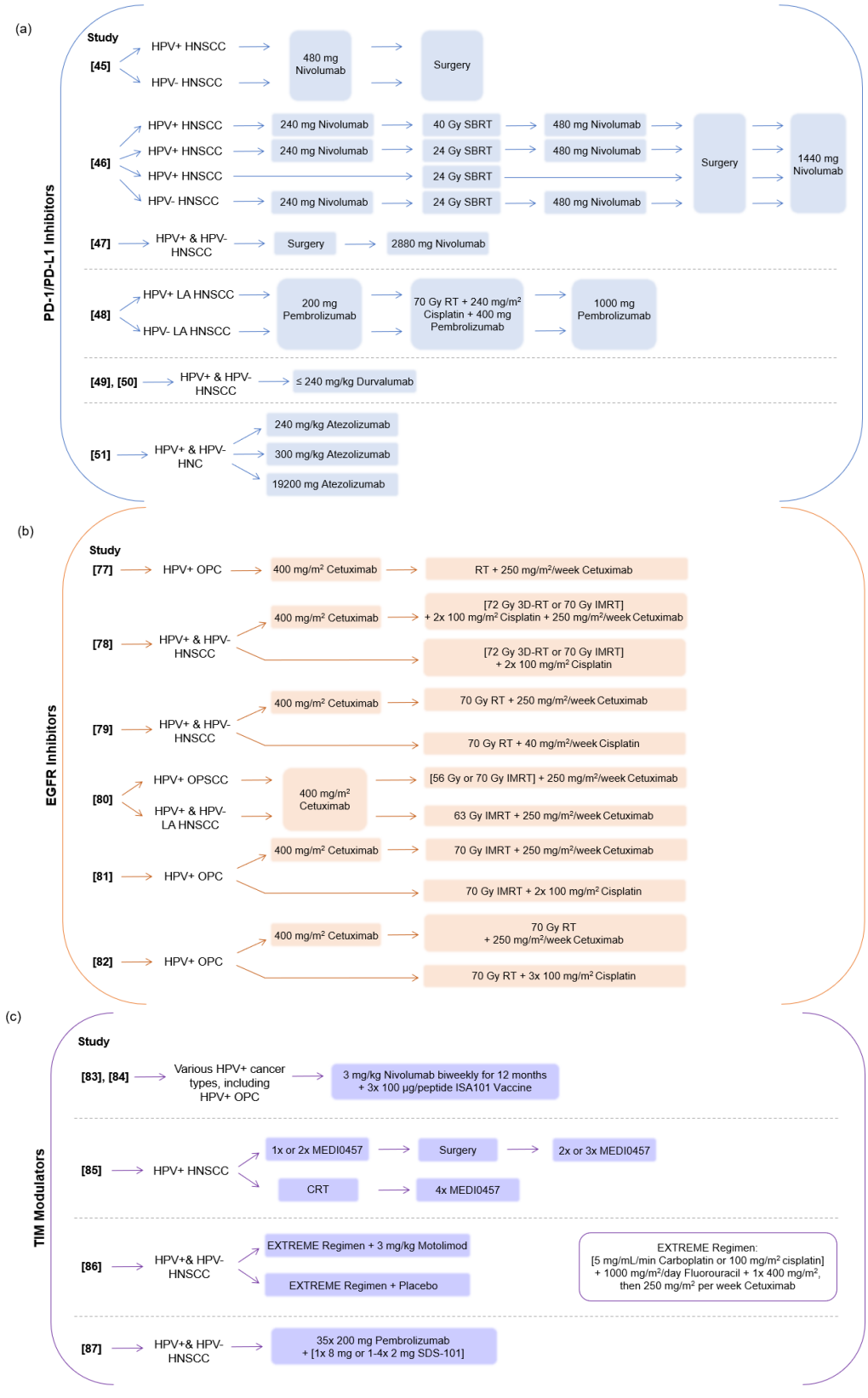

(a)
PD-1/PD-L1 Inhibitors
Study
[45]
HPV+ HNSCC
HPV- HNSCC
480 mg Nivolumab
Surgery
[46]
HPV+ HNSCC
HPV+ HNSCC
HPV+ HNSCC
HPV- HNSCC
240 mg Nivolumab
240 mg Nivolumab
240 mg Nivolumab
40 Gy SBRT
24 Gy SBRT
24 Gy SBRT
24 Gy SBRT
480 mg Nivolumab
480 mg Nivolumab
480 mg Nivolumab
Surgery
1440 mg Nivolumab
[47]
HPV+ & HPV- HNSCC
Surgery
2880 mg Nivolumab
[48]
HPV+ LA HNSCC
HPV- LA HNSCC
200 mg Pembrolizumab
70 Gy RT + 240 mg/m² Cisplatin + 400 mg Pembrolizumab
1000 mg Pembrolizumab
[49], [50]
HPV+ & HPV- HNSCC
≤ 240 mg/kg Durvalumab
[51]
HPV+ & HPV- HNC
240 mg/kg Atezolizumab
300 mg/kg Atezolizumab
19200 mg Atezolizumab
(b)
EGFR Inhibitors
Study
[77]
HPV+ OPC
400 mg/m² Cetuximab
RT + 250 mg/m²/week Cetuximab
[78]
HPV+ & HPV- HNSCC
400 mg/m² Cetuximab
[72 Gy 3D-RT or 70 Gy IMRT] + 2x 100 mg/m² Cisplatin + 250 mg/m²/week Cetuximab
[72 Gy 3D-RT or 70 Gy IMRT] + 2x 100 mg/m² Cisplatin
[79]
HPV+ & HPV- HNSCC
400 mg/m² Cetuximab
70 Gy RT + 250 mg/m²/week Cetuximab
70 Gy RT + 40 mg/m²/week Cisplatin
[80]
HPV+ OPSCC
HPV+ & HPV- LA HNSCC
400 mg/m² Cetuximab
[56 Gy or 70 Gy IMRT] + 250 mg/m²/week Cetuximab
63 Gy IMRT + 250 mg/m²/week Cetuximab
[81]
HPV+ OPC
400 mg/m² Cetuximab
70 Gy IMRT + 250 mg/m²/week Cetuximab
70 Gy IMRT + 2x 100 mg/m² Cisplatin
[82]
HPV+ OPC
400 mg/m² Cetuximab
70 Gy RT + 250 mg/m²/week Cetuximab
70 Gy RT + 3x 100 mg/m² Cisplatin
(c)
TIM Modulators
Study
[83], [84]
Various HPV+ cancer types, including HPV+ OPC
3 mg/kg Nivolumab biweekly for 12 months + 3x 100 µg/peptide ISA101 Vaccine
[85]
HPV+ HNSCC
1x or 2x MEDI0457
Surgery
2x or 3x MEDI0457
CRT
4x MEDI0457
[86]
HPV+& HPV- HNSCC
EXTREME Regimen + 3 mg/kg Motolimod
EXTREME Regimen + Placebo
EXTREME Regimen:
[5 mg/mL/min Carboplatin or 100 mg/m² cisplatin] + 1000 mg/m²/day Fluorouracil + 1x 400 mg/m², then 250 mg/m² per week Cetuximab
[87]
HPV+& HPV- HNSCC
35x 200 mg Pembrolizumab + [1x 8 mg or 1-4x 2 mg SDS-101]

Fig. 8. Neoadjuvant immunotherapies administered in recent clinical trials for treatment de-escalation in HPV-positive HNC. The type of cancer, HPV status, and order of intervention administration is specified for each study. (**a**) Studies investigating PD-1/PD-L1 inhibitors administered as adjuvants or used as primary treatments in HPV-positive HNCs. The total dose of the intervention administered is listed. (**b**) Studies investigating the EGFR inhibitor cetuximab as an adjuvant in HPV-positive HNCs. (**c**) Studies investigating agents that modulate the tumor immune microenvironment in HPV-positive HNCs. Abbreviations: CRT, chemoradiotherapy; DCR, disease control rate; DFS, disease-free survival; DM, distant metastasis; EGFR, epidermal growth factor receptor; HNC, head and neck cancer; HNSCC, head and neck squamous cell carcinoma; HPV+, human papilloma virus positive; HPV, human papilloma virus negative; IMRT, intensity-modulated radiotherapy; LA, locally advanced; LRC, locoregional control; LRF, locoregional failure; mPR, major pathological response; NA, not applicable; pCR, pathological complete response; PD-1, programmed cell death 1 receptor; PD-L1, programmed cell death ligand 1; PFS, progression-free survival; pPR, pathological partial response; OPC, oropharyngeal cancer; ORR, objective response rate; OPSCC, oropharyngeal squamous cell carcinoma; OS, overall survival; RFS, recurrence-free survival; RT, radiotherapy; SBRT, stereotactic body radiation; TIM, tumor immune microenvironment.

3.6.1.2 Epidermal growth factor receptor (EGFR)

The epidermal growth factor receptor (EGFR) is a tyrosine kinase that, upon activation through ligand binding, triggers signaling pathways involved in regulating cell cycle progression, proliferation, and angiogenesis[52–55]. Due to its role in cell growth and survival, EGFR emerges as a crucial factor in cancer biology (**Fig. 7b**). Its upregulation in tumor cells allows them to expand, adhere, and metastasize to various tissues[52,54]. Indeed, EGFR is commonly overexpressed in cancer cells, particularly in HNCs[52,54–57], and has also been associated with radiotherapy resistance[52,53,58]. The predictive significance of EGFR in HNC has been previously assessed, revealing that gene overexpression or mutations correlate with unfavorable patient survival rates and clinical outcomes[53,54,59]. However, recent studies have shown that HPV status may influence treatment response and resistance in HNCs with overexpressed EGFR[53,58]. Contrarily to HPV-negative HNC, high EGFR expression does not lead to radiotherapy resistance in HPV-positive HNC cells, but rather increases treatment sensitivity *in vitro* and *in vivo*, and hinders DNA repair mechanisms[58].

EGFR overexpression also led to the re-establishment of p53 function in HPV-positive HNC cells following radiotherapy[58], which can likely be explained by the reduced expression of viral HPV oncogenes E6 and E7 in the presence of high levels of EGFR[60]. Thus, though EGFR expression cannot be denied as a potential biomarker for HNC, further research is still needed to assess its prognostic value and diversify treatment strategies in HPV-positive HNCs.

3.6.1.3 Tumor immune microenvironment

As HPV-positive HNC tumors possess a distinct immune microenvironment from HPV-negative cancers (**Fig. 7c**), which in turn influences survival, the tumor immune microenvironment has emerged as an important therapeutic target. Indeed, increased tumor infiltrating lymphocytes (TILs) due to viral HPV infection are speculated to improve clinical outcomes[61,62]. Although one study by Wansom *et al.* failed to show differences in types of TILs based on HPV status[63], other studies have shown that HPV-positive OPC tumors possess higher levels of CD8+ T cells and FoxP3+ regulatory T cells[62,64], and that elevated levels of TILs are closely correlated with ameliorated treatment responses[61,62,64]. HPV-positive OPC tumors were also found to have higher T cell, macrophage, and inflammatory gene expression, as well as stronger activation of immune processes[65]. Thus, differences in the composition of the tumor immune microenvironment observed in HPV-positive HNCs can help to predict patient's outcomes. This paves the way for further investigation into innovative treatment strategies that focus on harnessing the immune system's capabilities.

3.6.2 Neoadjuvant immunotherapy and targeted therapy

The use of targeted therapies and immunotherapies as adjuvants in the treatment of cancer is becoming a widespread therapeutic strategy. The most common biological target in HPV-positive HNC is the PD-1/PD-L1 axis, however, its clinical significance remains controversial[12,40]. Recent meta-analysis results suggest that responses to PD-L1 inhibition do not significantly differ based on HPV status in HNC[66]. Despite this finding, recent clinical trials yield promising outcomes in HPV-positive cohorts, particularly for the PD-1 inhibitor nivolumab (**Fig. 8a**; **Table S1**). In the CheckMate 358 trial investigating neoadjuvant nivolumab prior to surgical tumor resection in HNC, positive HPV status correlated with improved tumor response to treatment[45]. Although no pCRs were reported, patients with HPV-positive HNC had higher rates of pPR, along with improved RFS and OS compared to HPV-negative counterparts[45]. Another study evaluated the

efficacy of nivolumab when combined with low-dose stereotactic body radiation therapy (SBRT) prior to curative surgery for HPV-positive or -negative HNC[46]. This neoadjuvant treatment not only exhibited a favorable safety profile but also demonstrated increased efficacy in HPV-positive patients, evidenced by pPR and pCR rates of 100% and 90%, respectively, within this subgroup[46]. The use of nivolumab is further supported in a study by Leddon *et al*., where it resulted in significantly higher overall DFS when administered following salvage surgery[47]. However, this study failed to find differences in efficacy based on HPV status[47], indicating that nivolumab may be a potential adjuvant treatment for both HPV-positive and -negative HNCs.

Other inhibitors of the PD-1/PD-L1 axis have also been studied as adjuvants or primary treatments in HPV-positive HNC, but with mixed results (**Fig. 8a**; **Table S1**). Pembrolizumab was examined as both an adjuvant and a neoadjuvant to standard platinum CRT and resulted in higher rates of complete response, OS, and PFS in HPV-positive HNC compared to HPV-negative, though statistical significance was not assessed[48]. Durvalumab was administered as a first line of treatment in two different studies, reporting conflicting results in HPV-positive subgroups, with one study showing higher ORRs, median PFS and OS in HPV-positive patients[49], while another showed better ORRs and disease control rates in HPV-negative patients[50], though neither study assessed statistical significance[49,50]. Similarly, atezolizumab as a primary treatment in HNC resulted in a modest overall ORR and disease control rate (DCR), with no major differences in tumor response based on HPV status[51]. These varying results support the need for future investigation into these immunotherapies as adjuvants or neoadjuvants in addition to first-line treatments. Nevertheless, the literature has shown that the PD-1/PD-L1 axis constitutes an actionable biomarker in HPV-positive HNC, and though more research may be required, anti-PD-1 or PD-L1 immunotherapies can provide potential benefits to standard treatments as adjuvants.

The tyrosine kinase EGFR is another important biomarker directing the use of targeted therapies to treat HNC. Though high EGFR expression levels have been correlated with poor prognoses, EGFR overexpression has also been shown to result in different treatment responses based on HPV status[53,57]. The clinical value of EGFR inhibitors as treatments in HPV-positive HNC remains debated. Although they have shown clinical benefit in HPV-negative HNCs, HPV-positivity has been associated with worse outcomes under similar treatment regimens[58,60]. This is evidenced by recent studies on the EGFR inhibitor cetuximab (**Fig. 8b**; **Table S1**). Despite one

study finding improved immune signaling and tumor infiltration of CD8+ lymphocytes with neoadjuvant cetuximab prior to RT in HPV-positive OPC[67], many studies have reported negative outcomes among HPV-positive cohorts. Long-term results from a phase III trial assessing cetuximab as a neoadjuvant and concomitant treatment to platinum CRT in HNC found that the immunotherapy failed to improve PFS, locoregional failure, rates of distant metastases, and OS in the entire study population, without any significant differences in outcomes based on HPV status[68]. Similarly, when administered prior to and during RT for locally advanced HNC, cetuximab did not have a superior efficacy to cisplatin CRT, with no differences in local control, rates of metastases, and OS between the treatments[69]. In the same study, cancer-specific survival was unaffected by the therapy, and exploratory analyses of an HPV-positive subgroup revealed that cisplatin CRT resulted in better clinical outcomes than cetuximab, though statistical significance could not be assessed[69]. Conversely, one study found that administering cetuximab as a neoadjuvant and an adjuvant to RT resulted in promising 2-year DFS and OS rates of 81% and 95%, respectively, among patients with HPV-positive OPC[70]. However, this subgroup had higher rates of severe toxicities, failing to justify the use of cetuximab for treatment de-escalation[70]. Finally, two landmark studies, the NRG Oncology RTOG 1016 trial and the De-ESCALaTE HPV trial, examined the effects of cetuximab administered prior to and concurrently with RT in HPV-positive OPC[71,72]. Both studies reported lower rates of OS with cetuximab compared to cisplatin CRT[71,72], with the NRG Oncology RTOG 1016 trial also reporting significantly worse rates of PFS and locoregional failure, and the De-ESCALaTE HPV trial reporting significantly higher rates of recurrence and metastases with the immunotherapy[71,72]. Consequently, the use of the EGFR inhibitor cetuximab does not seem to aid in the treatment of HPV-positive HNC, contrarily to HPV-negative HNC. Thus, de-escalation strategies must be approached differently in this cancer type and require further investigation into other prognostic markers of the disease that may serve as better targets for neoadjuvant therapies.

Another treatment strategy for HPV-positive HNC includes modulating the tumor immune microenvironment. Although research in this area is still underway, studies have already shown that targeting the immune system on multiple fronts may affect the immune response and clinical outcomes (**Fig. 8c**; **Table S1**). A recent study examined the effects of the ISA101 HPV-16 vaccine on the tumor immune microenvironment and on tumor response when administered in combination with the PD-1 inhibitor nivolumab[73,74]. The adjuvant peptide vaccine resulted in an ORR of 33%,

with high PD-L1 expression correlating with better tumor responses[73]. Though this study included various HPV-positive cancer types, all patients that responded to the treatment had HPV-positive OPC[73]. Updated results showed significant increases in immune cells, as well as higher expression of immune and inflammatory genes were reported in tumors that had a clinical response to the treatment[74]. However, the study population included cancer types other than HNC, and reported low long-term PFS and OS rates[73,74], warranting larger-scale trials specifically in HPV-positive OPC.

As seen, proteins specific to HPV are promising targets for novel immunotherapies. MEDI0457, a DNA-based immunotherapy targeting the E6 and E7 HPV proteins, was studied as an adjuvant to surgery or standard CRT in HPV-positive HNC[75]. The adjuvant immunotherapy induced multiple changes in the tumor immune microenvironment, such as the production of long-lasting antibodies and cytotoxic T cells specific to HPV peptide antigens[75].

Toll-like receptor (TLR) agonists have also been explored in the treatment of HPV-positive HNCs. Motolimod, a TLR8 agonist, was studied as an adjuvant to standard platinum chemotherapy with cetuximab in HNC, and resulted in significantly longer PFS and OS in an HPV-positive subgroup, though no differences were seen in the entire study population compared to a placebo[76]. Similarly, when combined with pembrolizumab, the TLR9 agonist SD-101 resulted in a higher ORR in HPV-positive HNC[77]. As such, recent studies show the value of targeting the tumor immune microenvironment in HPV-positive HNC, though further research is needed on their efficacy as adjuvant or neoadjuvant treatments for their implementation in de-escalation strategies.

3.6.3 Combined therapy

Beyond their application as adjuvants to standard RT or surgical procedures, immunotherapies and targeted therapies have also been explored in combination with existing taxane chemotherapies, as adjuvant or neoadjuvant therapies, in hopes of eliciting greater anti-tumor responses by targeting multiple facets of the disease. However, due to the limited number of recent trials investigating these combined strategies, the findings remain preliminary and exhibit diverse outcomes.

A study conducted by Oppelt et al. examined the efficacy of combination nab-paclitaxel with cetuximab or cisplatin as adjuvant induction therapies prior to CRT in cases of locally advanced HNC[78]. Interestingly, HPV-positive patients had higher clinical complete response (cCR)

rates than HPV-negative patients, regardless of the treatment received, though HPV-positive patients exhibited a lower cCR rate with cetuximab than cisplatin[78]. Similarly, the cohort receiving cetuximab also showed lower rates of LRC, PFS, and OS, though statistical significance of these differences was not evaluated[78]. Despite these outcomes, the induction therapy led to a moderate number of severe grade 3–4 adverse events, with lower rates of toxicities observed throughout the study among patients receiving cetuximab[78].

In a recent study known as the CheckRad-CD8 trial, a combined therapy approach was examined, involving two chemotherapies (cisplatin and docetaxel) and two immunotherapies (PD-L1 inhibitor durvalumab and a CTLA-4 inhibitor tremelimumab) as a neoadjuvant induction therapy prior to radioimmunotherapy in HNC[79]. Administering this intervention before radioimmunotherapy showed promising 2-year PFS and OS rates of 72% and 84% among HPV-positive patients[79]. However, high rates of grade 3–4 adverse events were reported, though toxicities related to the immunotherapy were low[79].

Given these findings, the potential of combined adjuvant chemo-immunotherapies require further investigation to validate their efficacy and mitigate potential toxicities.

3.7 Conclusions and future directions

Recent studies have provided encouraging insights into the utilization of de-escalation strategies and neoadjuvant therapies for the treatment of HPV-positive HNC. When implementing these approaches, it is crucial to consider both their strengths and limitations. Innovative surgical methods, such as minimally invasive transoral surgery[22] and radiation de-escalation strategies, while showing promise, face challenges such as potential adverse events and limited accessibility[5,22]. These techniques often necessitate specialized equipment, training, and stringent patient selection criteria, which can affect their widespread use.

Neoadjuvant chemotherapies and immunotherapies have demonstrated their efficacy in de-escalating treatments for HPV-positive HNC. These innovative neoadjuvant approaches hold the potential to enhance clinical outcomes while concurrently minimizing morbidities and side effects. The use of docetaxel, an established chemotherapy for HNC, in combination with radiotherapy and surgery has been widely accepted in HNC treatment[80], highlighting its suitability for integration into de-escalation strategies. Similarly, recently approved immunotherapies targeting

the PD-1/PD-L1 axis, such as nivolumab and pembrolizumab, have displayed positive outcomes in recurrent or metastatic HNC[81], and their potential value extends to neoadjuvant strategies in HPV-positive HNC. However, caution is warranted when considering the EGFR inhibitor cetuximab, which was previously approved for use in HNC[81], but has recently demonstrated unfavorable survival outcomes in HPV-positive tumors compared to conventional chemoradiotherapy.

The evolving landscape of immunotherapies focusing on the tumor immune microenvironment and HPV proteins, along with combined chemo-immunotherapies, holds promise for HPV-positive HNC treatment. Nevertheless, further research is essential to refine these approaches and ensure their successful integration into de-escalated treatment regimens. As HPV-positive HNC gains recognition as a distinct cancer subtype, tailoring de-escalated treatment strategies to account for HPV status becomes paramount. While challenges persist, advancements in surgical techniques, radiation technologies, and neoadjuvant therapies are reshaping treatment paradigms for HPV-positive HNC, offering improved clinical outcomes with minimized toxicities.

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CRediT authorship contribution statement

Conceptualization, S.D.S. and J.B.; methodology, J.B.; software, not applicable; formal analysis, not applicable; investigation, J.B.; resources, S.D.S; data curation, J.B.; writing—original draft preparation, J.B.; writing—review and editing, K.S., A.M., M.M., N.K., M.A.J., M.H., N.S., L.P.K., S.D.S. and J.B.; visualization, J.B.; supervision, S.D.S.; project administration, S.D.S. and J.B.; funding acquisition, S.D.S. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

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Printing Considerations

Color should be used for all figures in print.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.critrevonc.2024.104326.

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Chapter 4: Bridging Statement

The manuscript presented in Chapter 3 provided a comprehensive summary of novel advancements in de-escalated treatments for HPV-related OPC, namely reduced-dose RT, neoadjuvant chemotherapy, and neoadjuvant or adjuvant immunotherapy, among others. The chapter explored results from recent clinical trials on these de-escalated treatment strategies, finding that established therapies like radiation, taxane chemotherapy, and PD-1 immunotherapy provide favorable outcomes when applied in a de-escalated setting, while the use of the chemotherapy cetuximab may not be beneficial in HPV-positive OPC patients. As previously mentioned in Chapter 2, a lack of evidence from large-scale clinical studies was one of the major barriers preventing the widespread acceptance of de-escalated treatments for HPV-positive OPC patients[9]. Chapter 3 addresses this knowledge gap, summarizing the outcomes from recent clinical trials, as well as introducing novel therapies that are currently under development. However, as discussed in Chapter 2, the implementation of de-escalated treatments for HPV-positive OPC is also hindered by the lack of standardized HPV testing in this cancer type. Since HPV-negative patients have worse prognoses and require more aggressive treatments, proper HPV testing is essential to ensure that de-escalated treatments are only allocated to HPV-positive patients. For this reason, hesitation surrounding the implementation of de-escalated treatment strategies remains despite the successful results seen in recent clinical trials. As explored in Chapter 2, multiple direct and indirect HPV testing methods are currently being used to identify HPV-positive OPC patients in clinical trials, with p16 IHC being the most common[4,23–25]. However, as OPC patients with discordant p16 and HPV statuses have worse prognoses and may not be good candidates for de-escalated strategies[4,23,26,27,88–90], direct molecular HPV testing has been recommended as an auxiliary technique to ensure proper prognostic stratification during treatment allocation[4,25–27,81,89,90], though variability in the methods selected remains without a standardized HPV testing regimen for OPC. As such, the study presented in Chapter 5 aims to address this issue by comparing the differences in HPV detection rates between p16 IHC and direct molecular methods, shedding light on the optimal method or combination of methods for HPV detection in OPC. Together, Chapters 3 and 5 will address the main issues impeding the acceptance of de-escalating treatments for HPV-positive OPC.

Chapter 5: Manuscript 2

Comparative Analysis of Histological and Molecular Methods for HPV Detection in Oropharyngeal Cancer

Differences in HPV Detection in Oropharyngeal Cancer

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5.1 Abstract

Standardized human papillomavirus (HPV) testing is lacking in oropharyngeal cancer (OPC), with p16 immunohistochemistry (IHC) being most used, though direct molecular detection is recommended. We assess the differences between p16 IHC and molecular HPV detection methods in OPC. HPV genotyping was performed on 124 FFPE OPC specimens using the Anyplex™ II HPV28 Detection (Anyplex II) kit. The INNO-LiPA® HPV Genotyping Extra II (INNO-LiPA) and an *in-house* qPCR assay were used for validation. p16 IHC was performed using antibodies E6H4, BC42, and JC8, and positivity thresholds of 70%, 50%, and 0.01%. HPV detection was significantly higher with the Anyplex II assay than the *in-house* qPCR (87.9% vs 29.5%, $p = 2.33 \times 10^{-13}$), though it was significantly lower than with INNO-LiPA. No differences were found in genotype-specific or co-infection detection. IHC antibody E6H4 significantly outperformed BC42 at all p16 thresholds, and JC8 at thresholds of 70% and 50%. The Anyplex II assay provided significantly more HPV detection than all p16 antibodies at positivity thresholds of 70% (E6H4: 92.5% vs 71.3%, $p = 1.59 \times 10^{-4}$; BC42: 92.5% vs 31.3%, $p = 4.49 \times 10^{-14}$; JC8: 93.0% vs 35.2%, $p = 3.06 \times 10^{-13}$) and 50% (E6H4: 92.5% vs 77.5%, $p = 0.00316$; BC42: 92.5% vs 36.3%, $p = 5.67 \times 10^{-13}$; JC8: 93.0% vs 52.1%, $p = 1.64 \times 10^{-9}$). High rates of HPV detection using a molecular HPV testing method support the use of direct testing to validate p16 IHC results in OPC and may help improve treatment allocation.

5.2 Introduction

Head and neck cancer (HNC) is the 6th most common cancer[1], affecting the oral cavity, tongue, pharynx, larynx, and oropharynx, among other sites[2–4], and accounts for around 4.6% of cancer-related deaths each year[3].Traditionally, excessive tobacco and alcohol consumption were the main risk factors for the disease[2–4]. However, human papillomavirus (HPV) infections have emerged as prominent risk factors for HNC, particularly for oropharyngeal cancer (OPC). Over 30% of global OPC cases can be attributed to HPV infections, though rates are higher in high-income countries like the United States of America, where over 70% of OPC cases are HPV-related[4]. Unlike HPV-negative OPC, HPV-related OPC is characterized by a lower mutational burden[2,4] and superior patient prognoses[5–7], leading it to gain recognition as a distinct HNC subtype[2,4,8].This classification has also led to a shift in treatment strategies for HPV-positive patients, with research focusing on the development of de-escalated therapies like reduced-dose radiotherapy, neoadjuvant

chemotherapy, and immunotherapy which maintain anti-cancer efficacy while minimizing the severe acute and chronic toxicities associated with the current standard of care[9]. Though these treatments have shown promise, their widespread implementation and approval has been hindered by a lack of standardized diagnostic methods for HPV in OPC[10–12].

Currently, the most common HPV detection method in OPC remains p16 INK4A (p16) immunohistochemistry (IHC), relying on the expression of the protein p16 which becomes elevated due to the activity of viral HPV proteins E6 and E7[10,11]. Due to its low cost, simple methodology, and compatibility with formalin-fixed paraffin-embedded (FFPE) tissues, p16 IHC has become widely adopted in clinical settings[10,11]. Despite its advantages, the use of p16 IHC as a standalone diagnostic method has been debated, as the reliability of p16 as a proxy for HPV can be influenced by non-viral factors, like *TP53* mutations[10,13], the use of different IHC antibodies and positivity thresholds[14], and the geographic prevalence of HPV-related disease[12], resulting in a risk of false positive HPV diagnoses[10–12].This is concerning, as OPC patients with discrepant p16 expression and HPV DNA integration have been found to have worse prognoses[15–19], meaning they may be undertreated if allocated a de-escalated treatment. Molecular methods that directly detect the presence of HPV, such as consensus multiplex polymerase chain reaction (PCR), real-time PCR (qPCR) and DNA or RNA hybridization, have been proposed in conjunction with p16 IHC[12,20–22], though a consensus on the best HPV testing method for OPC has yet to be reached[10,11,22].

As such, the objective of our study was to shed light on the differences in HPV detection rates between histological and molecular methods. To this end, we examined the discrepancies between the initial p16 IHC grading, the Anyplex™ II HPV28 Detection (hereafter referred to as Anyplex II, Seegene Inc., South Korea) multiplex qPCR assay, and an *in-house* HPV-targeted qPCR assay. The INNO-LiPA® HPV Genotyping Extra II (hereafter referred to as INNO-LiPA, Fujirebio, Belgium) reverse hybridization line probe assay was used to assess genotype-specific differences in HPV detection between molecular genotyping methods. Differences in HPV detection between three p16 IHC antibodies was also examined and compared against direct HPV detection methods.

5.3 Results

Molecular HPV Detection Methods Have Significantly Less HPV Detection than Initial p16 IHC Scoring

One hundred and twenty-four (n = 124) OPC FFPE tissue specimens were selected for molecular genotyping using the Anyplex II assay. While all specimens had been previously deemed HPV-positive by p16 IHC, the Anyplex II assay had significantly less HPV detection, failing to detect the presence of HPV in 12.1% of samples (**Figure 9A**, 87.9%, $p = 0.00142$). There were no invalid results obtained with the Anyplex II assay. As commercially available genotyping kits can be costly for high-throughput applications, the HPV status of 112 samples from the cohort was assessed using an *in-house* qPCR with the Gp5+/Gp6+ primers. However, 15.2% of samples run using the *in-house* qPCR produced invalid results, with the test failing to detect the β-globin control in these cases. This assay only detected the presence of HPV in 29.5% of samples (**Figure 9A**), which was significantly less than both the Anyplex II assay ($p = 2.33 \times 10^{-13}$) and the initial p16 IHC grading ($p < 2.2 \times 10^{-16}$). Conversely, the *in-house* qPCR failed to detect HPV in 55.4% of samples, of which 11.3% also had no detection by Anyplex II. In terms of overall HPV detection, these results show that qPCR-based molecular methods appear to have a lower HPV detection rate than p16 IHC, though a commercial test is preferred over an *in-house* protocol when comparing molecular methods.

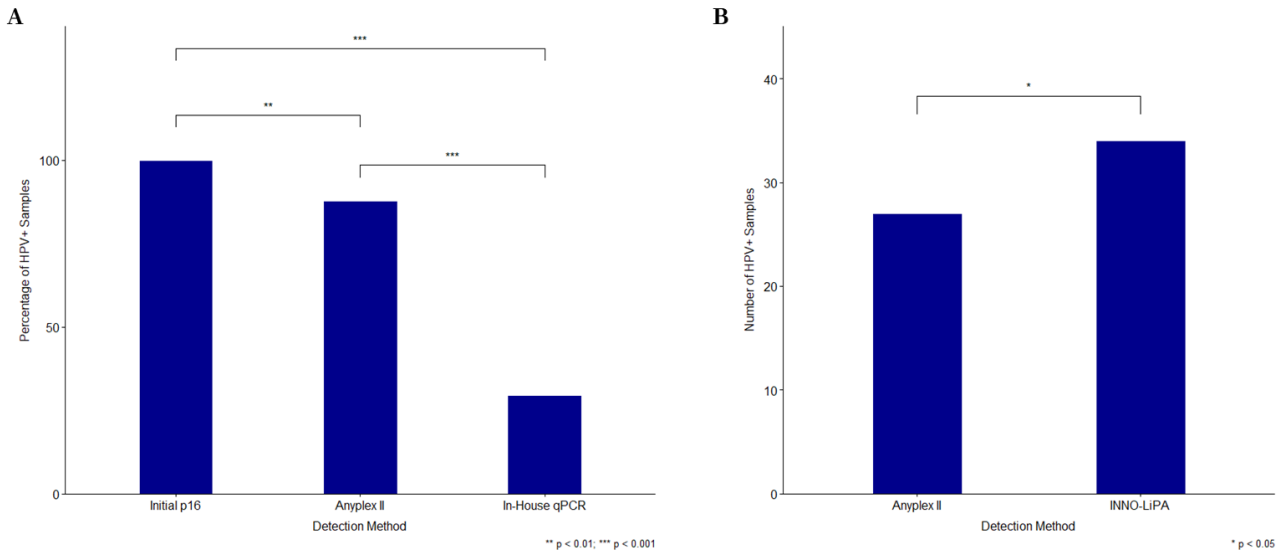

Figure 9. Overall HPV Detection in OPC FFPE Specimens by the Initial p16 IHC Assessment and Molecular Methods. (**A**) Differences in HPV detection between the initial p16 IHC

assessment, Anyplex II assay, and *in-house* qPCR. (**B**) Difference in HPV detection between the Anyplex II and INNO-LiPA assays for a subset of samples.

The INNO-LiPA Assay Outperforms Anyplex II for Overall HPV Detection

Since the Anyplex II HPV genotyping test was more sensitive than an *in-house* method for the detection of HPV, a subset of 34 samples was selected for further validation of HPV status using another commercial test, the INNO-LiPA HPV genotyping assay. Between both assays, INNO-LiPA had significantly greater HPV detection rate since all samples were HPV-positive, while only 27 (79.4%) were HPV-positive by the Anyplex II assay (**Figure 9B**, $p = 0.0233$).

The Anyplex II and INNO-LiPA Assays Do Not Differ in Genotype-Specific HPV Detection

Since both the Anyplex II and INNO-LiPA assays are optimized for HPV genotyping, detection rates of individual genotypes were also compared. Of all the genotypes detected by the Anyplex II assay (n =124), HPV 16 was the most common genotype being detected in 105 (84.7%) samples (**Figure 10A**). Other genotypes detected by the assay were HPV 33 (1.61%), 35 (4.03%), 51 (2.42%), 58 (0.806%), and 59 (0.806%) (**Figure 10A**). Similarly, in the 34 samples assessed using the INNO-LiPA assay, HPV 16 was also the most commonly detected genotype, appearing in all samples (**Figure 10B**). The assay also detected HPV 6 (2.94%), 18 (2.94%), 26 (2.94%), 33 (2.94%), 35 (11.8%), 51 (11.8%), and 58 (8.82%) (**Figure 10B**). Of the samples tested with both assays (n = 34), only 20.6% had discordant genotyping results, while 52.9% had the same genotype and 26.5% had at least 1 genotype detected which was the same in both tests in cases with multiple detection. Furthermore, no significant differences were found in the detection frequency of specific HPV genotypes between both assays, though the Anyplex II test failed to detect HPV significantly more often (**Figure 10C**, $p = 0.00301$).

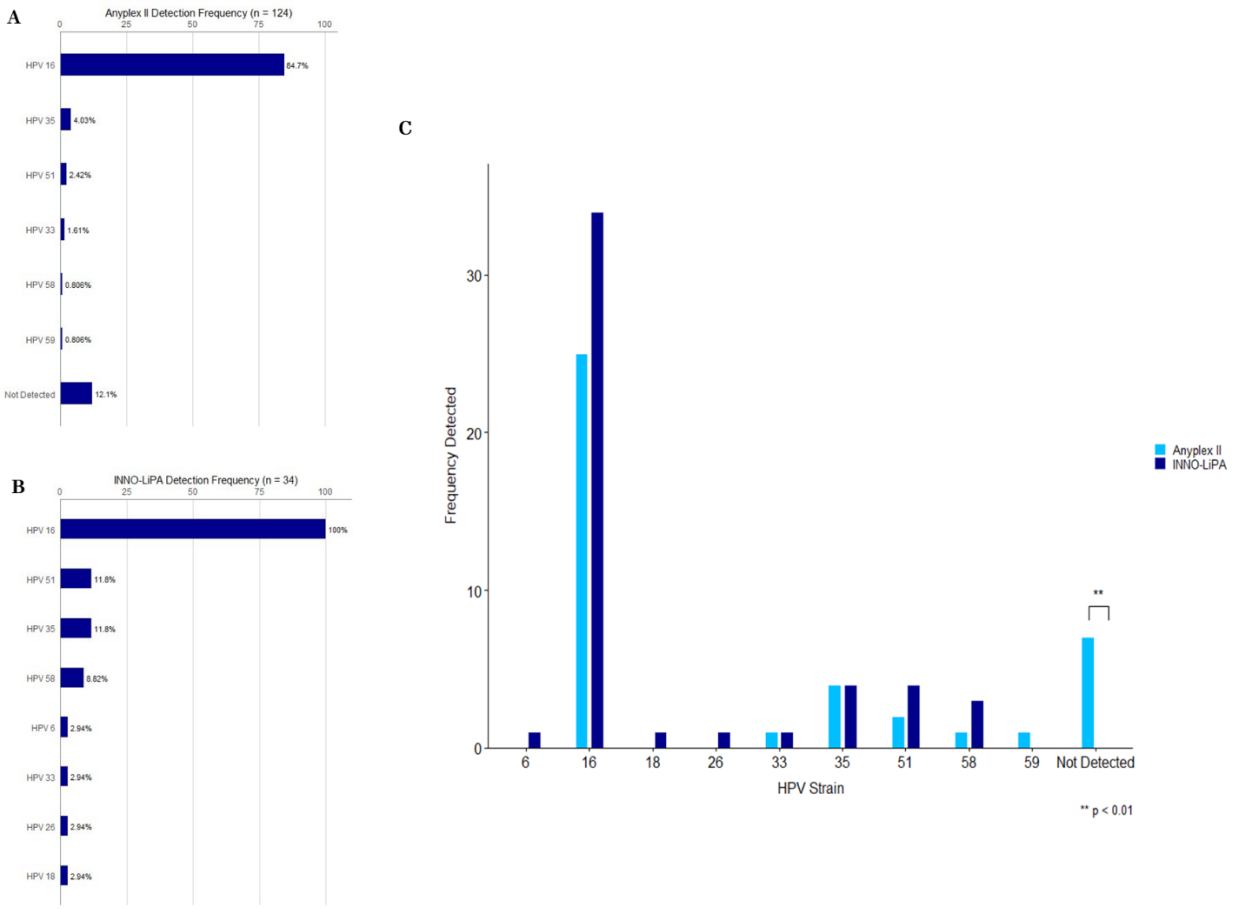

Figure 10. Differences in genotype-specific HPV detection between the Anyplex II and INNO-LiPA genotyping assays. (**A**) The frequency of HPV genotypes detected by the Anyplex II assay in all samples run (n = 124). (**B**) The frequency of HPV genotypes detected by the INNO-LiPA assay in all samples run (n = 34). (**C**) Differences in the detection of specific HPV genotypes between both genotyping tests.

The detection of single genotypes was similar to that of multiple genotypes for each test, and no significant differences were found between these assays (**Figure 11A**). Similarly, there were no differences in the detection of samples with 1, 2, or 3 genotypes between the Anyplex II and INNO-LiPA assays, though the former failed to detect any HPV genotypes in significantly more samples (**Figure 11B**, $p = 0.0233$). Together, these results show that although the INNO-LiPA assay appears to detect more HPV infections compared to the Anyplex II assay, both genotyping methods showed the predominance of HPV16 and have similar distribution of the other genotypes.

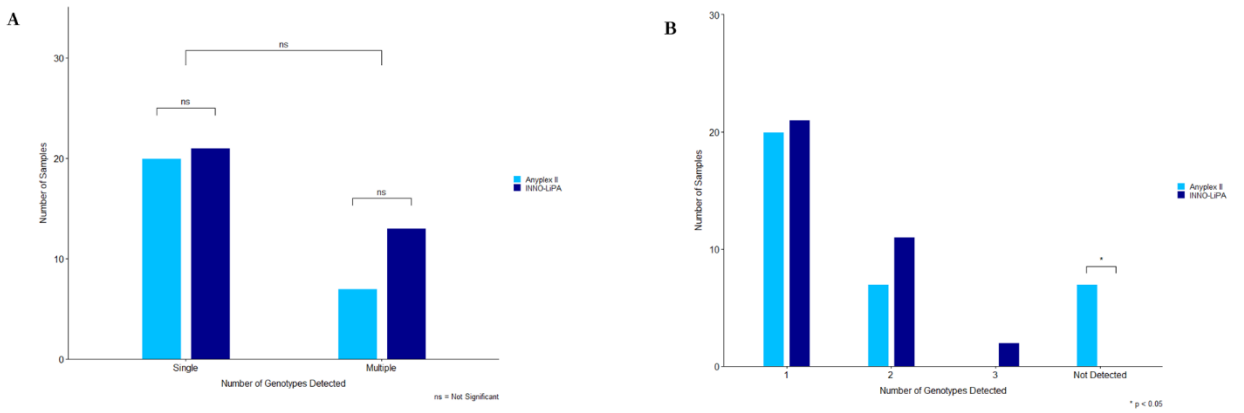

Figure 11. Differences in the detection of HPV co-infections between the Anyplex II and INNO-LiPA genotyping assays. (**A**) Differences in the detection of single or multiple HPV genotypes in patient samples between genotyping assays. (**B**) Differences in the detection of 1, 2, or 3 HPV genotypes in patient samples between genotyping assays.

The Clinical Antibody E6H4 Best Detects HPV, and p16 Positivity Thresholds Significantly Impact Detection Rates

Next, the accuracy of p16 IHC in HPV detection was assessed using different antibody clones: E6H4 and JC8, which are approved for clinical use, and BC42, which is only intended for use in research (**Figure 12A**). Samples with evaluable tumor sections were analyzed for differences in HPV detection between antibody clones (**Figure 12A**) at p16 positivity thresholds of 70%, 50%, and 0.01%. Overall, the clone E6H4 resulted in significantly higher HPV detection at all p16 thresholds compared to BC42, and at thresholds of 70% and 50% in comparison to JC8 (**Figure 12B**). At a p16 positivity threshold of 70% there was significantly more HPV detection with E6H4 compared to BC42 (71.6% vs 32.6%, $p = 9.68 \times 10^{-8}$) and JC8 (71.6% vs 35.9%, $p = 1.10 \times 10^{-6}$). Similarly, at a threshold of 50%, HPV detection with E6H4 was significantly higher than BC42 (77.0% vs 38.2%, $p = 6.30 \times 10^{-8}$) and JC8 (77.0% vs 51.3%, $p = 2.66 \times 10^{-4}$). However, using a lower threshold of 0.01%, differences in HPV detection were only seen between antibodies E6H4 and BC42 (95.4% vs 83.1%, $p = 0.0101$), while no differences were seen between the E6H4 and JC8 (**Figure 12B**). Conversely, antibodies BC42 and JC8 performed similarly at all p16 positivity thresholds, having no significant differences in HPV detection (**Figure 12B**). Thus, for p16 IHC, the antibody E6H4 provides a significantly superior HPV detection overall, while BC42 and JC8 perform similarly regardless of the p16 positivity thresholds used.

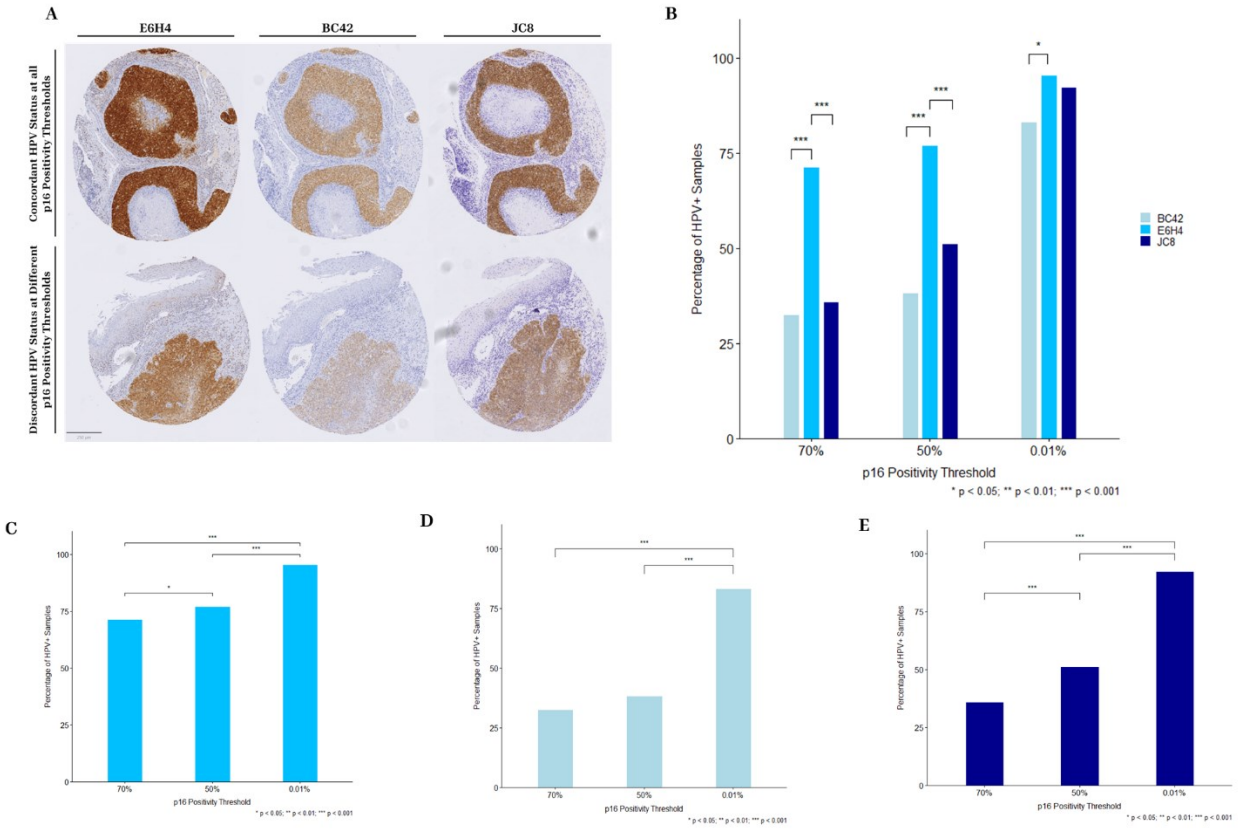

Figure 12. Differences in HPV Status Measured by p16 IHC with Different Antibodies at Various Positivity Thresholds. (**A**) Example of tissue staining by antibodies E6H4, BC42, and JC8. The sample depicted on top is HPV-positive with each antibody at all p16 positivity thresholds, while the sample on the bottom has a discordant HPV status based on the antibody and threshold used. This sample is HPV-positive at all thresholds for E6H4, while it is only HPV-positive using the 0.01% threshold for BC42, and 50% and 0.01% thresholds for JC8. (**B**) Differences in the percentage of HPV-positive samples between antibodies tested at different p16 positivity thresholds. (**C**) Differences in the percentage of HPV-positive samples at various p16 positivity thresholds using the antibody E6H4 (n = 87). (**D**) Differences in the percentage of HPV-positive samples at various p16 positivity thresholds using the antibody BC42 (n = 89). (**E**) Differences in the percentage of HPV-positive samples at various p16 positivity thresholds using the antibody JC8 (n = 78).

Furthermore, with all antibodies, HPV detection rates were also significantly influenced by the p16 positivity threshold chosen to define HPV status. Clinical antibodies E6H4 and JC8 had

the significantly higher HPV detection with a low p16 positivity threshold of 0.01%, compared to 50% (E6H4, 95.4% vs 77.0%,$p = 5.01 \times 10^{-5}$; JC8, 92.3% vs 51.3%, $p = 4.13 \times 10^{-10}$,) and 70% (E6H4, 95.4% vs 71.3%, $p = 2.48 \times 10^{-6}$; JC8,92.3% vs 35.9%, $p = 1.61 \times 10^{-13}$), allowing for HPV to be detected in a broader number of samples (**Figures 12C, E**). Similarly, significant differences were seen in HPV detection between p16 thresholds of 70% and 50%,with the latter allowing for more HPV-positive cases to be detected by IHC with E6H4 (71.3% vs 77.0%, $p = 0.0477$) and JC8(35.9% vs 51.3%, $p = 2.45 \times 10^{-4}$) (**Figures 2C, E**). Conversely, no differences were seen in HPV detection between 70% and 50% p16 thresholds for the research antibody BC42 (**Figure 12D**). However, with this antibody, HPV detection was significantly higher using a 0.01% p16 positivity threshold compared to 70% (83.1% vs 32.6%, $p = 3.17 \times 10^{-12}$) or 50% (83.1% vs 38.2%, $p = 5.57 \times 10^{-11}$) (**Figure 12D**). These results show that lower p16 positivity thresholds provide the widest coverage and most HPV detection with all antibodies, while the use of 50% or 70% thresholds will impact detection rates by the clinical E6H4 and JC8 antibodies.

<u>The Anyplex II Assay Significantly Outperforms Each p16 IHC Antibody</u>

Finally, HPV detection by p16 IHC was compared against that by the Anyplex II assay. When using high p16 positivity thresholds in IHC, all antibodies result in significantly lower HPV detection than the Anyplex II assay (**Figures 13A, B, C**).Indeed, using the antibody E6H4, there was significantly lower HPV detection at p16 thresholds of 50% (77.5% vs 92.5%, $p = 0.00316$) and 70% (71.3% vs 92.5%, $p = 1.59 \times 10^{-4}$) than the molecular assay (**Figure 13A**). Similarly, the antibody JC8 also had significantly lower detection at these thresholds (50% p16 positive, 52.1% vs 93.0%, $p = 1.64 \times 10^{-9}$; 70% p16 positive, 35.2% vs 93.0%, $p = 3.06 \times 10^{-13}$) (**Figure 13C**). Conversely, the antibody BC42 resulted in significantly lower HPV detection at 0.01% (82.5% vs 92.5%, $p = 0.0314$), 50% (36.3% vs 92.5%, $p = 5.67 \times 10^{-13}$), and 70% (31.3% vs 92.5%, $p = 4.49 \times 10^{-14}$) thresholds compared to the Anyplex II assay (**Figure 13B**), though no significant differences were found at the lowest threshold with the clinical antibodies (**Figures 13A, C**). As such, the molecular Anyplex II assay outperforms p16 IHC in HPV detection in OPC, regardless of the antibodies used.

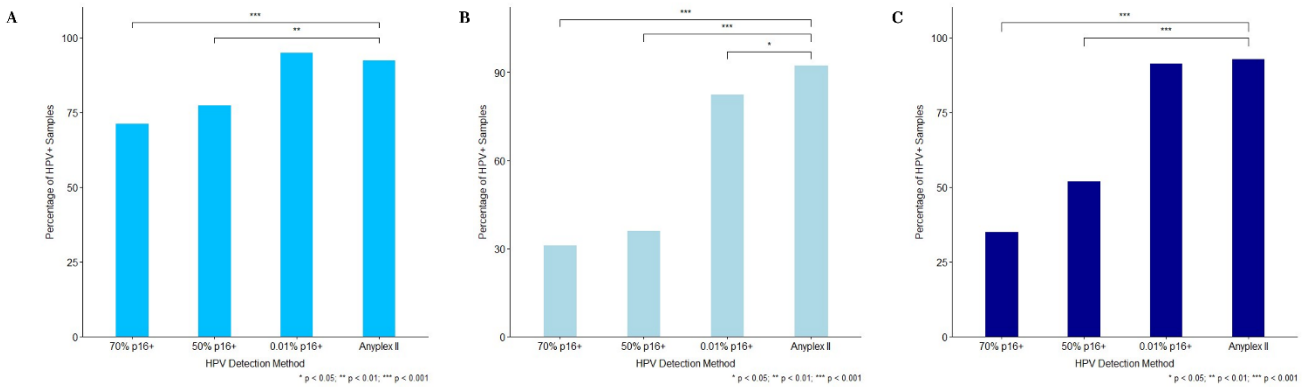

Figure 13. Differences in HPV Detection Between Various p16 IHC Antibodies and the Anyplex II Assay. (**A**) Differences in HPV detection between p16 IHC using the antibody E6H4 at different p16 positivity thresholds and the Anyplex II assay (n = 80). (**B**) Differences in HPV detection between p16 IHC using the antibody BC42 at different p16 positivity thresholds and the Anyplex II assay (n = 80). (**C**) Differences in HPV detection between p16 IHC using the antibody JC8 at different p16 positivity thresholds and the Anyplex II assay (n = 71). Abbreviations: p16+; p16 positive.

5.4 Discussion

This study investigated the differences in HPV detection rates between histological and molecular methods in OPC, providing critical insights into whether these methods are reliable and interchangeable. Our results may help inform clinical recommendations for HPV testing in OPC, at a time where standardization is needed to facilitate de-escalated treatment allocation[10–12].

Currently, p16 IHC remains the most used HPV detection method in OPC[10–12]. Despite its advantages, it remains limited by the non-viral upregulation of p16[10,23], which could lead to false positive results when compared to direct HPV testing. Many studies have shown that patients with discordant p16 expression and HPV DNA integration have worse prognoses[15–19], meaning that they may be undertreated if they are allocated a de-escalated therapy. For this reason, direct HPV testing using molecular detection methods has been recommended in addition to p16 IHC[12,19–21,24,25], though this implementation is not yet widespread. We assessed HPV detection using the commercialized Anyplex™ II HPV 28 genotyping test on archival samples that were HPV-positive by p16 IHC. Though detection was high at 87.9%, molecular testing captured significantly less

HPV-positive samples than the initial p16 grading. This is unusual, as the Anyplex II assay has been previously shown to have good concordance with p16 IHC[26]. This difference could be due to the sample quality and viral load, which can impact the efficacy of assays like Anyplex II[10,26,27], or differences in p16 antibodies and positivity thresholds used in IHC[14], which were not standardized in the initial grading. High variability in the antibodies used and the p16 positivity thresholds selected to define HPV status[14] may pose major barriers in HPV detection by p16 IHC. We assessed HPV detection using three different p16 antibodies and positivity thresholds. Our results showed that the Anyplex II assay provides significantly better HPV detection than any of the p16 IHC antibodies tested with positivity thresholds of 50% and 70%, though detection rates are similar between both methods at a low threshold of 0.01% with clinically approved antibodies. These results show that molecular HPV detection could be beneficial as an auxiliary testing method when high p16 positivity thresholds are used to determine HPV status in IHC.

Despite these promising findings, a consensus is needed on the best IHC antibodies, p16 positivity thresholds, and molecular assays for the standardization of HPV testing in OPC. Our results showed that at all p16 positivity thresholds, the clinical antibody E6H4 had superior HPV detection than the research antibody BC42, while at higher thresholds of 50% and 70% p16-positivity, it also outperformed JC8. With its high staining intensity, clone E6H4 has been previously found to be most reliable due to its low risk of partial and non-specific staining, reducing variability in HPV detection[14]. Though E6H4 appears to have the best performance, the use of other approved antibodies, such as JC8, might be preferred for high throughput testing due to the high cost of the former or due to equipment availability, as manufacturers may optimize antibodies for use with specific instruments. In this case, the threshold of p16 positivity must be carefully chosen, as discrepancies are still seen between antibodies at higher cut-offs. Likewise, the research-use only antibody BC42 resulted in similar HPV detection to JC8, though investigations in larger cohorts are needed before it can be recommended for use in clinical settings as this clone still significantly underperformed compared to E6H4 at low thresholds. Optimizing the p16 positivity threshold used to define HPV status is essential for the standardization of HPV testing in OPC. Currently, a p16 positivity threshold of 70% is recommended to classify oropharyngeal tumors as HPV-positive[10,28,29]. We found that this cutoff was too stringent, having significantly worse HPV detection with all antibodies compared to molecular testing, while lower p16 positivity thresholds increased HPV detection. Many studies have corroborated these findings,

proposing a reduction in the threshold to 50%[10,30], 15%[31] or even 10%[32] to reduce the rate of false negative classifications. However, low p16 positivity thresholds run the risk of introducing false positive cases that are p16-positive but HPV-negative, which occur in around 5% to 25% of patients depending on geographic location[12,17]. Thus, molecular testing in addition to p16 IHC can help validate HPV status and ensure accurate patient classification.

Though molecular HPV testing is recommended, many different assays are available and have yet to be approved for clinical use in OPC. As such, we also assessed the reliability of an *in-house* qPCR for HPV detection, finding that it had a significantly lower detection rate than the commercial Anyplex II and the initial p16 IHC grading, despite the HPV-specific Gp5+/Gp6+ primers being widely studied[21,24,33]. Though commercialized assays have yet to be clinically approved for use in OPC, multiple studies have reported favorable HPV detection rates with PCR-based and line probe assays[26,27,34–37]. In a subset of samples, we assessed the differences in HPV detection between the Anyplex II test with another commercialized genotyping assays, the INNO-LiPA® HPV Genotyping Extra II test, with our results showing that both assays had similar genotype-specific prevalence, though the Anyplex II test had lower overall HPV detection. This difference should take into consideration the difference in the number of genotypes detected with each assay that is different and favors INNO-LiPA. Detection in fixed tissue is improved when PCR assays amplify a short length amplicon because of the process of fixation that causes fragmentation of DNA in biopsies. INNO-LiPA amplifies a very short segment of DNA. However, the length of amplicons in Anyplex II is not known. However, these results should be approached with caution, due to our small sample size and reports of superior HPV detection by Anyplex II in other studies[26,27]. Nevertheless, the use of commercially developed molecular assays is favored over *in-house* methods, and until one becomes clinically approved, the choice of assay will likely depend heavily on resource availability.

5.5 Conclusions

The standardization of HPV detection methods is needed in OPC to support the widespread implementation of de-escalated treatment strategies for HPV-positive patients. Though p16 IHC remains the most used in OPC, it has inferior HPV detection than direct molecular testing depending on the antibody and the p16 positivity threshold used. The use of molecular methods in addition to p16 IHC will help validate HPV status and ensure proper treatment allocation.

5.6 Methods

Patient Samples

Tissue biopsies were collected from OPC patients at two healthcare institutions in Montreal, Quebec, Canada, between 2009 and 2019. HPV status was assessed by p16 IHC, and samples were stored as FFPE tissues blocks. One hundred and twenty-four (n = 124) FFPE specimens were obtained for use in this study. All samples were deemed HPV positive by p16 IHC assessment at each respective institution. The study was conducted following the Declaration of Helsinki; Scientific Research Ethics Committees of the Centre intégré universitaire de santé et de services sociaux du Centre-Ouest-de-l'Île-de-Montréal (MEO-37-2022-2938) reviewed and approved this study.

DNA Extraction

According to tissue availability, between 5 and 20 scrolls were sliced from FFPE samples at a thickness of 5 µm using a microtome. DNA was extracted from tissues using either the QIAamp® DNA FFPE Tissue or DNeasy® Blood & Tissue kits (QIAGEN, Germany) with minor modifications to the kit protocols. Tissue deparaffinization and ethanol washes were repeated twice to ensure complete removal of paraffin wax, and subsequent steps of the extraction were performed according to manufacturer recommendations. DNA was eluted by centrifugation following a 5-minute incubation at room temperature with 50 µL ATE buffer (QIAGEN), quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific, United States of America (USA)), and stored at -20 °C until used.

Molecular HPV Detection and Genotyping

Anyplex™ II HPV28 Detection Assay

The Anyplex™ II HPV28 Detection kit, is a commercially available assay which detects 28 different high- and low-risk HPV genotypes (**Table 3**)[26,27]. Using multiplex qPCR, the assay amplifies genotype-specific regions of the HPV L1 gene, allowing for viral detection[27]. Genotyping was performed on DNA extracted from all FFPE tissues (n = 124) according to manufacturer protocols. The assay was run and analyzed on the CFX96™ Real-time PCR Detection System (C1000 Touch™ Thermal Cycler, CFX Manager™ Software 3.1), and results

were considered invalid if a negative result was obtained with both the internal control and HPV genotyping assessment.

Table 3. HPV Genotypes Detected by Each Molecular Method.

Method	High-Risk Genotypes	Possibly High-Risk Genotypes	Low-Risk Genotypes	Undetermined
Anyplex™ II HPV28 Detection[27,38,39]	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	26, 53, 66, 69, 70, 73, 82	6, 11, 40, 42, 43, 44, 54, 61	-
INNO-LiPA® HPV Genotyping Extra II[27,38,39]	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	26, 53, 66, 67, 70, 73, 82	6, 11, 40, 42, 43, 44, 54, 61, 81	62, 83, 89
In-house qPCR (Gp5+/Gp6+ primers)[33,38,39]	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59	66, 30	6, 11, 40, 43, 54	13, 32, 55

INNO-LiPA® HPV Genotyping Extra II Assay

The INNO-LiPA® HPV Genotyping Extra II kit is a reverse hybridization line probe assay that allows for the detection of 32 different HPV genotypes, including high- and low-risk types (**Table 3**), by targeting the SPF10 region of the HPV L1 gene[27,40]. Extracted DNA from 34 FFPE samples was genotyped and scored according to manufacturer protocols. HPV genotypes were identified by the presence of a colored band on a genotype-specific line and at least one of the positive control lines on the testing strip. Samples were considered HPV negative if bands failed to develop on both the HPV control lines and the type-specific lines, while results were invalid if bands failed to appear for the kit's internal controls.

In-House qPCR

Following Anyplex II and INNO-LiPA genotyping, samples with enough remaining extracted DNA (n = 112) were assessed for HPV-positivity using an *in-house* qPCR. The GoTaq® qPCR system (Promega, USA) was used with slight modifications to kit recommendations. Briefly, 1X GoTaq® qPCR Master Mix and 4 µL extracted DNA were used in the reaction. To assess HPV status, 0.5 µM of a premixed reaction-ready pool containing the Gp5+/Gp6+ primers[33] (Integrated

DNA Technologies (IDT), **Table S2**) were used. These primers have been shown to detect the presence of 22 different HPV genotypes (**Table 3**)[33]. Each sample was run with a matched internal control targeting β-hemoglobin, using 0.5 μM of a premixed reaction-ready primer pool (IDT) or 5 nM of GH20 and PC04 primers[41] (**Table S2**).The reaction was run on the 7500 Fast Real-Time PCR System or QuantStudio 7 Flex machines (Applied Biosystems, USA) for 40 cycles at 60 °C. Samples were considered HPV positive if both Gp5+/Gp6+ and β-hemoglobin amplification passed threshold before 35 cycles, while HPV was not detected in those without Gp5+/Gp6+ amplification. Samples without β-hemoglobin amplification before 35 cycles were re-run and considered invalid if no changes in amplification occurred.

p16 Immunohistochemistry

To assess histological detection of HPV, most samples (n = 121) underwent p16 IHC using the clinically approved p16 antibody E6H4 (CINTEC p16 Histology Kit, Roche Diagnostics, USA), JC8 (Dako Omnis, Agilent Technologies, USA), and the research antibody BC42 (1:50; Cell Signaling Technologies, USA). IHC was performed at the Segal Cancer Centre Research Pathology Facility (Jewish General Hospital, Canada), according to institutional procedures. Briefly, 2 mm cores from FFPE tissues were mounted in tissue microarrays (TMAs), sliced into5 μm sections and dried overnight on TOMO slides before being loaded onto the Discovery XT Autostainer (Ventana Medical System) for automated IHC. The antibodies E6H4, BC42, or JC8 were then used with the Omnimap anti-Mouse HRP (Roche) or ChromoMap-DAB (Roche) kits for p16 detection, alongside positive and negative controls. Slides were counterstained with Hematoxylin, blued, and mounted before being scanned for analysis in QuPath (version 0.2.3)[42]. Tumor sections were identified in each TMA core by a pathologist, and the percentage of p16 positive cells in tumor-containing cores was determined using QuPath's positive cell detection function. HPV status was assessed for each antibody based on the percentage of p16-positive cells at thresholds of 70%, 50%, and 0.01%. HPV detection was compared against that of the Anyplex II assay for samples that underwent testing by both methods.

Statistical Analyses

All statistical comparisons were performed with R Studio (version 4.4.0). McNemar's Chi-Squared Test was used for paired comparisons with equal sample sizes, with continuity correction. In the

case of unpaired comparisons with different sample sizes, Pearson's Chi-Squared Test with Yates' Continuity Correction or Fisher's Exact Test were used where appropriate.

5.7 Data Availability

Source data for the analyses performed in this article are available from the corresponding author upon request. Please contact Dr. Sabrina Wurzba (sabrina.wurzba@mcgill.ca) to request a copy of the data.

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Conflict of Interest Disclosures

The authors have no conflicts of interest to declare.

Author Contributions

Conceptualization: Sabrina Wurzba, Michael Hier; Methodology: Sabrina Wurzba, Michael Hier, Naser Karimi, Jenna Bouassaly; Data collection: Jenna Bouassaly, Lilianny Querino Rocha de Oliveira, Megan Araujo, Naser Karimi, Sabrina Wurzba; Data analysis: Jenna Bouassaly;

Resources: Sabrina Wurzba; Writing—original draft preparation: Jenna Bouassaly; Writing—review and editing, Jenna Bouassaly, Sabrina Wurzba, Lilianny Querino Rocha de Oliveira, Megan Araujo, Naser Karimi, Michael Hier, Alex Mlynarek, Marco Mascarella; Supervision: Sabrina Wurzba; Funding acquisition: Sabrina Wurzba. All authors have read and agreed to the published version of the manuscript.

5.8 Supplemental Materials

Supplementary information accompanies the manuscript on the *International Journal of Oral Science* website http://www.nature.com/ijos.

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Chapter 6: Discussion

Through a narrative literature review and an original research investigation, this thesis addresses two of the most prevalent barriers to the implementation of de-escalated treatment strategies for HPV-positive OPC, namely the need for more evidence on their efficacy and the lack of a standardized HPV testing regimen in this cancer type.

6.1 The Benefits of De-Escalated Treatments for HPV-Positive OPC

As discussed in Chapters 1 and 2, the use of established HNC therapies like surgery, radiation, chemotherapy, and immunotherapy in a de-escalated manner may provide alternative treatment strategies for HPV-positive OPC patients, who historically have superior prognoses[61–66]. These de-escalated modalities include minimally invasive surgery like TORS or TLM, reduced-dose radiation, neoadjuvant systemic chemotherapy, and neoadjuvant or adjuvant immunotherapy[69]. If successful, de-escalated treatments would maintain the anti-cancer efficacy of these treatments while minimizing the severe acute and chronic side effects that negatively impact patient quality of life. However, despite their initial promise, hesitation remains surrounding the implementation of these treatments, in part due to the need for further evidence supporting their efficacy from large-scale trials[9]. Chapter 3 summarizes the findings from recent clinical trials on various de-escalated therapies for OPC, highlighting their successes and downfalls in HPV-positive cohorts.

The review found that many studies reported favorable outcomes with combinations of de-escalated surgical techniques, reduced-dose RT, and neoadjuvant chemotherapy or immunotherapy[69]. Notably, high survival rates and tumor responses were observed with transoral surgery (TOS) modalities when combined with reduced-dose postoperative radiation[16], or neoadjuvant taxane chemotherapy[18,139]. Similarly, induction or adjuvant chemotherapy using docetaxel or paclitaxel with reduced-dose RT also resulted in lower toxicity rates while maintaining anti-cancer efficacy[17,140,144,145]. However, in many of these studies, a fraction of patients failed to respond to the de-escalated intervention[17,18]. While the number of partial- or non-responders is often small, it is important to investigate these patients further to uncover any demographic, biological, or molecular differences that could be driving treatment resistance. For instance, confounding lifestyle factors like tobacco and alcohol consumption have been found to worsen prognoses[146,147] and increase the risk of recurrence[147] in HPV-positive OPC patients. As

treatment de-escalation may not be recommended in these cases, the incorporation of confounding risks in allocation guidelines will be essential for the widespread acceptance of these therapies.

In the same manner, immune involvement may play a considerable role in de-escalated treatment responses. As introduced in Chapter 2, persistent HPV infections may modulate components of the immune system, particularly taking advantage of the PD-1/PD-L1 axis[54–57]. While the prognostic value of PD-L1 expression remains debated[55,148–150], this marker is often elevated in HPV-positive OPC[55,148,150], which may contribute to immune evasion and tumor persistence[150]. High cytotoxic immune cell recruitment and activation, which can be influenced by the PD-1/PD-L1 axis, has been associated with superior outcomes in HPV-positive OPC[150–152]. Thus, as these biomarkers may influence tumor responses and patient outcomes, the use of immunotherapy in de-escalated treatments may be preferred for patients with high immune cell recruitment in the tumor microenvironment, which is common in HPV-positive OPC, to help improve immune cell activation[141]. Indeed, the review in Chapter 3 highlighted the successes of PD-1 inhibitors nivolumab and pembrolizumab as neoadjuvant or adjuvant agents in de-escalated treatments for HPV-positive OPC[69]. As such, further investigations on the factors that may influence treatment efficacy and resistance would allow for the development of robust guidelines for the allocation of de-escalated treatments, utilizing an individualized approach to improve the stratification and treatment of HPV-positive OPC patients. These studies would also pave the way for the development of new strategies and treatment plans for refractory tumors, and together with proper guidelines, may help address some additional doubts surrounding the acceptance of de-escalated treatments.

Nevertheless, the findings presented in Chapter 3 provide overwhelming support for the implementation of a separate standard of care for HPV-positive OPC. Particularly, worse outcomes under the targeted treatment cetuximab in these patients[69] should bring into question its use in this cancer subtype, while the successes of reduced-dose RT, neoadjuvant taxane chemotherapy, and neoadjuvant or adjuvant PD-1 immunotherapy[69] justify a re-evaluation of the status of de-escalated therapies.

6.2 The Need for Standardized HPV Detection in OPC

Although de-escalated treatment strategies have shown success in recent clinical trials, their acceptance is still limited by the need for standardized HPV testing in OPC. As discussed in

Chapter 2, p16 IHC remains the most used HPV detection method in OPC, though the method's reliability varies based on the p16 antibody and positivity threshold used, and is limited by a low specificity, which together, could lead to false-negative or -positive diagnoses if IHC alone is used for HPV detection[23,25,82,83]. For this reason, direct molecular HPV testing has been recommended in addition to p16 IHC[4,25,26,81,87–89], though without standardized guidelines for HPV testing in OPC, this practice is not widespread and is usually applied as needed[87].

With recent studies debating the use of p16 IHC as a standalone method, the original investigation presented in Chapter 5 was undertaken to compare HPV detection rates between p16 IHC and molecular methods, providing a better understanding of the differences between these methods and their interchangeability. The study also examined differences within each technique, evaluating the sensitivity of various p16 antibodies, positivity thresholds, and molecular detection assays. Initially, molecular tests appeared to be less sensitive than p16 IHC, with the commercial Anyplex II assay and an *in-house* qPCR assay failing to detect HPV DNA in a significant number of cases that were previously deemed positive by p16 IHC. These discrepancies may be explained by the p16 antibodies or thresholds used[23,82], which, as mentioned in Chapter 5, were not accounted for in the initial p16 IHC analysis. To this end, differences in HPV detection between three different clones of p16 antibodies, E6H4, BC42, and JC8 were compared, with E6H4 having the highest staining sensitivity. Conversely, clone JC8 had poor sensitivity at p16 positivity thresholds of 50% and 70%, performing similarly to clone BC42, which in turn had the lowest HPV detection overall. Differences in staining sensitivity can impact HPV detection rates, potentially mis-classifying positive cases. In clinical settings, false-negative IHC diagnoses will lead to eligible patients being excluded from de-escalated treatment regimens, potentially receiving unnecessarily intensive therapies with lasting side effects. Currently, comparative analyses between various p16 antibodies are limited, particularly in OPC[82,153], though E6H4 is most often used in IHC applications[153]. These findings highlight the need for a dedicated large-scale investigation assessing multiple p16 clones in OPC, which could facilitate the establishment of a gold-standard in p16 IHC applications[153].

Similarly, Chapter 5 showed that the p16 positivity threshold used to define HPV status also impacted HPV detection rates, with lower thresholds increasing detection. While a p16 positivity threshold of 70% is currently recommended by the ASCO and College of American Pathologists[23,87], many studies have questioned whether this threshold is too strict, with lower

positivity cutoffs being suggested instead[23,83–85]. This is supported by the findings from Chapter 5, where HPV detection was significantly lower with p16 IHC than the Anyplex II assay at a p16 positivity threshold of 70%, regardless of the antibody used. However, lower p16 positivity thresholds may introduce false-positive cases[82], which is an inherent issue with the use of p16 IHC as a standalone detection method. Up to 26% of OPC patients have been found to have high p16 expression without HPV DNA integration[25], meaning they are at risk of a false-positive HPV diagnosis by p16 IHC. This is concerning, as patients with discordant p16 and HPV status have been found to have worse prognoses[4,23,26,27,88–90] and would not be good candidates for treatment de-escalation. For this reason, the addition of molecular HPV testing as a supplement to p16 IHC may help minimize rates of false-positive diagnoses and allow for HPV status to be determined with more certainty[4,25,26,81,88–90]. Indeed, combining these detection methods has shown much success in improving HPV diagnoses and prognostic assessments of OPC patients in the Netherlands[25,95,96], Sweden[25,89], and the UK[25,81], among others[25].

Multiple molecular HPV tests are available with different sensitivities, as seen with discrepancies in overall HPV detection between the Anyplex II and INNO-LiPA assays in Chapter 5. However, these results are to be interpreted with caution, due to a small sample size. Molecular HPV detection is greatly limited by sample quality, including the availability of genomic material and the viral load present in tissues[23]. OPC biopsies are most often preserved as FFPE specimens, making them prone to DNA and RNA degradation[23]. Thus, the optimization and validation of molecular HPV tests for FFPE tissues in large-scale studies is important for their application in OPC. As many commercial HPV detection assays are already implemented in the diagnosis of cervical cancer[92], their clinical approval in OPC would help streamline the diagnosis of HPV and allocation of de-escalated treatments in this cancer type. Thus, further dedicated studies are needed to refine and validate these assays for use in OPC.

With the variation in sensitivity and specificity between different HPV detection methods, it is unsurprising that the lack of a standardized testing regimen in OPC is limiting the implementation of de-escalated treatments. As these therapies are only recommended for HPV-positive patients based on p16-positive status due to their superior prognoses, proper HPV diagnoses are crucial to minimize patient undertreatment and ensure appropriate treatment de-escalation. The results reported in Chapter 5 highlight the need for standardized HPV detection

procedures that include both p16 IHC and molecular HPV detection. However, reaching a consensus on the best antibody and positivity threshold to use in p16 IHC, as well as validating molecular HPV detection kits in OPC is essential if these methods are to be used in the stratification of HPV-positive OPC patients for de-escalated treatments.

Chapter 7: Conclusions & Future Directions

Currently, the standard of care for HPV-positive OPC remains combinations of surgery, high-intensity RT, and systemic chemotherapy, which often result in severe and lasting side effects. However, HPV-related OPC has recently been recognized as a distinct HNC subtype due to superior patient prognoses. For this reason, much research has been undertaken to develop de-escalated treatments that can minimize adverse effects, although these strategies have yet to be widely implemented in HPV-related OPC. Through a narrative literature review and original research, this thesis aimed to address the major barriers preventing the acceptance of de-escalated treatments in HPV-positive OPC, namely the need for further evidence on their efficacy and the lack of standardized HPV testing in this cancer type. Chapter 3 summarized recent advances in de-escalated therapies for HPV-positive OPC, highlighting clinical trials that showcased the benefits of reduced-dose radiation, neoadjuvant taxane chemotherapy, and neoadjuvant or adjuvant immunotherapy, while also bringing attention to the downfalls of some targeted therapies in these patients. While these findings support the use of de-escalated strategies in the standard of care of HPV-related OPC, further studies on the lifestyle or biological factors that may be influencing treatment resistance is warranted, and the integration of confounding prognostic risk factors into allocation guidelines may help improve the acceptance of these treatments. Conversely, Chapter 5 tackled the need for standardized HPV testing guidelines by comparing direct and indirect detection methods, shedding light on the differences in p16 IHC HPV detection based on antibodies and positivity thresholds used. This chapter also uncovered differences in overall HPV detection rates between commercial genotyping tests, warranting further investigations on the discrepancies between multiple direct molecular tests with a larger sample size. Finally, Chapter 5 also placed a spotlight on the significant differences in HPV detection with p16 IHC compared to direct molecular testing, supporting the recommendation of combining these methods to minimize false-positive detections and improve the reliability of HPV detection in OPC. Together, the findings presented in Chapters 3 and 5 show that although much research has successfully demonstrated the benefits of de-escalated treatment strategies for HPV-positive OPC, studies aiming to improve and standardize HPV detection in this cancer type are still needed to facilitate their implementation.

Chapter 8: References

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Chapter 9: Appendices

9.1 Appendix A: Supplemental Material for Manuscript 1 (Chapter 3)

Supplemental Material:

Table S1. Recent Clinical Trials Investigating Neoadjuvant Immunotherapies in HPV-positive head and neck cancer***.

Study	Cancer Type & HPV Status	Primary Therapy & Dose	Neoadjuvant Therapy & Dose	Biomarker Targeted	Outcomes
[45]	HPV+ and HPV- HNSCC	Surgery	2x 240 mg nivolumab prior to surgery	PD-1	- Overall pathologic response rate of 23.5% in HPV+ patients vs 5.9% in HPV- - 2-year RFS rate of 88.2% in HPV+ patients vs 54.2% in HPV- - 3-year OS rate of 100% in HPV+ patients vs 63.5% in HPV-
[46]	HPV+ and HPV- HNSCC	Surgery	40 Gy or 24 Gy SBRT ± 3x 240 mg nivolumab before surgery 3x 480 mg nivolumab after surgery for all groups	PD-1	- Low rates of grade 3 treatment-related toxicities, moderate to high rates of toxicities of any grade - In HPV+ groups, mPR rate of 100% and pCR rate of 90% with SBRT and nivolumab vs pCR of 50% with SBRT alone - Lower pCR and mPR rates of 20% and 60%, respectively, in HPV- group with SBRT and nivolumab
[47]	HPV+ and HPV- HNSCC	Salvage surgery	Total 2880 mg nivolumab (12x 240 mg or 6x 480 mg)	PD-1	- Significantly higher 2-year DFS of 71.4% overall vs 41% from historic controls

			prior to surgery		- Higher, but not significant, 2-year OS overall (77.7%) vs historic controls (57.8%) - No difference in DFS and OS based on HPV status
48	HPV+ and HPV- LA HNSCC	CRT (70 Gy RT with 6x 40 mg/m^2 cisplatin)	Total 8x 200 mg pembrolizumab before, during, and after CRT	PD-1	- Overall CR rate of 85.3% in the HPV+ cohort vs 78.3% in HPV- - 2-year OS of 97.1% and PFS of 92.8% in the HPV+ cohort - 1-year OS of 86.5% and PFS of 72.6% in HPV- cohort, estimate of 2-year survival was limited
49	HPV+ and HPV- HNSCC	10 mg/kg durvalumab	NA	PD-L1	- Overall ORR of 16.2% and DCR of 23.4% - Higher ORR in HPV+ subgroup (29.4%) vs HPV- (10.8%), significance not assessed - Longer median PFS in HPV+ subgroup (3.6 months) vs HPV- (1.8 months), significance not assessed
50	HPV+ and HPV- HNSCC	10 mg/kg durvalumab	NA	PD-L1	- Overall ORR of 6.5% and DCR of 12.9% - Higher ORR and DCR in HPV- patients than HPV+, statistical significance not assessed - Similar median PFS between HPV+ and HPV- patients, statistical significance not assessed

51	HPV+ and HPV- HNC	15 mg/kg, 20 mg/kg, or fixed 1200 mg atezolizumab	NA	PD-L1	- Overall ORR of 22% and DCR of 31% - No difference in response rate based on HPV status
67	HPV+ OPC	RT with concurrent 250 mg/m^2 cetuximab	Induction 400 mg/m^2 cetuximab	EGFR	- Improved CD8+ T cell tumor infiltration after cetuximab induction in 62.5% of patients - Increased RNA transcripts associated with responses to interferon signaling and antigen recognition in tumors that could be evaluated
68	HPV+ and HPV- HNSCC	CRT (72 Gy 3D-RT or 70 Gy IMRT, with 100 mg/m^2 cisplatin)	± 400 mg/m^2 cetuximab prior to CRT, then 250 mg/m^2 per week during CRT	EGFR	- No difference in 5-year and 10-year PFS, LRF, DM, and OS overall with cetuximab - HPV+ patients had significantly improved PFS and LRF than HPV- patients, regardless of treatment - No difference in PFS in HPV+ or HPV- patients with cetuximab vs cisplatin - Similar rates of grade 3-4 late toxicities with both treatments
69	HPV+ and HPV- LA HNSCC	RT (70 Gy)	40 mg/m^2 cisplatin per week vs 400 mg/m^2 cetuximab prior to RT, then 250 mg/m^2 per week concomitantly	EGFR	- No differences in grade 3+ late toxicities overall between treatments - No differences in local control, metastasis-free survival, OS, and cancer-specific survival overall between treatments - Higher local control, metastasis-free

					survival, OS, and cancer-specific survival with cisplatin than cetuximab in HPV+ patients, statistical significance not assessed due to small sample size
70	Arm A: HPV+ OPSCC Arm B: HPV+ and HPV- LA HNSCC not eligible for platinum therapy	IMRT (Arm A: 70 Gy or 56 Gy, Arm B: 63 Gy)	400 mg/m^2 cetuximab prior to IMRT, then 250 mg/m^2 per week concomitantly	EGFR	- Higher rates of grade 3+ toxicities in arm A (67%) vs arm B (48%) - 2-year DFS of 81% and OS of 95% in arm A, higher than estimated 37.2% DFS and 47.6% OS in arm B
71	HPV+ OPC	IMRT (70 Gy)	2x 100 mg/m^2 cisplatin vs 400 mg/m^2 cetuximab prior to IMRT, then 250 mg/m^2 per week concomitantly	EGFR	- Adjuvant cetuximab did not meet the criteria for non-inferiority to adjuvant cisplatin - Significantly worse 5-year OS and PFS with cetuximab (77.9% and 67.3%, respectively) than cisplatin (84.6% and 78.4%, respectively) - Significantly higher LRF with cetuximab (17.3%) vs cisplatin (9.9%) - No differences in distant metastases and acute grade 3-4 toxicities between treatments
72	HPV+ OPC	RT (70 Gy)	3x 100 mg/m^2 cisplatin vs 400 mg/m^2 cetuximab	EGFR	- No differences in severe acute and late toxicities between treatments, but significantly higher

			prior to RT, then 250 mg/m^2 per week concomitantly		serious adverse events with cisplatin than cetuximab - 2-year OS significantly higher with cisplatin (97.5%) than cetuximab (89.4%) - Significantly higher 2-year overall recurrence rate, locoregional recurrence rate, and distant metastasis rate with cetuximab than cisplatin
[73]	Various HPV+ cancer types, including HPV+ OPC	3 mg/kg nivolumab	3x 100 µg/peptide ISA101 vaccine	TIM	- ORR of 33%, all responders had HPV+ OPC - 1-year PFS of 25% and OS of 70% in entire population, no differences in OPC subgroup - Tumor response was significantly correlated to PD-L1 expression
[74]	Various HPV+ cancer types, including HPV+ OPC	3 mg/kg nivolumab	3x 100 µg/peptide ISA101 vaccine	TIM	- 3-year PFS and OS of 12.5% in entire population - Significantly higher activated T cells, activated cytotoxic T cells, and macrophages in responders - No correlation between PD-L1 expression in tumor, stroma, or combined areas and tumor response in long-term analyses - Differential gene expression between responders and non-responders

					- CD68 expression significantly higher in responders and associated with better survival
[75]	HPV+ HNSCC	Surgery or CRT externally to the trial	Total 4x MEDI0457 prior to and after surgery or following CRT	TIM	- Immunotherapy induced the production of antibodies specific to the E6 and E7 proteins of HPV16 and HPV18 that persisted for at least 3 months - Significantly increased interferon γ production against HPV16 and HPV18 antigens from baseline - Increased HPV-specific T cells from baseline, differences not statistically significant due to small sample size - Non-significant increase in the CD8/FoxP3 ratio following treatment, change in tumor infiltrating lymphocytes
[76]	HPV+ and HPV- HNSCC	EXTREME Regimen (carboplatin (5 mg/mL/min) or cisplatin (100 mg/m^2) with fluorouracil (1000 mg/m^2/day), and cetuximab (1x 400 mg/m^2, then 250 mg/m^2 per week))	3 mg/m^2 motolimod vs placebo	TIM	- No differences in PFS, OS or ORR with motolimod overall - Significantly improved PFS and OS with motolimod vs placebo in HPV+ patients - HPV status was significantly associated with treatment survival outcomes

[77]	HPV+ and HPV- HNSCC	35x 200 mg pembrolizumab	11x 2 mg/lesion (up to 8 mg total) or 8 mg SD-101	TIM	- Total ORR of 24% and DCR of 47% overall - 9-month PFS of 21.2% in 2 mg group and 17.4% in 8 mg group overall - 9-month OS of 79.9% in 2 mg group and 57.2% in 8 mg group overall - Higher ORRs in HPV+ patients (44%) vs HPV- patients (12%), statistical significance not assessed - Increase in immune gene expression, particularly in responders

*** Abbreviations: CRT, chemoradiotherapy; DCR, disease control rate; DFS, disease-free survival; DM, distant metastasis; EGFR, epidermal growth factor receptor; HNC, head and neck cancer; HNSCC, head and neck squamous cell carcinoma; HPV+, human papilloma virus positive; HPV, human papilloma virus negative; IMRT, intensity-modulated radiotherapy; LA, locally advanced; LRC, locoregional control; LRF, locoregional failure; mPR, major pathological response; NA, not applicable; pCR, pathological complete response; PD-1, programmed cell death 1 receptor; PD-L1, programmed cell death ligand 1; PFS, progression-free survival; pPR, pathological partial response; OPC, oropharyngeal cancer; ORR, objective response rate; OPSCC, oropharyngeal squamous cell carcinoma; OS, overall survival; RFS, recurrence-free survival; RT, radiotherapy; SBRT, stereotactic body radiation; TIM, tumor immune microenvironment.

9.2 Appendix B: Supplemental Material for Manuscript 2 (Chapter 5)

Comparative Analysis of Histological and Molecular Methods for HPV Detection in Oropharyngeal Cancer

Supplemental Material

Table S2. Primers used for *in-house* qPCR HPV detection.

Name	Sequence
Gp5+[33]	TTTGTTACTGTGGTAGATACTAC
Gp6+[33]	GAAAAATAAACTGTAAATCATATTC
PC04[41]	CAACTTCATCCACGTTCACC
GH20[41]	GAAGAGCCAAGGACAGGTAC