

Prevalence and clinical characterization of HPV-induced oropharyngeal cancers: the experience of the Portuguese Oncological Institute in Porto

Ana Teresa Perdigão Vaz Fernandes

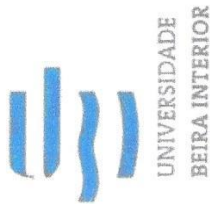
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ANEXO

Declaração de Integridade

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Universidade da Beira Interior, Covilhã 21/06/2023.

Ana Teresa Fernandes

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I dedicate this work and the end of my course to all of you. Thanks.

Abstract

Introduction: Head and neck squamous cell carcinoma (HNSCC) represents the eighth most common cancer worldwide. Alongside traditional risk factors such as smoking and excessive alcohol consumption, HPV is now recognized as the etiologic factor driving carcinogenesis for HNSCCs of the oropharynx.

Recently, multiple investigational groups have found that in the last two decades there has been a rising incidence of oropharyngeal squamous cell carcinoma (OPSCC) with a decrease in the incidence of other head and neck cancers, likely due to declines in alcohol and tobacco abuse.

There is robust evidence in the literature supporting the etiologic role of HPV in a subset of OPSCC that have a distinct epidemiologic profile, and also, a strong evidence to show that HPV positive status is an independent marker of favorable prognosis for OPSCC, with an improved response to treatment and survival.

Objectives: To review the current scientific evidence about the new entity of head and neck cancer: human papillomavirus positive (HPV+) oropharyngeal squamous cell carcinoma (OPSCC) and to retrospectively analyze the set of patients diagnosed in the period between 2018 till 2021 with OPSCC in Portuguese Oncological Institute Porto (IPO-Porto), in order to determine the prevalence of HPV+ OPCSS and to make a clinical characterization of patients with oropharyngeal cancers.

Methods: A bibliographic search and a review of the scientific literature about the topic were carried out using the PubMed, ClinicalKey, Jama, NCCN and other scientific platforms. Regarding the IPO study, the medical records of each patient were analyzed, and p16 status, age, gender, TNM-stage, treatment and survival were recorded. Statistical analysis was performed.

Results: In total, 280 patients were included. Immunohistochemically (IHC), p16 protein overexpression was only present in 57 (20.4%) of the 280 cases. The vast majority of patients (223, 79.6%) were HPV negative. According to the gender the patients were mostly male (254, 90.7%). 80 (28.6%) patients had low-T-stage (T1/T2) OPSCC tumors, and the others 197 (70.4%) had high-T-stage (T3/T4) OPSCC tumors. 74.5% of the stage I/II tumors were HPV +, and only 25.5% were HPV-. Regarding patients with advanced stages III/IV, only 7.1% were HPV+ and 92.9% were HPV-. 3 (1.1%) cases were To.

226 (80.7%) patients showed clinically positive lymph node metastasis (cN+). According to the 8th UICC/AJCC TNM classification, 55 (19.6 %) patients were at a low clinical stage (I/II).

The overall 2-years survival rate was 63.5%. The 2-years survival rate in stage I-II was 89.2% and 57.2 % in stage III-IV. The HPV+ group had a better prognosis than the HPV– group (OS: $p = 0.014$, DFS: $p = 0.45$).

Discussion: There are few reports about HPV-related cancers prevalence in Portugal. One of the strengths of this study was the evaluation of HPV status using p16 immunohistochemistry (IHC) for HPV detection. p16 protein overexpression is a surrogate marker for HPV-related oropharyngeal carcinoma and has been researched in all patients (n=280).

In contrast to most European countries and the USA, only a minority of patients (20.4%) in the sample were p16 positive. It may reflect differences in sexual behaviors (type of sex, age at onset of sex, number of sexual partners) of the Portuguese population for 6-7 decades ago “less liberal” compared to other Western societies.

The HPV+ group had a significantly better prognosis than de HPV – group in terms of overall survival (OS) and disease- free survival (DFS), and this is in line with the published studies.

This study has some limitations, the retrospective analysis might have hampered the accurate characterization of some patient risk factors, such as tobacco and alcohol use and there is a chance that p16 could have been inactivated by mutation or promoter methylation. This may be one of the possible explanations for the low rate of p16+ in our sample.

Conclusion: The prevalence of HPV-related cancers in IPO-Porto is low, when compared to other studies focused on developed countries.

Accordingly to literature review, the HPV-related OPSCC had a significantly better prognosis than de non-HPV – related group in terms of overall survival (OS) and disease-free survival (DFS).

Resumo

Introdução: O carcinoma de células escamosas da cabeça e pescoço representa o oitavo cancro mais comum em todo o mundo. Além de fatores de risco, como o tabagismo e o consumo excessivo de álcool, o HPV é atualmente reconhecido como um fator etiológico que conduz à carcinogénese destes carcinomas da orofaringe.

Recentemente, vários grupos de investigação epidemiológica mostraram que nas últimas duas décadas houve um aumento na incidência de carcinoma de células escamosas da orofaringe, com uma diminuição na incidência de outros cancros da cabeça e pescoço, provavelmente devido ao declínio do consumo excessivo de álcool e tabaco.

Na literatura há fortes evidências que suportam o papel etiológico do HPV num subgrupo de doentes com carcinomas de células escamosas da orofaringe, com um perfil epidemiológico distinto, e também uma forte evidência que mostra que o *status* HPV positivo é um fator prognóstico independente favorável para estes carcinomas, com melhor resposta ao tratamento e sobrevivência.

Objetivos: Revisão da evidência científica atual acerca do carcinoma de células escamosas da orofaringe HPV-positivo e análise retrospectiva do conjunto de doentes diagnosticados com carcinoma de células escamosas da orofaringe, no período de 2018 a 2021, no Instituto Português de Oncologia do Porto (IPO-Porto), com o objetivo de determinar a prevalência do carcinoma de células escamosas da orofaringe HPV-positivo e fazer uma caracterização clínico-patológica dos doentes com cancro da orofaringe.

Métodos: Foi realizada uma pesquisa bibliográfica e revisão da literatura científica sobre o tema, utilizando a PubMed, ClinicalKey, Jama, NCCN e outras plataformas científicas. Em relação ao estudo do IPO, foram analisados os registos clínicos de cada doente, registando-se a respetiva idade, sexo, status p16, estadio TNM, o tipo de tratamento e a sobrevivência. Realizou-se uma análise estatística a partir dos dados recolhidos.

Resultados: No total, foram incluídos 280 doentes. O estudo imuno-histoquímico mostrou uma sobreexpressão da proteína p16 em apenas 57 (20,4%) dos 280 casos. A grande maioria dos doentes (223, 79,6%) eram HPV- negativo. Quanto ao sexo, a maioria era do sexo masculino (254, 90,7%). 80 (28,6%) doentes tinham tumores em estadio T precoce (T1/T2), e os outros 197 (70,4%) tinham tumores em estadio T avançado (T3/T4). 3 (1,1%) casos eram T0.

226 (80,7%) doentes apresentaram-se com metástases regionais clinicamente positivas (cN+).

De acordo com a 8ª edição da classificação TNM da UICC/AJCC, dos 280 doentes, 55 (19,6%) apresentavam-se em estadio clínico precoce (I/II) e desses 55 doentes, 41 (74,5%) eram HPV+, e apenas 14 (25,5%) eram HPV-. 225 doentes apresentavam doença em estadio avançado III/IV, sendo que destes, apenas 16 (7,1%) eram HPV+ e 209 (92,9%) eram HPV-.

A taxa de sobrevivência global aos 2 anos foi de 63,5%. A taxa de sobrevivência aos 2 anos no estadio I-II foi de 89,2% e 57,2% no estadio III-IV. O grupo HPV-positivo teve um prognóstico melhor do que o grupo HPV-negativo.

Discussão: Existem poucos estudos sobre a prevalência de cancros associados ao HPV em Portugal. Um aspeto mais importantes deste estudo foi a avaliação do *status* HPV usando a imuno-histoquímica do p16 para a deteção do HPV. A sobreexpressão da proteína p16 é um marcador indireto do carcinoma orofaríngeo associado ao HPV e foi pesquisado em todos os doentes (n=280).

Ao contrário da maioria dos países europeus e dos EUA, apenas uma minoria dos doentes (20,4%) desta amostra eram p16 positivos. Isto pode refletir diferenças nos comportamentos sexuais (tipo de sexo, idade de início das relações sexuais, número de parceiros sexuais) da população portuguesa há 6-7 décadas, “menos liberais”, em comparação com outras sociedades ocidentais, à época “mais liberais”.

O grupo HPV+ teve um prognóstico significativamente melhor do que o grupo HPV- em termos de sobrevivência global e sobrevivência livre de doença, o que está de acordo com os estudos publicados.

Este estudo apresenta algumas limitações, como seja a análise retrospectiva, que pode ter dificultado a caracterização precisa de alguns fatores de risco de cada doente, como consumo tabágico e de álcool, bem como a possibilidade de ter ocorrido a inativação do p16 por mutação ou por metilação. Essa pode ser uma das possíveis explicações para a baixa taxa de p16+ nesta amostra.

Conclusão: A prevalência de cancros relacionados com o HPV no IPO-Porto é baixa, quando comparada com outros estudos centrados em países desenvolvidos.

O estudo foi de encontro ao que se verifica na revisão da literatura, na medida em que o carcinoma de células escamosas da orofaringe associado ao HPV teve um prognóstico significativamente melhor do que o grupo não associado ao HPV em termos de sobrevivência global e sobrevivência livre de doença.

Key Words

“Oropharynx”; “human papillomavirus”; “HPV”; “squamous cell carcinoma”; “head and neck cancer”; “oropharyngeal carcinoma”; “p16”.

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Acronyms list

AJCC - American Joint Committee on Cancer
BOTSCC - Base of Tongue Squamous Cell Carcinoma
DFS - Disease-Free Survival
DNA - Deoxyribonucleic Acid
HPV - Human Papillomavirus
HR-HPV - High-Risk HPV
IHC – Immunohistochemistry
T - Tumor
N- Node
M - Metastasis
NCCN - National Comprehensive Cancer Network
OPSCC - Oropharyngeal Squamous Cell Carcinoma
OS – Overall survival
PCR - Polymerase Chain Reaction
pRb – protein of retinoblastoma
RNA - Ribonucleic Acid
RT - Radiotherapy
TNM – Tumor Node Metastasis (classification)
TSCC- Tonsillar Squamous Cell Carcinoma
UICC - Union for International Cancer Control
USA – United States of America

1- Introduction

1.1- Epidemiology of head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) represents the eighth most common cancer worldwide and contribute nearly 600,000 new cases diagnosed, and over 300,000 deaths each year [1]. Alongside traditional risk factors, such as smoking and excessive alcohol consumption [2], HPV is now recognized as an etiologic factor driving carcinogenesis for HNSCCs of the oropharynx.

The oropharynx (tonsils, soft palate, base of tongue and lateral/posterior pharyngeal walls) is an important region of the head and neck. Recently, multiple investigational groups have revealed in the last two decades a rising incidence of oropharyngeal squamous cell carcinoma (OPSCC) (Fig.1) in their respective country [3-5], particularly in caucasian men and they also showed there has been a decrease in the incidence of other head and neck cancers likely due to declines in alcohol and tobacco abuse [5].

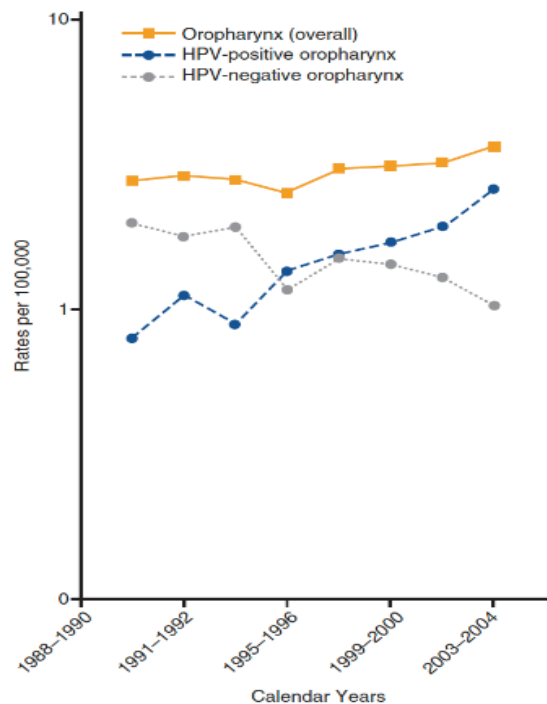


Figure 1 - Worldwide incidence of oropharyngeal carcinoma (1988-2004) [40].

There is robust evidence in the literature supporting the etiologic role of HPV in a subset of OPSCC that have a distinct epidemiologic profile [6,7-10].

Since the turn of the 21st century, a new entity of head and neck cancer has surfaced: human papillomavirus-positive (HPV+) OPSCC.

1.2- Epidemiology of oropharyngeal cancer

Strongly divergent results have been reported regarding the extent of HPV16, infection in OPSCC in different countries [11-13].

Whether these divergent geographic results represent important differences in the etiology of HNC or whether they are explained by differences in laboratory practices is unknown.

In many Western countries, a large proportion of OPSCC, which is dominated by tonsillar and base of tongue (Fig.2) squamous cell carcinoma (TSCC/BOTSCC), is human papillomavirus positive (HPV+) [14-18]. In Figure 3, is demonstrated the incidence rates of OPSCC in different countries between 1970 till 2000.

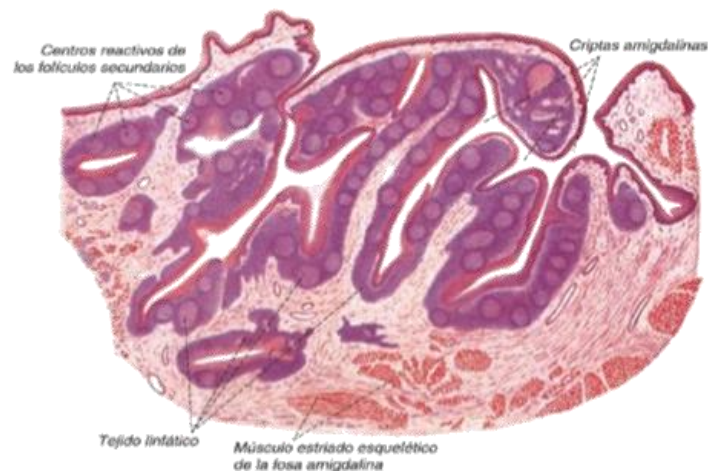


Figure 2 – Tonsils and base of tongue: privileged site of infection by HPV and immunological evasion. Downloaded from a figure by unknown author.

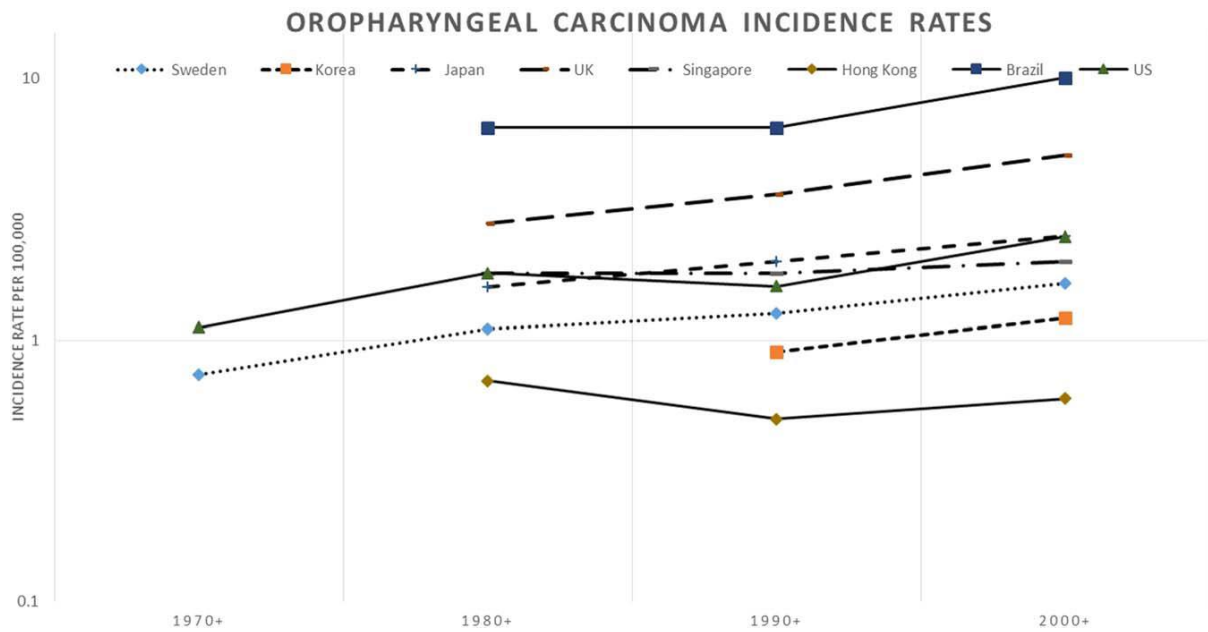


Figure 3 - Incidence rates of oropharyngeal carcinoma in different countries by decade [67].

1.3- HPV virology

HPVs are small, 50–55 nm in diameter, non-enveloped, double stranded DNA viruses (Fig.4). Belong to *papillomaviridae* family, and carry out their life cycle in either mucosal or cutaneous epithelia. Infection may result in an asymptomatic carrier state or a variety of both benign and malignant neoplasia. These viridae have icosahedral capsids composed of 72 capsomeres, surrounding a circular DNA genome capsid protein, L1 [19].

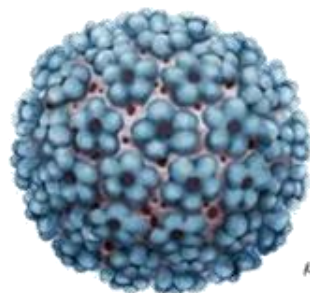


Figure 4 - HPV: 50–55 nm in diameter, non-enveloped double stranded DNA viruses [69].

The genome can be divided into an early (E) region (containing genes E1, E2, E4, E5, E6 and E7), late (L) region (containing genes L1 and L2), and a URR (upstream regulatory region) (Fig.5,6).

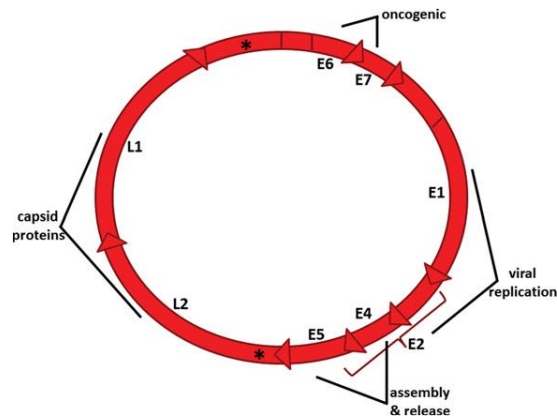


Figure 5 - HPV genome organization [68].

The viral genome of HPV is a double-stranded circular genome of approximately 8 kb transcribed as polycistronic mRNAs with eight ORFs. High-risk HPV genomes contain two viral promoters (*) encoding early (E) and late (L) genes.

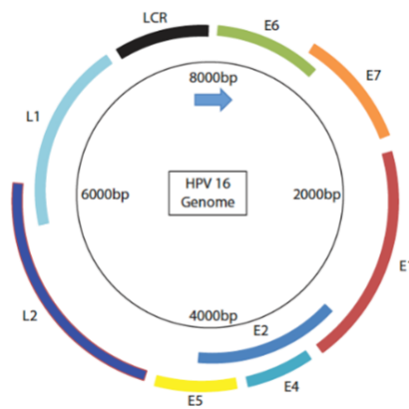


Figure 6 - HPV Genome. Three functional regions: LCR- regulation of gene expression; Early genes- transcription, replication, viral release; Late genes- structural proteins (capsid). Downloaded from a figure by unknown author.

The early genes E1–E7 play a role in regulating, promoting and supporting viral DNA transcription and replication. The late genes, L1 and L2, are transcribed only in productively infected cells and encode the major and minor capsid proteins required for assembly of progeny virions and eventual accumulation and release into the environment.

E6/E7, when overexpressed, disrupt the function of wild-type Rb and p53, leading to the development of a malignant phenotype (Fig.7,8,9).

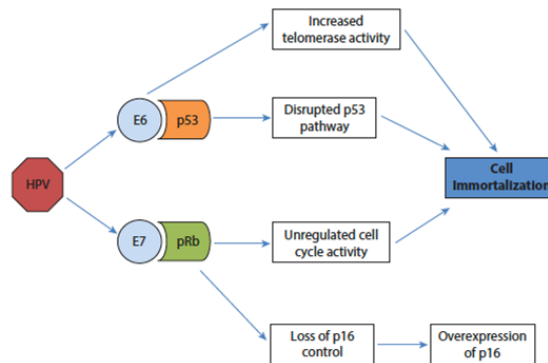


Figure 4 - HPV oncogenesis. Downloaded from a figure by unknown author.

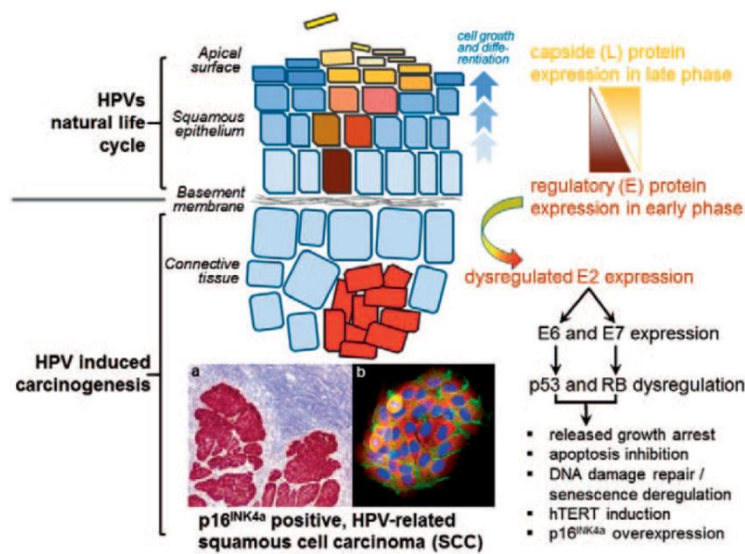


Figure 5 - HPV oncogenesis [70].

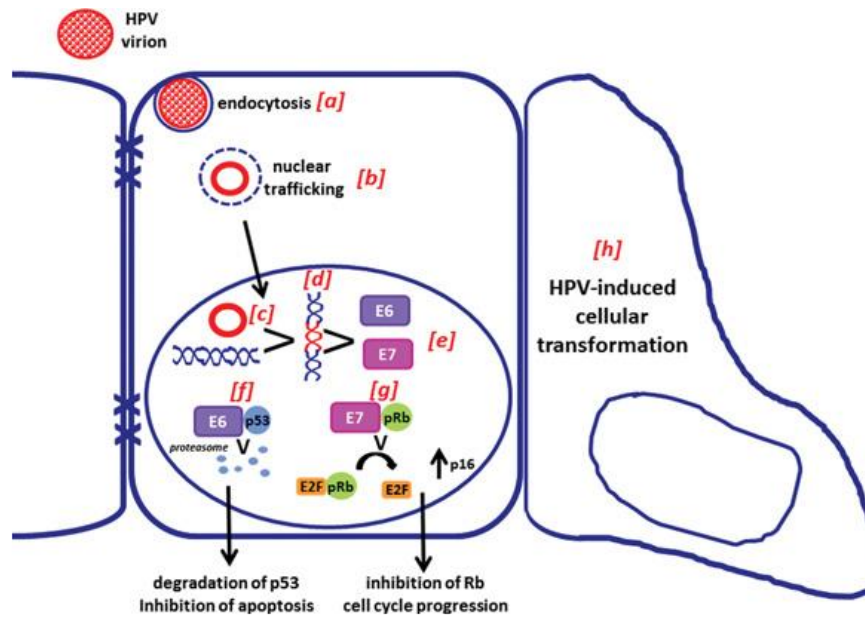


Figure 6 - HPV E6 and E7 promote cellular transformation and development of malignant phenotype [68]. Virions enter the cell via endocytosis (a) and are trafficked to the nucleus (b) where they persist in episomal form (c) or are integrated into the host genome (d). Both episomal and integrated viral DNA produce E6 and E7 (e). Interaction of E6 with p53 and the ubiquitin ligase E6-associated protein target p53 for proteasomal degradation (f) and prevents apoptosis. Rb family tumour-suppressor proteins including Rb (pRb), p130 and p107 interact with E7 (g) and are inactivated, resulting in release of E2F and promoting cell-cycle progression. Together, these functions of E6 and E7 promote cellular transformation (h).

HPV is a sexually transmitted virus with over 150 unique types. Although there are 15 known high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) and 12 low-risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108), types 16, 18 and 31 are the major types associated with mucosal epithelial cancers.

Although there is a broad distribution of high-risk HPV types responsible for cervical cancer, few are associated with oropharyngeal squamous cell carcinoma (OPSCC). Type 16 (85–95%) and, to a lesser extent, type 18 are the subtypes most commonly identified among HPV+ OPSCC. HPV16 accounts for the majority of OPSCCs in the United States and Europe [11].

1.4- Oral HPV infection and natural course in the general population

In HPV infection, the life cycle of the virus is linked to the differentiation state of the host cell and requires that the host cell remains active in the cell cycle. The highest level of viral replication occurs in the granular layer of stratified epithelia, where keratinocytes are terminally differentiated and are in the process of enucleation and death.

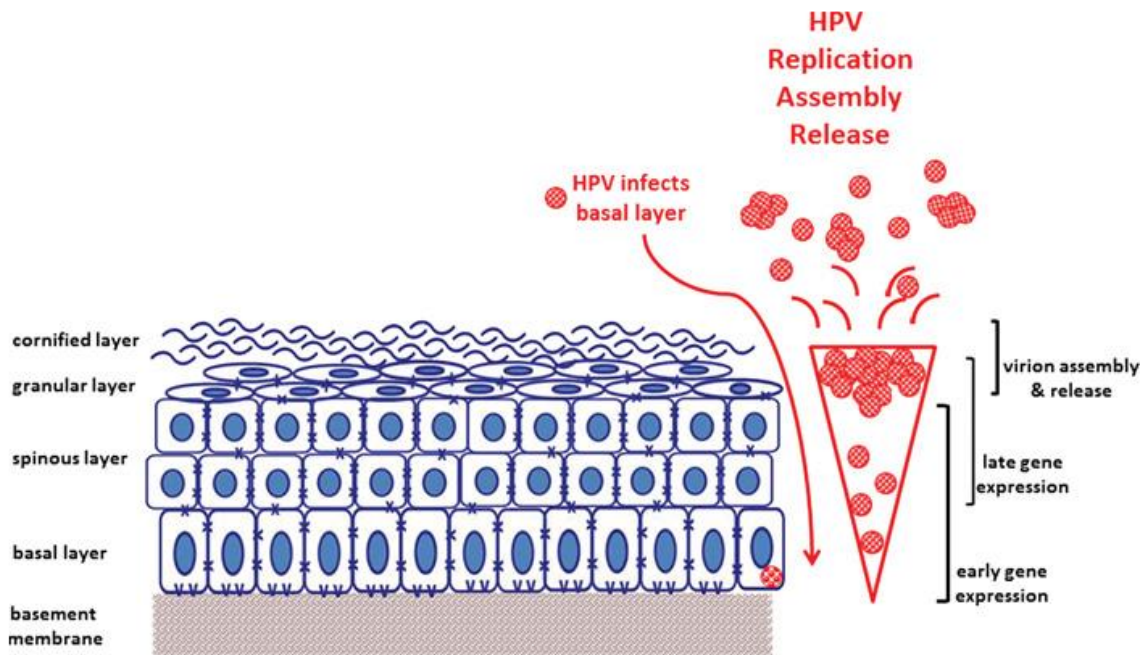


Figure 7 - HPV infection of oral mucosa [68].

HPV (red) infects proliferating cells in the basal layer that are exposed by wounding. The virus replicates in synchrony with cellular DNA replication. The highest level of viral replication occurs in the granular layer.

The basal cell is fundamental to papillomavirus infection and may be the only cell within epithelia capable of establishing infection. It is thought that infection occurs at sites of injury in the proliferating basal layer of epithelial surfaces (Fig.10). This proliferation due to microtrauma induces basal cell migration and enhanced cell division, therefore increasing the probability of a productive infection [20].

In most cases (90%) the infection is transient and self-limited.

Strongly divergent results have been reported regarding the extent of HPV16 infection in OPSCC in different countries (Fig.11) [11-13]. Studies in the USA suggest that the majority of OPSCC are now caused by HPV16, [18, 21-22] although proportions of

<10% have been reported in the few studies completed in South America, [7,23-24] with European estimates being in between [25-26]. Whether these divergent geographic results represent important differences in the etiology of OPSCC or whether they are explained by different criteria for patient inclusion/exclusion and utilized different detection methods for defining HPV-positivity employed by each study is unknown.

Geographic differences in the proportion of HPV16-positive OPSCC may in part be explained by differences in tobacco use [27]. As performing oral sex is the primary risk factor for HPV-positive OPSCC, differences in oral sexual behavior likely contribute to geographic differences in incidence. It has been suggested that changing sexual practices, in particular increasing oral sexual behavior, may have led to higher rates of oral HPV infection and ultimately HPV-positive OPSCCs. The United States is the only country with significant studies reporting time-based trends of oral sexual behavior, and studies spanning from the 1940s to the present day appear to support the notion of increasing oral sexual behavior [28]. These changing sexual practices could help explain the observed trends in the prevalence of HPV-positive OPSCCs in North America and Europe.

The prevalence of oral HPV infections in the healthy general population has been studied in cross-sectional studies. A systematic review estimated a prevalence of 4.5% for any HPV infection and 1.3% for oral HPV16 [29].

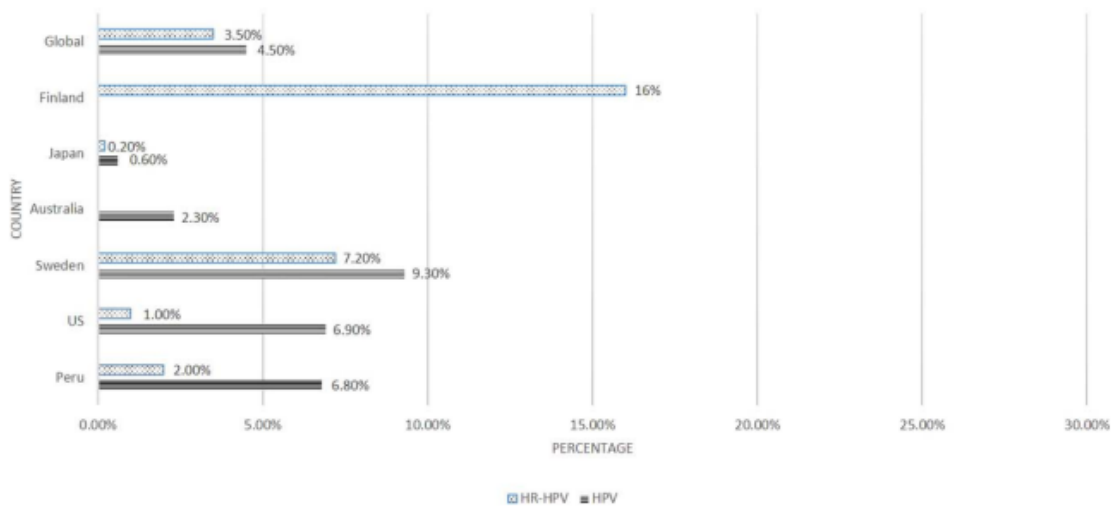


Figure 8 - Prevalence of oral HPV infection in different countries. HPV = human papillomavirus; HR-HPV = high-risk human papillomavirus [67].

Significant risk factors for oral HPV infection include an increasing number of recent and lifetime oral sex, open mouth kissing, vaginal, and any sex partners, aged <18 years at the time of first oral sexual intercourse, current tobacco use, and a personal history of cervical HPV infection [30-31]. The prevalence of oral HPV infection increases in a dose-response fashion with increased number of sexual partners.

The natural history of oral HPV infection is a subject of interest. Such data are clinically necessary to contextualize what the presence of a one-time infection means.

The largest prospective study to date examined oral HPV infection among 1,626 adult males with a median follow-up of 12.7 months [32]. Only 4.4% of participants had a new oral HPV infection, and the median duration of infection was 6.9 months [32]. In addition, 0.6% of men developed an oral HPV16 infection with a median duration of infection lasting 7.3 months [32]. By 18 months the vast majority of infections cleared (or were below the threshold of detection). A further indication that most oral HPV infections are cleared is that the prevalence of high-risk oral HPV infection in partners of patients with biopsy-proven HPV-related OPSCC is equivalent to the prevalence of the general population (1.2%) [33].

Another study demonstrated that HIV seropositivity did not impact persistence of oral HPV infections [34]. Active smoking, age >44 years, and CD4 count <500 were associated with persistence of oral HPV infection.

On the other hand, oral HPV infection is higher among men and Caucasian, and the men had significantly higher lifetime oral and vaginal sexual partners compared to women. Men and women aged 30 to 59 years were more likely to have performed oral sex compared to those aged 60 to 69 years [35]. Additionally, white males had the highest number of lifetime oral sex partners and the youngest age of initial oral sexual intercourse compared to other ethnic groups [35].

In multivariate analysis, an increasing number of oral sexual partners was associated with increasing odds of oral HPV16 infection but not age or ethnicity, thus indicating that the observed epidemiologic differences in oral HPV16 infection are due to differences in oral sexual behaviors [35]. Oral sex on a woman results in a higher level of oral exposure compared to oral sex on a male.

In HPV-positive carcinoma the viral life cycle is interrupted and cancer cells remain in an undifferentiated state and infectious particles are not released.

1.5- Carcinogenesis

HPV+ tumors have a unique profile of protein expression and genetic and epigenetic alterations characterized by p16 overexpression, absence of somatic inactivating mutations of the p53 tumor suppressor gene, decrease and dysregulation of the cell cycle mediated by the retinoblastoma tumor suppressor gene (pRb), when compared to HPV-, where chemical carcinogenesis of tobacco and alcohol prevails, associated with a mutant p53, inactivated p16 and a normal or overexpressed pRb (Fig.12).

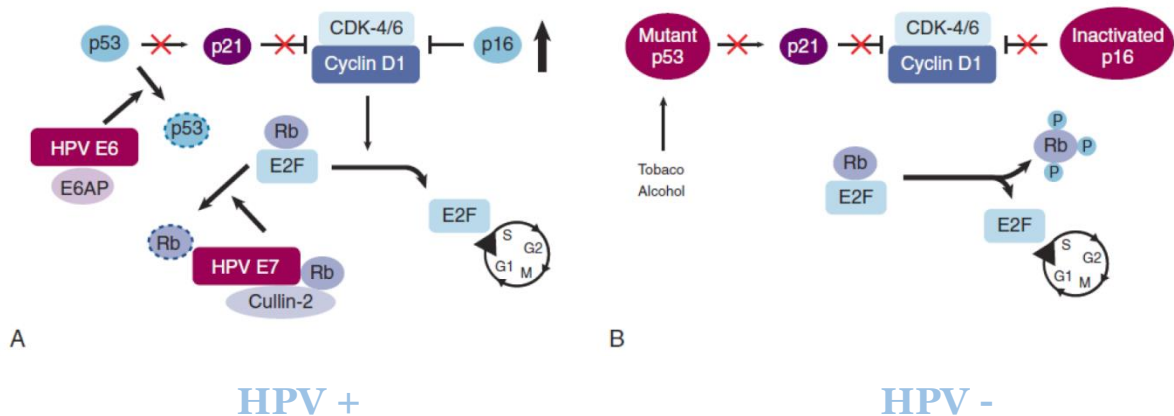


Figure 9 - Viral carcinogenesis versus chemical carcinogenesis. Downloaded and adapted from a figure by unknown author.

1.6- Clinicopathological characteristics of patients with HPV-related OPSCC

HPV-related OPSCC, represents a novel disease that occurs more often in younger, healthier individuals with little or no tobacco exposure [36], more commonly male, caucasian and with a higher socioeconomic status compared to HPV-unrelated OPSCC patients (Table 1).

HPV+ OPSCC has phenotypic characteristics that distinguish it from HPV- OPSCC, including low differentiation, poor keratinization, and basaloid phenotype (Table 1).

Characteristics	HPV+ OPSCC	HPV- OPSCC
Patient characteristics		
Average age at diagnosis (years)	59 ^a	60 ($P < 0.001$) ³⁵
Sex	86.9% male	76.8% male ($P < 0.001$) ³⁵
Ethnicity	90% white	75.9% white ($P < 0.001$) ³⁵
Role of smoking	Rising incidence of HPV+ OPSCC in smokers, as well as in nonsmokers ³⁵	
Role of alcohol	HPV- OPSCC associated with greater alcohol consumption ⁷	
Role of sexual history	High number of sexual partners a risk factor for HPV+ OPSCC ⁷	
Tumour characteristics		
Incidence per 100,000	4.62	1.82 (REF. ³⁶)
Anatomical location	More prevalent in oropharynx (94.2% HNSCC); specifically the base of tongue and tonsils ⁷	Less prevalent in the oropharynx (72.8% HNSCC) ³⁵
Stage (AJCC 7th edn)	Early stage (T1–2); frequently with nodal metastasis at presentation ¹⁵⁶	All stages (T1–4) ³⁵
Histopathological appearance	Immature, basal-like/basaloid, non-keratinizing ¹⁵⁶	Frequently keratinizing SCC
Cancer-specific mortality	HPV+ OPSCC associated with a more favourable prognosis (aHR 0.40, $P < 0.001$) ³⁵	
Biological characteristics		
Genetic alterations	More frequent alterations in genes encoding DNA damage response proteins, FGF and JAK–STAT signalling proteins, as well as immune-related genes such as <i>HLA-A/B</i> ; <i>PIK3CA</i> mutations more commonly observed ⁷⁵	Aberration of <i>TP53</i> and cell-cycle pathways (such as <i>CDKN2A</i> loss); oxidative stress regulation more frequently mutated ⁷⁵
Other aberrations	p53 and Rb degradation by E6 and E7, respectively ¹⁴³	NR

Table 1 - Comparison of the key characteristics of HPV+ and HPV– OPSCCs [71].

1.7- Survival outcomes

There is strong evidence that HPV positive status is an independent marker of favorable prognosis for OPSCC, with an improved response to treatment and survival. The figure 13 represents an example of that evidence, in a study about the impact of HPV-associated p16-expression on radiotherapy outcome in advanced oropharynx, published in *Radiotherapy and Oncology* journal, in December 2014 (Fig.13).

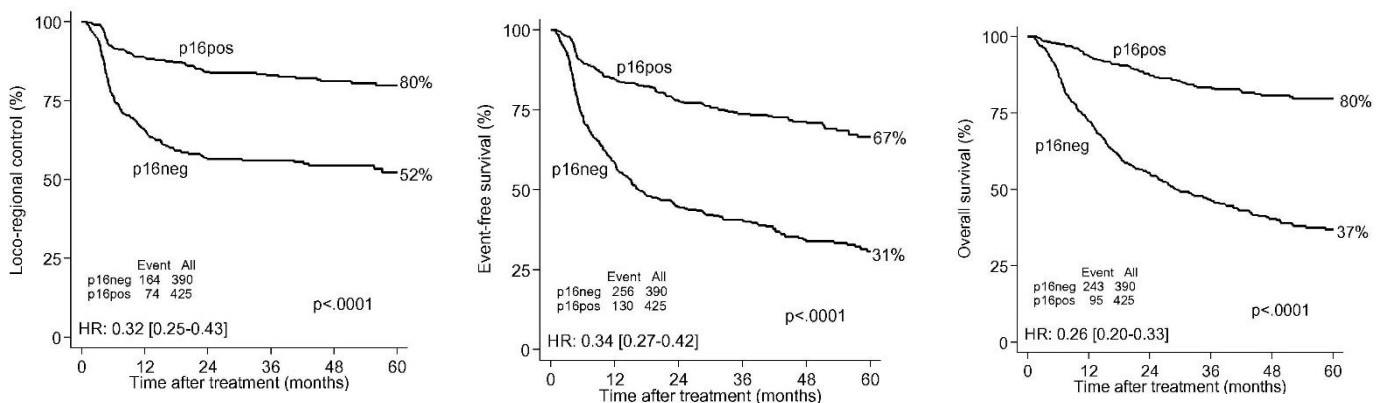


Figure 10 - Impact of p16 on locoregional control (LRC), event-free survival and overall survival (OS) [72].

The prognostic significance of HPV tumor status and tobacco use were combined into a risk stratification model. The low-risk group (HPV-positive with <10 pack-years), intermediate-risk group (HPV-positive with >10 pack-years or HPV-negative with <10 pack-years) and high-risk group (HPV-negative with >10 pack-years) were shown to have distinct 3-year OS rates of 93%, 70.8%, and 46.2%, respectively [37].

Despite a favorable initial response to therapy, up to 30% of HPV-related HNSCC patients experience recurrence [37-38].

The majority of recurrences occurred within 1 year regardless if patients were p16-positive or negative (65% vs. 63%) [39].

HPV types 16 and 18 are the most commonly detected, transcriptionally active HR-HPV types in head and neck cancer [40].

The entity is highly responsive to treatment and carries an excellent prognosis.

1.8- TNM staging

While the seventh edition TNM staging adequately reflects the behavior of those cancers typically associated with tobacco and alcohol abuse (not caused by HR-HPV), it does not properly describe HR-HPV disease with respect to prognosis or behavior [11, 40-41].

Therefore, a new staging system was needed for HR-HPV OPSCC.

HPV+ cancers have a different biology from that of HPV- cancers, with distinct phenotypic features, including poor differentiation, scant keratinization and basaloid phenotype, compared with the typically keratinizing morphology of HPV- TSCCs (Fig.14).

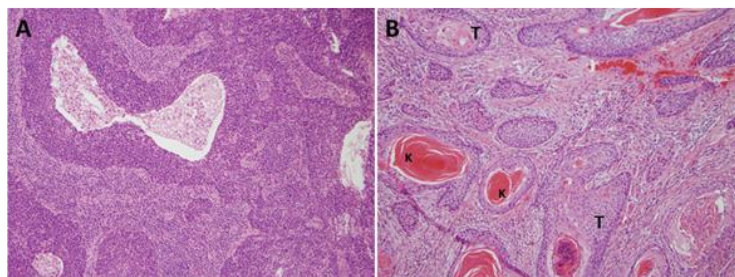


Figure 11 - Distinct phenotypic features characteristic of (A) HPV+ OPSCC relative to (B) HPV- OPSCC [68].

Non-keratinizing hyperchromatic tumor cells with ill-defined borders, abundant mitoses and areas of necrosis (A). Keratinizing tumor cells with abundant pink cytoplasm composed in discrete nests (B).

As because site or histology alone cannot differentiate the two entities, it was imperative to identify an accurate or characteristic test to distinguish the 2 types of OPSCC.

The test should be simple, inexpensive, and reproducible. One option was to consider tobacco exposure versus no tobacco exposure to define the 2 types of oropharynx disease. However, tobacco use is found among patients with HR-HPV-associated tumors, and non HR-HPV-associated tumors emerge in nontobacco users (yet behave like classical tobacco-associated tumors). Hence, tobacco exposure fails as a differentiating characteristic. HPV mediation of oropharyngeal cancer (direct HR-HPV detection) can be ascertained by testing for the presence of HPV DNA or mRNA in the tissue samples by use of PCR-based methods or in-situ hybridization, but it is expensive and is not universally available, rendering suboptimal for worldwide adoption.

Active transcription by HR-HPV types, in turn, leads to overexpression of the tumor suppressor protein, p16, which may act as a useful surrogate marker for active HPV transcription in OPSCC, because the HPV early protein E7 results in p16 overexpression in HPV-related cancers. In HPV-unrelated oropharyngeal cancer, the *CDKN2A* gene encoding p16 is mutated or lost in almost all cases, and so p16 is usually not expressed in these tumors.

IHC for overexpression of the tumor suppressor protein p16 (cyclin-dependent kinase 2A) is an established, robust, surrogate biomarker for HPV-mediated carcinogenesis. It is also an independent positive prognosticator in the context of OPSCC [11, 20-41]. IHC staining for p16 is inexpensive, has near universal availability, and is relatively straightforward to interpret.

HPV-associated OPSCC have a remarkably better prognosis [42-43], but the HPV status has had no impact on the treatment decision to date. Therefore, patients with HPV-associated OPSCC might be overtreated and treatment de-escalation is under investigation in clinical trials. Due to a more favorable prognosis observed in HPV+ OPSCCs in comparison with HPV-negative (HPV-) OPSCCs, p16 has recently been included in the eighth edition of the Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) TNM-classification of OPSCCs [11].

Hence, OPSCCs are now staged according to 2 distinct systems, depending on whether or not they overexpress p16 [44]. Staging by the HR-HPV-associated OPSCC system should only be assigned when p16 overexpression is determined using established criteria [45-47]. Specifically, the cutoff point for p16 overexpression is diffuse

(>70%) tumor expression, with at least moderate (+2/3) staining intensity. Overexpression of p16 is usually localized to tumor cell nuclei and cytoplasm, and p16 staining localized only to the cytoplasm is considered nonspecific and thus not diagnostic (negative).

p16 INK4a overexpression (p16+) is used as a surrogate marker for presence of HPV in the eighth edition of the AJCC/UICC staging system for OPSCC, which separates the TNM classification of HPV mediated (p16+) and HPV unrelated (p16-) OPSCC.

1.8.1- Clinical and Pathologic T category for Oropharyngeal Cancer

T CATEGORY	T CRITERIA
T0	No primary identified
T1	Tumor 2 cm or smaller in greatest dimension
T2	Tumor larger than 2 cm but not larger than 4 cm in greatest dimension
T3	Tumor larger than 4 cm in greatest dimension or extension to lingual surface of epiglottis
T4	Moderately advanced local disease; tumor invades the larynx, extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible or beyond ^b

^aTable 1 is used with the permission of the American Joint Committee on Cancer (AJCC), Chicago, Illinois. The original source for this material is the AJCC Cancer Staging Manual, Eighth Edition (2017) published by Springer Science and Business Media LLC (springer.com) (Amin MB, Edge SB, Greene FL, et al, eds. AJCC Cancer Staging Manual. 8th ed. New York: Springer; 2017, with permission²). ^bMucosal extension to lingual surface of epiglottis from primary tumors of the base of the tongue and vallecula does not constitute invasion of the larynx.

Table 2 - Clinical and Pathologic T category for Human Papillomavirus-Associated (p16-positive) Oropharyngeal Cancer [73].

T CATEGORY	T CRITERIA
Tx	Primary tumor cannot be assessed
Tis	Carcinoma in situ
T1	Tumor 2 cm or smaller in greatest dimension
T2	Tumor larger than 2 cm but not larger than 4 cm in greatest dimension
T3	Tumor larger than 4 cm in greatest dimension or extension to lingual surface of epiglottis
T4	Moderately advanced or very advanced local disease
T4a	Moderately advanced local disease; tumor invades the larynx, extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible ^b
T4b	Very advanced local disease; tumor invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases carotid artery

^aTable 2 is used with the permission of the American Joint Committee on Cancer (AJCC), Chicago, Illinois. The original source for this material is the AJCC Cancer Staging Manual, Eighth Edition (2017) published by Springer Science and Business Media LLC (springer.com) (Amin MB, Edge SB, Greene FL, et al, eds. AJCC Cancer Staging Manual. 8th ed. New York: Springer; 2017, with permission²). ^bMucosal extension to lingual surface of epiglottis from primary tumors of the base of the tongue and vallecula does not constitute invasion of the larynx.

Table 3 - Clinical and Pathologic T category for Non-Human Papillomavirus-Associated (p16-negative) Oropharyngeal Cancer [73].

1.8.2- Clinical and Pathologic N category for Oropharyngeal Cancer

N CATEGORY N CRITERIA	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	One or more ipsilateral lymph nodes, none larger than 6 cm
N2	Contralateral or bilateral lymph nodes, none larger than 6 cm
N3	Lymph node(s) larger than 6 cm

^aTable 3 is used with the permission of the American Joint Committee on Cancer (AJCC), Chicago, Illinois. The original source for this material is the AJCC Cancer Staging Manual, Eighth Edition (2017) published by Springer Science and Business Media LLC (springer.com) (Amin MB, Edge SB, Greene FL, et al, eds. AJCC Cancer Staging Manual. 8th ed. New York: Springer; 2017, with permission²).

Table 4 - Clinical N category for Human Papillomavirus-Associated (p16-positive) Oropharyngeal Cancer [73].

N CATEGORY N CRITERIA	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or smaller in greatest dimension and ENE-negative
N2	Metastasis in a single ipsilateral lymph node larger than 3 cm but not larger than 6 cm in greatest dimension and ENE-negative; or metastases in multiple ipsilateral lymph nodes, none larger than 6 cm in greatest dimension and ENE-negative; or metastasis in bilateral or contralateral lymph nodes, none larger than 6 cm in greatest dimension and ENE-negative
N2a	Metastasis in a single ipsilateral lymph node larger than 3 cm but not larger than 6 cm in greatest dimension and ENE-negative
N2b	Metastasis in multiple ipsilateral lymph nodes, none larger than 6 cm in greatest dimension and ENE-negative
N2c	Metastasis in bilateral or contralateral lymph nodes, none larger than 6 cm in greatest dimension and ENE-negative
N3	Metastasis in a lymph node larger than 6 cm in greatest dimension and ENE-negative; or metastasis in any lymph node(s) and clinically overt ENE-positive
N3a	Metastasis in a lymph node larger than 6 cm in greatest dimension and ENE-negative
N3b	Metastasis in any node(s) and clinically overt ENE-positive

Abbreviations: ENE, extranodal extension. ^aTable 4 is used with the permission of the American Joint Committee on Cancer (AJCC), Chicago, Illinois. The original source for this material is the AJCC Cancer Staging Manual, Eighth Edition (2017) published by Springer Science and Business Media LLC (springer.com) (Amin MB, Edge SB, Greene FL, et al, eds. AJCC Cancer Staging Manual. 8th ed. New York: Springer; 2017, with permission²).

Table 5 - Clinical N category for Non-Human Papillomavirus-Associated (p16-negative) Oropharyngeal Cancer [73].

N CATEGORY	N CRITERIA
NX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastasis
pN1	Metastasis in 4 or fewer lymph nodes
pN2	Metastasis in more than 4 lymph nodes

^aTable 5 is used with the permission of the American Joint Committee on Cancer (AJCC), Chicago, Illinois. The original source for this material is the AJCC Cancer Staging Manual, Eighth Edition (2017) published by Springer Science and Business Media LLC (springer.com) (Amin MB, Edge SB, Greene FL, et al, eds. AJCC Cancer Staging Manual. 8th ed. New York: Springer; 2017, with permission²).

Table 6 - Pathologic N category for Human Papillomavirus-Associated (p16-positive) Oropharyngeal Cancer [73].

T CATEGORY	N CATEGORY			
	N0	N1	N2	N3
T0	NA	I	II	III
T1	I	I	II	III
T2	I	I	II	III
T3	II	II	II	III
T4	III	III	III	III

^aAny M1 is stage IV.

Table 7 - Anatomic Stage and Prognostic Groups for Clinical TNM grouping of HPV-associated (p-16 positive) Oropharyngeal Cancer [73].

T CATEGORY	N CATEGORY		
	N0	N1	N2
T0	NA	I	II
T1	I	I	II
T2	I	I	II
T3	II	II	III
T4	II	II	III

^aAny M1 is stage IV.

Table 8 - Anatomic Stage and Prognostic Groups for Pathologic TNM grouping of HPV-associated (p-16 positive) Oropharyngeal Cancer [73].

T CATEGORY	N CATEGORY			
	N0	N1	N2a,b,c	N3a,b
T1	I	III	IVA	IVB
T2	II	III	IVA	IVB
T3	III	III	IVA	IVB
T4a	IVA	IVA	IVA	IVB
T4b	IVB	IVB	IVB	IVB

^aAny M1 is stage IVC.

Table 9 - Anatomic Stage and Prognostic Groups for Clinical and Pathologic TNM grouping of Non-HPV-associated (p-16 negative) Oropharyngeal Cancer [73].

1.8.3 - p16 and HPV discordance in OPSCC

For that staging system, and for most of the de-escalation clinical trials done so far [48-49], HPV-related oropharyngeal cancer is therefore usually defined on the basis of overexpression of p16 alone, without other HPV biomarker confirmation [50]. However, up to 20% of patients who have p16- positive tumors test negative for HPV DNA or RNA [51-52].

In some studies, outcomes in patients with p16-positive and HPV-negative oropharyngeal cancer resembled the improved outcomes of patients with double positive (p16-positive and HPV-positive) cancer, but results of other studies show a poorer prognosis, similar to that in patients with double-negative (p16-negative and HPV-negative) cancer [52-61]. If the poorer prognosis is confirmed, then the use of p16 alone for the TNM staging system and for inclusion in clinical trials of treatment de-escalation might not be appropriate, since patients with p16-positive and HPV-negative cancer, who respond less well to treatment and are at higher risk of recurrence than patients with p16-positive and HPV-positive cancer, would be misclassified as having HPV-related tumors and could undergo de-escalation of treatment, which could be detrimental to their overall survival [62].

Few studies have described the characteristics and prognosis of patients with discordant combinations of oropharyngeal cancer (ie, p16-/HPV+ or p16+/HPV-) [52-61]. There are robust evidence that p16 and HPV discordance exists in some patients, with a prevalence that varies by geographical region, and that discordance between p16 and HPV biomarker status affects patient prognosis in terms of disease-free and overall survival. Moreover, the prognosis of patients with discordant p16+/HPV- oropharyngeal cancer depends on their smoking status. Never smokers have a significantly better prognosis than ever smokers, and their outcomes are similar to (but slightly worse than) p16+/HPV+ (double-positive) patients. p16+/HPV- patients who smoke have a significantly worse survival than p16+/HPV+ patients, with outcomes that are similar to (but slightly better than) p16-/HPV- patients.

Different p16+/HPV- oropharyngeal cancer tumors appear to overexpress p16 due to different mechanisms. Patients with p16+/HPV- tumors who do not smoke might mostly have HPV-mediated tumors, but possibly at lower copy numbers than all p16+/HPV+ patients, and therefore can only be detected by techniques that have the highest sensitivity, such as HPV RNA PCR. These could also relate to the group of so-called copy number silent tumors, a potentially separate genetic subgroup of HPV-negative tumours with a more favorable prognosis.

However, in patients who smoke, worse outcomes are in part driven by an increase in cancer-related deaths and not simply by an increase in deaths from non-cancer, smoking-related comorbidities. In these tumors, p16 expression might be due to causes that are not related to HPV, but to other molecular causes such as genomic alterations of genes active in the retinoblastoma protein pathway [63].

If p16 immunohistochemistry is used alone to determine HPV mediation a significant number of p16-positive patients worldwide are HPV-negative ever smoker patients and thus would be incorrectly classified as having HPV-related tumors.

Based on p16 immunohistochemistry positivity alone, thereby introducing potential bias to the results of the studies and so dual testing with p16 immunohistochemistry and an HPV DNA or RNA test should therefore be implemented as standard in the future.

For example, the prevalence of p16+/HPV– in non-TSCC or BOTSCC primary was much higher than in tonsil and base of tongue subsites [54]. Therefore, for a p16-positive oropharyngeal cancer arising from non-TSCC or BOTSCC, confirmatory HPV testing is particularly recommended.

Some findings indicate that classification of patients with oropharyngeal cancer based on p16-positive IHC alone is likely to be insufficient in routine clinical practice, both for predicting prognosis and when selecting treatment. Routine HPV testing alongside p16 evaluation, or at least following a positive result on p16 IHC, should be recommended in the clinical setting for more accurate counselling on prognosis, and in future circumstances in which treatment de-escalation or intensification are being considered and this approach is particularly important in patients with oropharyngeal cancer who smoke. So the best prognostic measure of survival out-comes, and a more accurate indication of HPV-infection, is combined HPV/p16-positivity.

In USA and in many European countries, a large proportion of OPSCC, which is dominated by TSCC/BOTSCC, is human papillomavirus positive [14-18]. In addition, patients with HPV+ TSCC/BOTSCC have a more favorable clinical outcome than those with corresponding HPV negative cancer. This has also been proposed for all HPV+ OPSCC as compared to HPV- OPSCC.

However, an estimated 10–20% of all OPSCCs are p16-positive, but HPV-, being most apparent in OPSCC arising outside the tonsils and base of tongue, such as e.g. other sites include the uvula/soft palate/pharyngeal wall, here defined as *other OPSCC*. The combination of HPV DNA and p16+ was much less common in *other OPSCC*, and that

presence of HPV DNA or p16+ in these tumors did not correlate to better clinical outcome.

It has been suggested that it should be possible to de-escalate today's more intensified treatment, i.e. chemo-radiotherapy, targeted therapy, and surgery either alone or in combination for patients with HPV+ OPSCC, in order to reduce therapy-related side effects and complications.

Patients with other OPSCC are often included into the same studies and treatment protocols as patients with TSCC/BOTSCC, even though earlier studies have indicated that prevalence, clinical significance and the correlation between HPV and p16+ is markedly lower in other OPSCC. Since TSCC/BOTSCC dominates OPSCC with roughly 90% of all cases, there is an obvious risk that the results from patients with other OPSCC are concealed and misinterpreted.

1.9- NCCN Guidelines

In generic and simplistic terms, the treatment of early stages of OPSCC is unimodal (surgery or RT) and in advanced stages it is multimodal (chemoradiotherapy or surgery followed by RTQT or induction chemotherapy followed by chemoradiotherapy).

1.9.1- Treatment of OPSCC HPV negative (p16-negative)

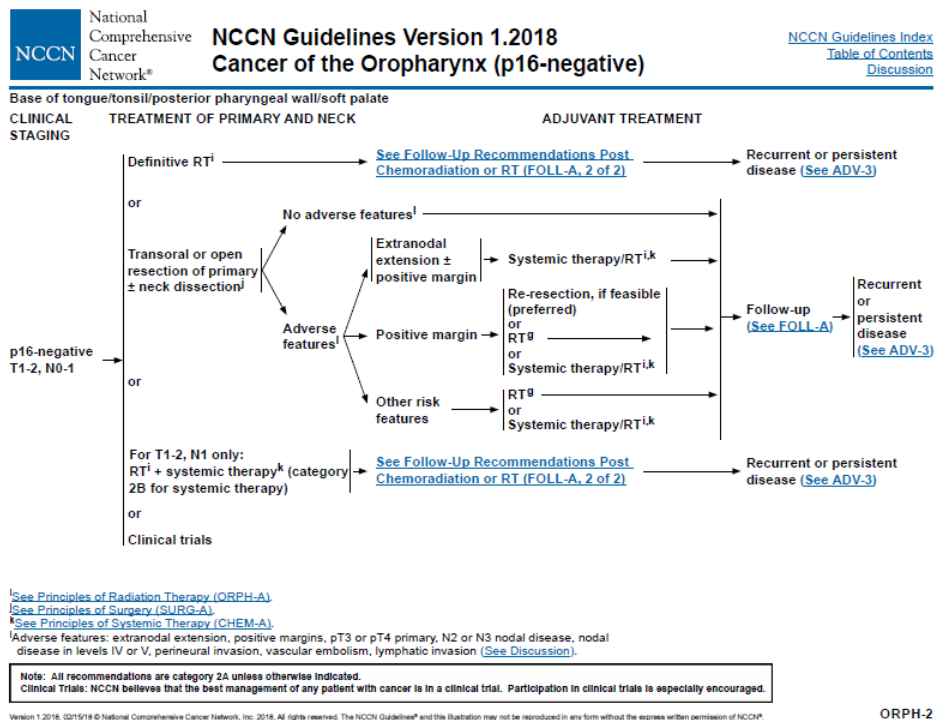


Figure 12 - Treatment of OPSCC HPV negative (p16-negative) [74].

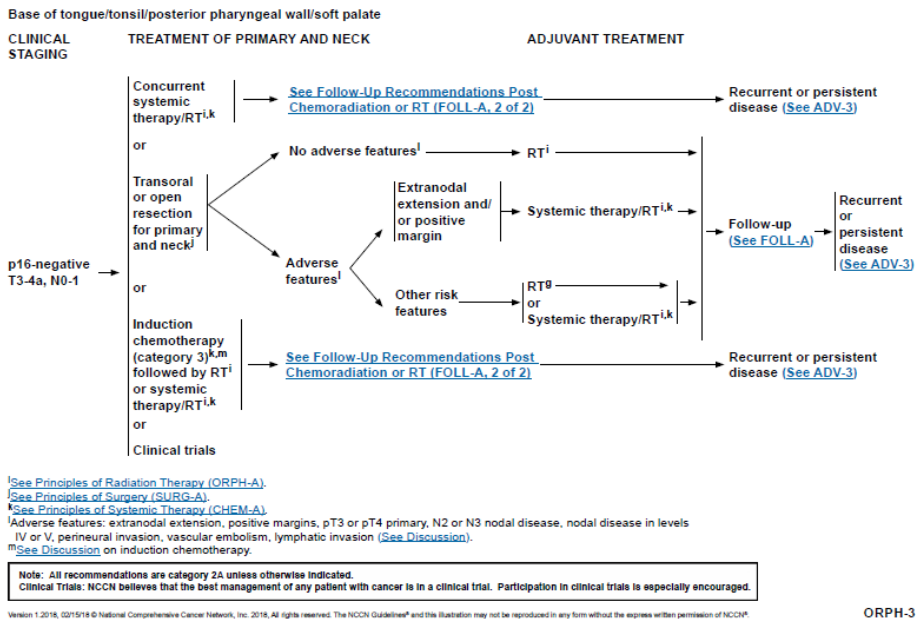


Figure 13 - Treatment of OPSCC HPV negative (p16-negative) [74].

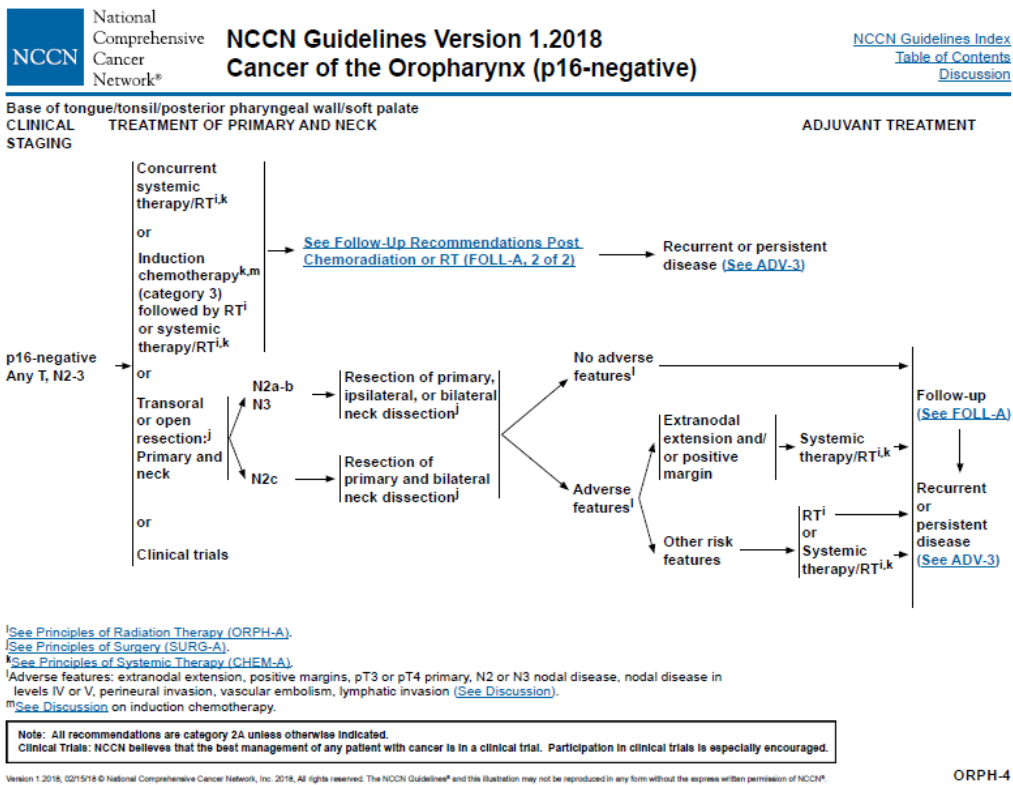


Figure 14 - Treatment of OPSCC HPV negative (p16-negative) [74].

1.9.2- Treatment of OPSCC HPV positive (p16-positive)

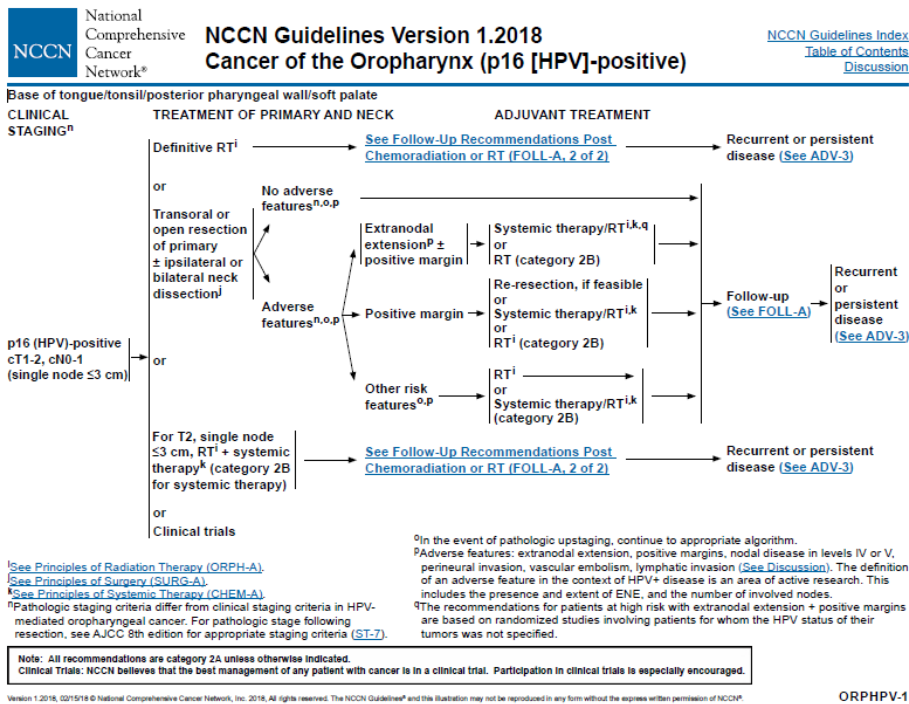


Figure 15 - Treatment of OPSCC HPV positive (p16-positive) [74].

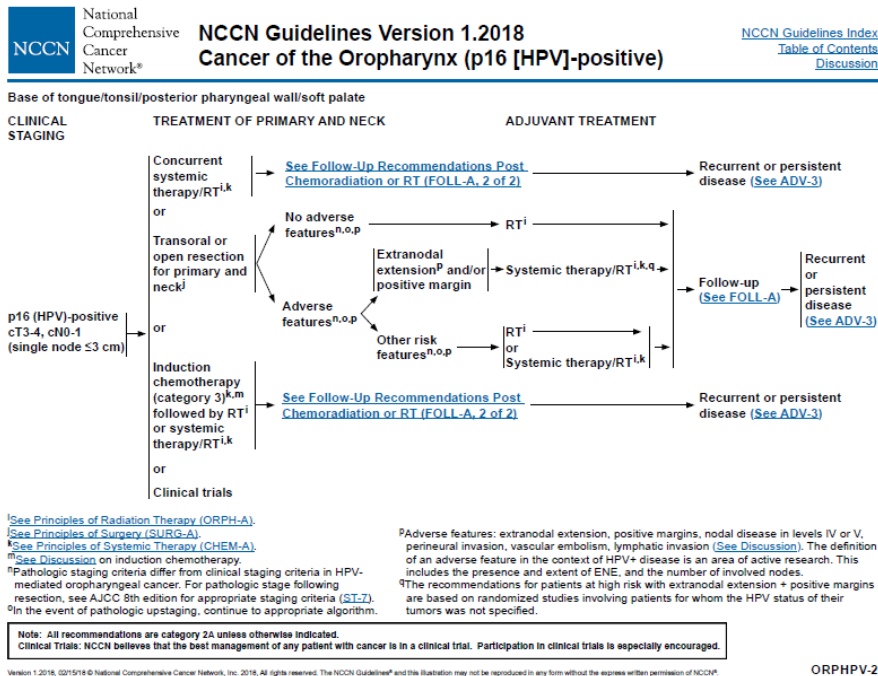
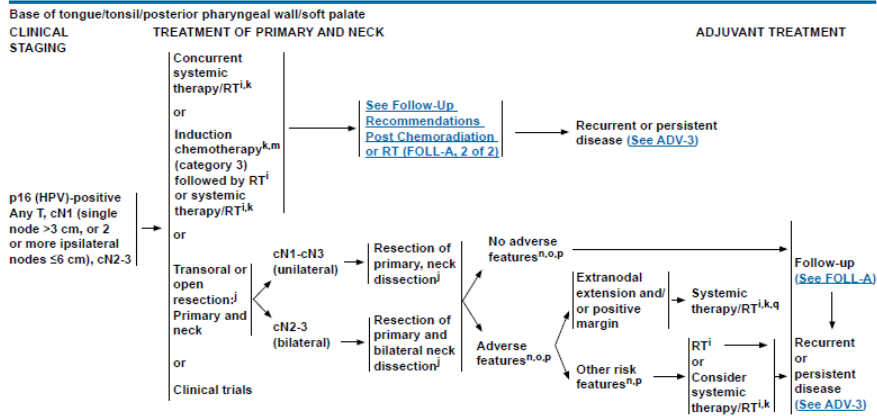


Figure 16 - Treatment of OPSCC HPV positive (p16-positive) [74].



^kSee Principles of Radiation Therapy (ORPH-A).

^lSee Principles of Surgery (SURG-A).

^mSee Principles of Systemic Therapy (CHEM-A).

ⁿSee Discussion on induction chemotherapy.

^oPathologic staging criteria differ from clinical staging criteria in HPV-mediated oropharyngeal cancer. For pathologic stage following resection, see AJCC 8th edition for appropriate staging criteria (ST-7).

^pIn the event of pathologic upstaging, continue to appropriate algorithm.

^qAdverse features: extranodal extension, positive margins, nodal disease in levels IV or V, perineural invasion, vascular embolism, lymphatic invasion (See Discussion). The definition of an adverse feature in the context of HPV+ disease is an area of active research. This includes the presence and extent of ENE, and the number of involved nodes.
^rThe recommendations for patients at high risk with extranodal extension + positive margins are based on randomized studies involving patients for whom the HPV status of their tumors was not specified.

Note: All recommendations are category 2A unless otherwise indicated.
Clinical Trials: NCCN believes that the best management of any patient with cancer is in a clinical trial. Participation in clinical trials is especially encouraged.

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ORPHPV-3

Figure 17 - Treatment of OPSCC HPV positive (p16-positive) [74].

2- Material and Methods

2.1 - Patient Eligibility

315 patients were diagnosed and/or treated in the period between 2018 till 2021 with OPSCC, which is dominated by TSCC/BOTSCC, but also other OPSCC, (including cancer of the uvula, the soft palate and the pharyngeal walls), at IPO-Porto.

Only the patients with histological diagnostic of OPSCC and p16-IHC were included in the analysis. The patients with any other histological types like lymphoma, adenocarcinoma and adenoid cystic carcinoma were excluded from the study.

35 patients diagnosed in other hospitals from the North of Portugal and referenced to IPO-Porto to treatment, with OPSCC histological diagnostic, but without p16-IHC, were excluded from the analysis. Therefore, only 280 patients fulfilled the inclusion criteria.

The diagnosis and TNM stage was established at a multidisciplinary conference.

The diagnosis was based on physical examination, computed tomography and/or magnetic resonance imaging scans and biopsy material and excised tumor material were subjected to pathological diagnostics.

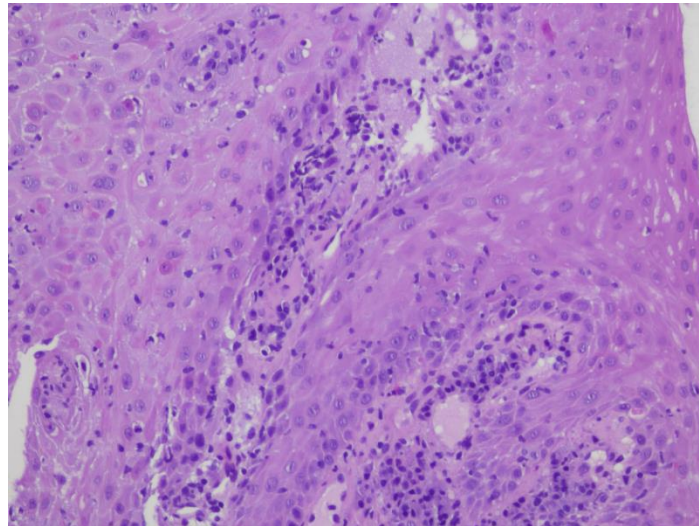


Figure 20 - HE 200x Conventional pattern squamous cell carcinoma. (Cortesy of the Doctor Manuel Jácome, anatomopathologist from IPO-PORTO).

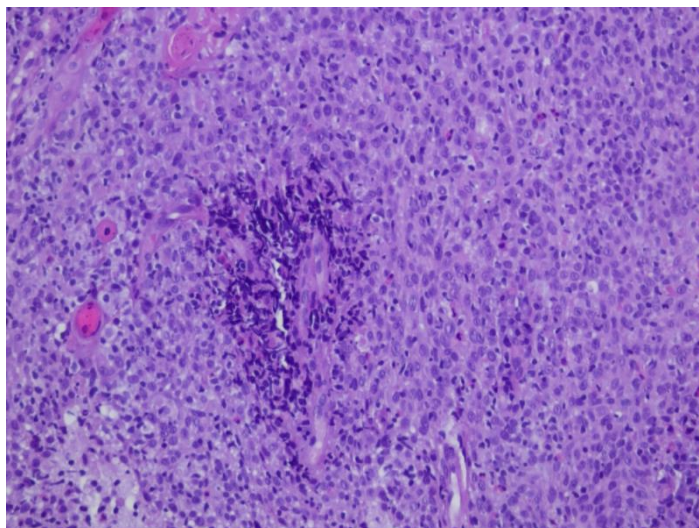


Figure 21 - HE 200x poorly differentiated squamous cell carcinoma with basaloid appearance. (Cortesy of the Doctor Manuel Jácome, anatomopathologist from IPO-PORTO).

2.2 – Methodology

In all cases (n=280) p16 expression was tested by immunohistochemistry using the monoclonal anti-body CINtec p16 Histology (Fig.22, 23). Were considered p16 overexpression (p16+) if >70% of the tumor cells being strong cytoplasmic and nuclear p16 positive.

In the cases where p16-IHC was inconclusive (if 50-70% of the tumor cells being strong cytoplasmic and nuclear p16 positive), was performed the search of the HPV-DNA in the tissue samples, by using PCR based methods.

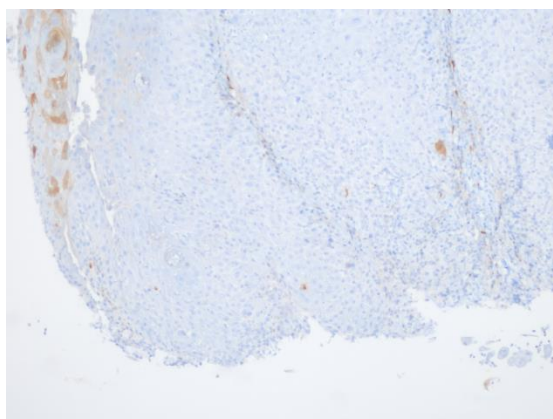


Figure 22 - IHC OPSCC p16-. (Cortesy of the Doctor Manuel Jácome, anatomopathologist from IPO-PORTO).

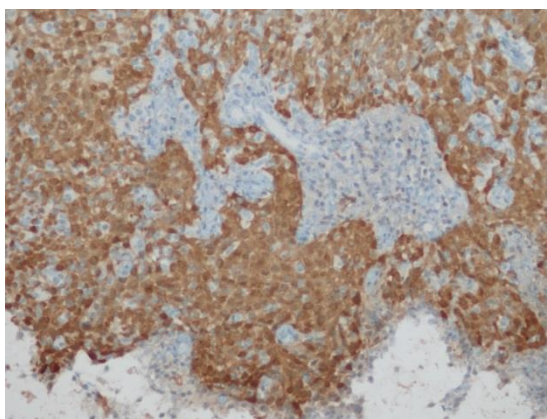


Figure 23 - IHC OPSCC p16+. (Cortesy of the Doctor Manuel Jácome, anatomopathologist from IPO-PORTO).

Patient case reports were analyzed, and age, gender, TNM-stage, treatment and survival were recorded.

Treatment was categorized as surgery, radiotherapy or chemoradiotherapy.

Patients were evaluated for treatment response 12 weeks after final treatment by a PET-scan and a follow-up every 3 months in the first 2 years was performed, and then every 6 months.

The follow-up period was calculated from the date of end the treatment of until the last appointment or death. The average follow-up was 24 months, ranging from a minimum 6 months to a maximum of 54 months.

The study was performed according to permissions from the IPO-Porto Ethical Review Board.

The outcome was analyzed as disease free survival (DFS) or overall survival (OS). DFS was defined as day of final treatment until day of any relapse. Patients never tumor-free were censored day 0, and patients dying without recurrence were censored at the time-point, when assessing DFS. OS was defined as day of final treatment until day of death irrespective of cause of death. Survival curves with DFS, and OS were calculated using the Kaplan–Meier method. Differences in survival were calculated using the log-rank test.

Only patients treated with curative intent, that completed their treatment, and with a minimum follow-up of 6 months were included in the survival analysis.

2.3 - Data Analysis

All statistical tests were performed using SPSS (SPSS Statistics for Mac, Version 25. Armonk, NY: IBM Corp. USA).

3- Results

3.1 - Clinical findings

The clinicopathological findings of the 280 cases with OPSCC are summarized in Table 10.

	Total no. (%)	HPV+ no. (%)	HPV- no. (%)
Age median	59	60	58
Gender			
Male	254 (90,7%)	40 (15,7%)	214 (84,3%)
Female	26 (9,3%)	17 (65,4%)	9 (34,6%)
Stage/TNM classification			
I/II	55 (19,6%)	41 (74,5%)	14 (25,5%)
III/IV	225 (80,4%)	16 (7,1%)	209 (92,9%)
T classification*			
Low-T-stage	80 (28,6%)	18 (22,5%)	62 (77,5%)
High-T-stage	197 (70,4%)	37 (18,8%)	160 (81,2%)
N classification			
No	54 (19,3%)	8 (14,8%)	46 (85,2%)
N+	226 (80,7%)	49 (21,7%)	177 (78,3%)
Status HPV per study period			
2018-2019	132 (47,1%)	22 (16,7%)	110 (83,3%)
2020-2021	148 (52,9%)	35 (23,6%)	113 (76,4%)

* To - 3 patients (1.1%)

Table 10 - Clinicopathological findings of 280 cases of OPSCC.

Most of the patients were male ($n = 254, 90.7\%$).

80 (28.6%) patients had low-T-stage (T1/T2) OPSCC tumors, and the others 197 (70.4%) had high-T-stage (T3/T4) OPSCC tumors. 3 (1.1%) cases were T0. 226 (80.7%) patients showed clinically positive lymph node metastasis (N+). According to the 8th UICC/AJCC TNM classification, 55 (19.6 %) patients were at a low clinical stage (I/II) and 225 (80.4%) were at high clinical stages (III/IV). 74.5% of the stage I/II tumors were HPV +, and only 25.5% were HPV-. Regarding patients with advanced stages III/IV, only 7.1% were HPV+ and 92.9% were HPV-.

22.5% of T1/T2 tumors were HPV + and 77.5% were HPV-. Comparatively, the local advanced tumors (T3/T4), 18.8% were HPV + and 81.2% were HPV -.

21.7% of N+ tumors were HPV+, while 78.3% were HPV-.

The prevalence of HPV+ tumors in the period of 2018-2019 was 16.7%. While in the period of 2020-2021 it was 23.6%.

For the initial therapy, all cases of stage I/II (55 cases) were treated by surgery alone or chemoradiotherapy/radiotherapy or surgery combined with RT after surgery.

Regarding the 225 cases of stage III/IV, 64 (28.4%) were treated by chemoradiation/radiation therapy in some cases after induction chemotherapy (cisplatin/docetaxel/5-fluorouracil). The other 161 (71.6%) cases of stage III/IV were treated by surgery combined with chemoradiotherapy. Chemoradiation therapy used cisplatin or cetuximab as concurrent drugs, and the radiation dose ranged from 30 to 70 Gy, with an average of 63.7 Gy.

In total, 53 (18.9 %) died due to their tumors.

52 (18.6%) patients were alive with their disease at the end of the follow-up period. 4 (1.8%) were alive with unknown status of disease. The remaining 171 (61.1 %) patients showed no evidence of disease at the end of the follow-up (Table 11).

p16 / Status at last observation date	
p16 / status	Total no. (%)
Positive	57
Alive with disease	11 (19,3%)
Died with evidence of disease	3 (5,3%)
Alive without evidence of disease	42 (73,7%)
Died without evidence of disease	1 (1,8%)
Negative	223
Alive with unknown status	4 (1,8%)
Alive with disease	41 (18,4%)
Died with evidence of disease	50 (22,4%)
Alive without evidence of disease	126 (56,5%)
Died without evidence of disease	2 (0,9%)

Table 11 - Status at last observation date

3.2- p16 immunohistochemistry and HPV infection

Immunohistochemically, p16 protein overexpression was present in 57 (20.4%) of the 280 cases (Table 12). In p16+ cases, strong and diffuse nuclear and cytoplasmic p16 staining was detected in the vast majority of the carcinoma cells. However in 4 (1.4 %) cases IHC was inconclusive for the positivity of the p16 and were positive for the presence of HPV DNA in the tissue samples by use of PCR-based methods and therefore were considered p16+.

The correlation between p16-IHC and clinicopathological variables is summarized in Table 12.

p16 status		
Resultado p16	No. of patients	% of patients
Positive	57	20,4%
Negative	223	79,6%
Total	280	100,0%

p16 by gender		
Result p16 / Gender	No. of patients	% of patients
Positive	57	
Female	17	29,8%
Male	40	70,2%
Negative	223	
Female	9	4,0%
Male	214	96,0%

p16 by Age Group		
Result p16 / Age Group	No. of patients	% of patients
Positive	57	
Under 40 years old	2	3,5%
41 - 50 years old	7	12,3%
51 - 60 years old	20	35,1%
61 - 70 years old	17	29,8%
71 - 80 years old	11	19,3%
Over 80 years old	0	0,0%
Negative	223	
Under 40 years old	1	0,4%
41 - 50 years old	42	18,8%
51 - 60 years old	85	38,1%
61 - 70 years old	68	30,5%
71 - 80 years old	22	9,9%
Over 80 years old	5	2,2%

Table 12 – Correlation between p16-IHC and clinicopathological variables.

p16+ was not significantly associated with lymph node metastasis ($p = 0.143$) and was not associated with a younger age ($p = 0.832$). In addition, low 8th UICC clinical stage was significantly associated with HPV infection ($p < 0.05$).

3.3- Evolution of the Prevalence of HPV-related OPSCC

The proportion of HPV-related carcinomas to total oropharyngeal carcinomas was compared chronologically (Table 10): from 2018 to 2019, 132 new cases and 22 HPV related cases, whereas 148 new cases and 35 HPV+ cases from 2020 to 2021, showing a considerable increase in the number of new cases (12.12%) and a significant increase in the prevalence rate of HPV-related carcinomas (59.09%).

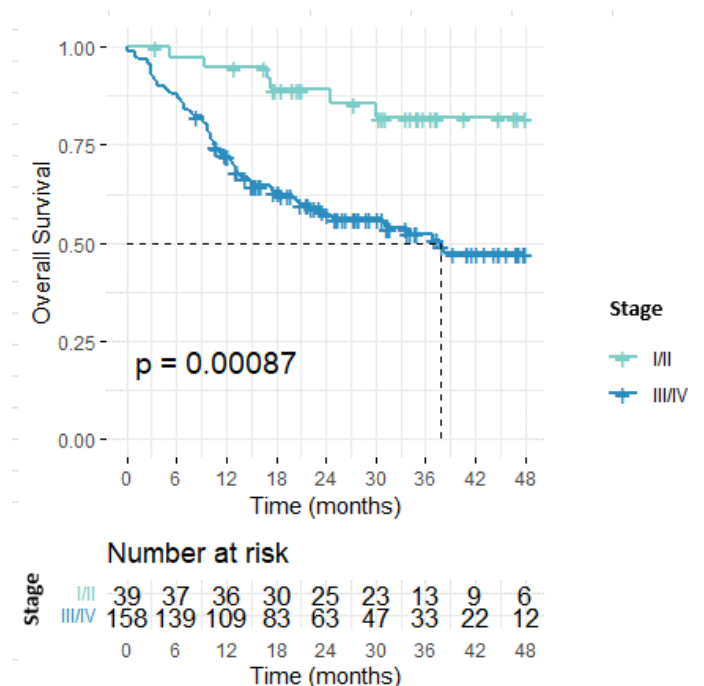
3.4- Prognostic analyses

Only 270 (96.4%) patients were included in the survival analysis.

The overall 2-years survival rate was 63.5% and was 89.2 %, in stage I-II and 57.2 % in stage III-IV (Fig. 24). These results indicate that the observed difference between stage I/II patients OS and stage III/IV patients OS is statistically significant ($p=0.00087$).

The 2-year disease-free survival (DFS) rate was 69.9%. For stage I-II, the DFS rate was 88.5%, while for stage III-IV, it was 65.4%. There was a statistically significant association between stage I-II and stage III-IV ($p=0.015$).

The HPV+ group had a significantly better prognosis than the HPV- group in terms of overall survival (OS) ($p=0.014$). However, there was no statistically significant difference between the two groups in terms of DFS ($p=0.45$) (Fig. 25).



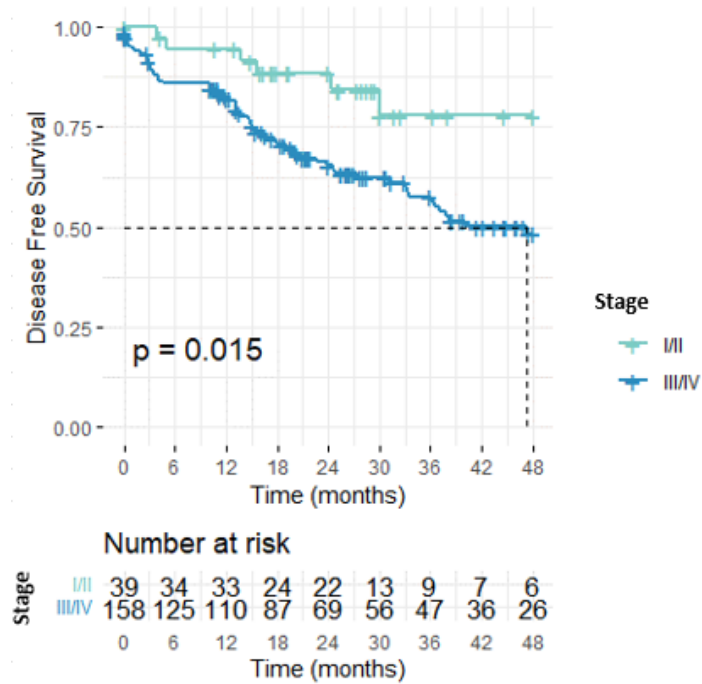
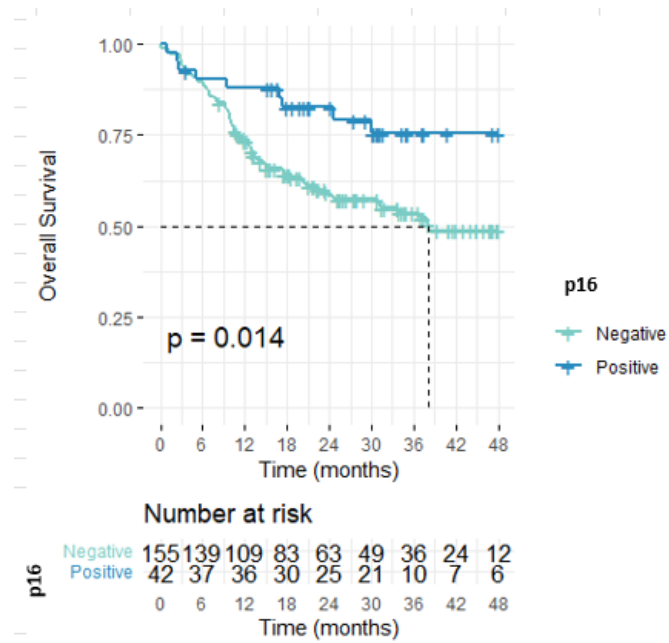


Figure 24 - The Kaplan–Meier analysis for the overall survival (OS) and Disease-free survival (DFS) by the 8th UICC clinical stage.



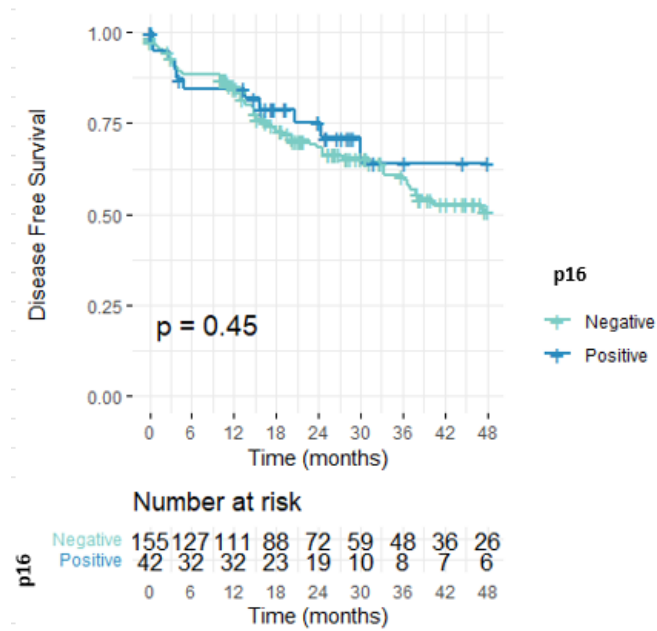


Figure 25 - The Kaplan–Meier analysis for the overall survival (OS) and Disease-free survival (DFS) by p16 expression.

Only patients on treatment with curative intent were considered for OS and DFS analysis (270 patients), but 3 patients were excluded because they did not finish treatment and 70 were lost to follow-up. Thus, only 197 patients were included in the analysis of overall survival and disease-free survival.

In our study, 96 (35.6%) of 270 patients treated with curative intent recurred. 82 of 213 (38.5 %) HPV– OPSCC cases recurred. In contrast, 14 of 57 (24.6 %) HPV+ OPSCC cases recurred.

3-5- Double cancers in HPV-related and non-related OPSCC

Of the 280 patients, 3 (1.1 %) had double cancers in the hypopharynx, including 2 of HPV+ OPSCC cases and 1 of HPV– OPSCC case.

3.6- Cases treated as primary unknown cancer at the first therapy

There were 3 (1.1 %) cases with unknown primary cancer at the first treatment, which turned out to be oropharyngeal cancer after surgery. Of these, 2 cases were HPV+, and 1 were HPV–.

4- Discussion

In many Western countries oropharyngeal cancer is increasing due to HR-HPV infection. However, there are few reports on the prevalence of the HPV-related cancers in Portugal, and none have examined the prevalence in HPV-related cancers using HPV-specific tests.

In the present study, we analyzed the total number of oropharyngeal cancers and the number of cases of HPV-related cancers diagnosed in the IPO-Porto in the period of 2018 to 2021 using HPV-IHC for HPV detection and so p16 protein overexpression has been used as a surrogate marker for HPV-related oropharyngeal carcinoma. Only when p16-IHC was inconclusive (if 50-70% of the tumor cells being strong cytoplasmic and nuclear p16 positive), was performed the search of the HPV-DNA in the tissue samples, by using PCR based methods.

In contrast to most European countries and the USA, only a minority of patients (20.4%) in the sample were p16+.

The significant risk factors for oral HPV infection include an increasing number of recent and lifetime oral sex, open mouth kissing, vaginal, and any sex partners, aged <18 years at the time of first oral sexual intercourse. We can speculate that the observed epidemiologic differences in OPSCC in the IPO study may reflect differences in sexual behaviors (type of sex, age at onset of sex, number of sexual partners) of the Portuguese population for 6-7 decades ago “less liberal” compared to other Western societies.

There has been controversy regarding the different techniques and biomarkers used to determine whether a tumor is related to HPV infection. Sustained and persistent high-risk HPV E6/E7 viral oncogene expression is essential for a HPV-driven malignant tumor [12]. The detection of HPV E6/E7 mRNA transcripts correlates with cellular genotoxic damage and gene expression changes that are the hallmarks of cancer. However, the detection of mRNA in the clinical setting is difficult and expensive [64].

Another approach is using p16 as a surrogate for HPV infection and could utilize the cheaper and more available immunohistochemistry stains. For prediction of outcome, the doubly positive p16/HPV DNA test had the best predictive ability.

Therefore, it has been proposed that the combination of p16 immunohistochemistry and the detection of HPV DNA by PCR is required [65].

Few studies have described discordant combinations of oropharyngeal cancer (ie, p16–/HPV+ or p16+/HPV–). There are robust evidence that p16 and HPV discordance exists in some patients, with a prevalence that varies by geographical region, and that

discordance between p16 and HPV biomarker status affects patient prognosis in terms of disease-free and overall survival.

Discordant p16–/HPV+ patients showed significantly worse recurrence rates, survival, and prognosis than did p16+/HPV+ patients, with similar outcomes to p16–/HPV– patients, regardless of smoking status, anatomical site, or HPV testing method. p16+/HPV– patients who smoke have a significantly worse survival than p16+/HPV+ patients, with outcomes that are similar to p16–/HPV– patients.

p16 and HPV discordant rates could also be related to the prevalence of other risk factors. For example, in a population of patients who smoked more, as the population of our study would be a greater probability that p16 is inactivated by mutation or promoter methylation. This may be one of the possible explanations for the low rate of p16+ in our sample. Therefore, we can speculate that eventually the prevalence of HPV-related OPSCC may be in reality superior than the prevalence calculated in the study.

The discrepancy between p16 positivity and HPV being most apparent in OPSCC arising outside the tonsils and base of tongue, such as e.g. other sites include the uvula/soft palate/pharyngeal wall. This may be another possible explanation for the low rate of p16+ in our study, since in a significant number of patients the tumor site is not de tonsil/base of the tongue.

The low representativeness of p16+ patients (20.4%) in IPO thereby introducing potential bias in our study in relation to the extensive published literature of the current epidemiological/clinical profile of OPSCC in the Western world.

The HPV+ group had a significantly better prognosis than de HPV – group in terms of overall survival (OS) and disease-free survival (DFS), and this is in line with the published studies.

Our study has some limitations, namely the low representativeness of p16+ patients in the total sample and, on the other hand, the retrospective nature of our study might have hampered the accurate characterization of some patient risk factors, such as tobacco and alcohol use.

Some findings indicate that classification of patients with oropharyngeal cancer based on p16-positive immunohistochemistry alone is likely to be insufficient in routine clinical practice.

On the other hand, one potential way to reduce the future burden of HPV-positive OPSCCs would be to prevent the initial infection in young men and women by vaccinating against HPV. Currently, Gardasil (targets HPV6, 11, 16, and 18) and Cervarix (targets

HPV16 and 18) represent the two available vaccines. Unfortunately, minimal information exists regarding the efficacy of these vaccines against OPSCC. However, a recent trial revealed a significant decrease in oral HPV infection in women receiving the vaccine versus control [66]. This trial, coupled with the fact that greater than 90% of HPV-positive OPSCCs are due to HPV16 and 18, suggests both vaccines could effectively prevent OPSCC.

5- Conclusion

The prevalence of HPV-related cancers in IPO-Porto is low, when compared to other studies focused on developed countries.

In this study, most of the patients with OPSCC were male. Only a minority were p16+. According to the 8th UICC/AJCC TNM classification, the majority of the patients were at high clinical stages (III/IV). The majority of the stage I/II tumors were HPV +, and only 25.5% were HPV-. Regarding patients with advanced stages III/IV, only 7.1% were HPV+ and 92.9% were HPV-.

The HPV-related OPSCC had a significantly better prognosis than de non-HPV – related group in turns of overall survival (OS) and disease- free survival (DFS).

The incidence of HPV-related cancers continue to increase in many developed countries and it is expected that the global trend will continue to increase in the coming decades and is expected that in Portugal the future incidence of HPV-related OPSCCs will increase and represent the majority of OPSCCs.

One potential way to reduce the future burden of HPV-positive OPSCCs would be to prevent the initial infection in young men and women by vaccinating against HPV (HR-HPV 16 and 18).

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