Universitat de Girona
Facultat de Medicina

INCIDENCE TRENDS AND SURVIVAL OF HUMAN PAPILLOMAVIRUS-RELATED OROPHARYNGEAL CANCER IN THE PROVINCE OF GIRONA (1994-2018)

A population-based study of oropharyngeal cancer in Southern Europe

END-OF-DEGREE THESIS

November 2021

AUTHOR: Elna de Ciurana Montiel

CLINICAL TUTOR: Jordi Rubió Casadevall

METHODOLOGICAL TUTOR: Rafael Marcos Gragera

Les pràctiques clíniques associades a aquest treball de final de grau van ser realitzades al Servei d'Oncologia del *Zealand University Hospital*, Dinamarca, a través d'un programa d'"Erasmus Plus for Traineeship" i gaudint de la "Beca Practicum 2021" concedida pel Col·legi Oficial de Metges de Girona.

Gràcies a tot el Grup d'Epidemiologia Descriptiva, Genètica i Prevenció del Càncer (GEDGPC) de l'Institut d'Investigació Biomèdica de Girona (IdIBGi), en especial a la Montserrat Puigdemont i a l'Arantza Sanvisens, per ajudar-me en la realització d'aquest projecte.

Gràcies a la Dra. Rosa Ortiz, del Servei d'Anatomia Patològica de l'Hospital Dr. Josep Trueta, per ajudar-me en la part histològica del treball.

Gràcies a la família i als/les amics/gues pel seu recolzament constant.

Thank you to the Oncology Department of the Naestved Hospital, Denmark, specially to Dr. Zaza Ujmajuridze and Dr. Julie Gehl, for giving me the opportunity to be a part of their team and acquire new skills and learning in oncology and patientcare.

Gràcies al Dr. Rafael Marcos, per haver-me guiat en el desenvolupament metodològic i per haver-me animat en tot el procés.

I, finalment, gràcies especialment al Dr. Jordi Rubió per haver-me endinsat en el món de l'epidemiologia del càncer, per la seva implicació, dedicació i suport durant totes les etapes d'aquest estudi i per fer-me retrobar l'entusiasme en la recerca de la medicina.

Girona, November 2021

CONTENTS

1. ABBREVIATIONS

APC: Annual Percent Change

$ASIR_E$: Age-Standardized to the European Standard Population Incidence Rate

$ASIR_W$: Age-Standardized to the WHO World Standard Population Incidence Rate

CEIm: Comitè d'Ètica d'Investigació amb Medicaments de Girona

CI: Confidence Interval

CR: Crude Rate

CRG: Cancer Registry of Girona

DAB: 3,3'-Diaminobenzidine

DCN: Death-Certificate Notification

DCO: Death-Certificate-Only

E5: Early Oncoprotein 5

E6: Early Oncoprotein 6

E7: Early Oncoprotein 7

EGFR: Epidermal Growth Factor Receptor

ENCR: European Network of Cancer Registries

FFPE: Formalin-Fixed Paraffin-Embedded

G1-phase: Growth 1 Phase

GEDGPC: Grup d'Epidemiologia Descriptiva, Genètica i Prevenció del Càncer

HNC: Head and Neck Cancer

HNSCC: Head and Neck Squamous Cell Carcinoma

HPV: Human Papillomavirus

HR HPV: High-Risk Human Papillomavirus

hTERT: Human Telomerase Reverse Transcriptase

IACR: International Association of Cancer Registries

IARC: International Agency of Research on Cancer

ICD-O-3: International Classification of Diseases for Oncology Edition 3

IDESCAT: Insitut d'Estadística de Catalunya

IdIBGi: Institut d'Investigació Biomèdica de Girona

IHC: Immunohistochemistry

IQR: Interquartile Range

ISH: In Situ Hybridization

M: Metastasis

N: Lymph Node

NOS: Not Otherwise Specified

OPC: Oropharyngeal Cancer

OPSCC: Oropharyngeal Squamous Cell Carcinoma

OS: Observed Survival

5-y OS: 5-year Observed Survival

p16: p16INK4a protein

PCR: Polymerase Chain Reaction

PD-1: Programmed Cell Death 1

pRb: Retinoblastoma Family Member proteins

REDECAN: Red Española de Registros del Cáncer

SCC: Squamous Cell Carcinomas

SD: Standard Deviation

S-phase: Synthesis Phase

SPN: Secondary Primary Neoplasm

T: Tumor

TIL: Tumor-Infiltrating Lymphocyte

UK: United Kingdom

US: United States of America

2. ABSTRACT

Background: A shift in etiology in oropharyngeal cancer (OPC) has caused a change in the epidemiological trends of head and neck cancer (HNC): whilst overall HNC incidence has decreased, OPC incidence has sharply increased, giving rise to an epidemic of a new subtype of OPC related to human papillomavirus (HPV), which has been attributed better prognosis and distinct clinicopathological characteristics. The emergence of this entity has predominantly been studied in regions of high burden of oral HPV infection, such as North America and Northern/Western Europe, but little is known about the onset of HPV-related OPC in Southern Europe.

Main objective: To determine the trends in the incidence of OPC, overall and in relation with p16 expression, between 1994 and 2018 in the population of the province of Girona.

Design: A population-based retrospective observational cohort study conducted between October 2020 and November 2021.

Methods: 509 cases of patients diagnosed with primary OPC from 1994 to 2018 were identified from the Cancer Registry of Girona (CRG) database. Incidence trends for overall OPC were calculated subsequently. All identified OPC cases from calendar periods 1998-1999, 2003-2005, 2009-2011, and 2016-2018, 227 cases in total, were selected for a p16 expression-based analysis to assess trends in incidence and survival of HPV-related OPC. Tumor samples of these cases were identified and determination of immunohistochemical expression of p16INK4a protein, which served as a surrogate biomarker for HPV involvement, was performed.

Results: A significant increase in incidence (Annual Percentage Change (APC): 4.1) was observed between 2001 and 2018 in overall OPC. In the p16 expression-based analysis, a sharp, significant increase in incidence was observed as well in HPV-related OPC (APC: 12.8) from 1998 to 2018, whilst the incidence in HPV-unrelated OPC in this same period experienced no significant changes. Significant differences were found between HPV-related and HPV-unrelated OPC regarding 5-year observed survival, which was determined to be higher in HPV-related OPC (64.8% versus 43.4%), and distribution among sexes, but none regarding age at diagnosis.

Conclusions: There has been a significant increase in the incidence of overall OPC from 2001 to 2018 and in that of HPV-related OPC from 1998 to 2018 in the province of Girona.

Key words: Oropharyngeal cancer, human papillomavirus, p16 expression, head and neck cancer, trends, incidence, survival, sexual behavior, tobacco use.

3. BACKGROUND

3.1. GENERAL ASPECTS OF HEAD AND NECK CANCER

Head and neck cancer (HNC) comprises a heterogeneous group of cancers located between the base of the skull and the clavicles, which include those tumors that arise from the sinonasal tract, the lips and oral cavity, the oropharynx, the larynx, the hypopharynx, and the salivary glands (1). They all together represent the sixth-seventh most common type of cancer worldwide[1] (2,3), but overall constitute 5% of all tumors in humans (4). Even though most of these entities are histologically similar, being 90% of head and neck cancers squamous cell carcinomas (SCC) (2), their epidemiological and clinical features vary considerably depending on the anatomical tumor site as well as the geographical region.

Therefore, the classification of HNC according to tumor topographical sites is of relevance when studying these malignancies, although it is conditioned by the lack of concordance between current standardized classifications and the clinical and epidemiological behavior of these neoplasms, a trait that depicts the heterogeneity within this cancer cluster. Recently, traditionally accepted classification approaches have been modified, but when studying HNC data there is still a limitation derived from this issue that needs to be considered. On the one hand, HNC may be classified as so according to the International Classification of Diseases for Oncology edition 3 (ICD-O-3) coding system, which may be consulted in *Annex 1*, into the following groups: malignant neoplasm of the **lip**, malignant neoplasm of the **base of the tongue**, malignant neoplasm of the **tongue** -excluding the base-, malignant neoplasm of the **gum**, of the **floor of the mouth** and of **other or not otherwise specified (NOS) mouth locations**, malignant neoplasm of the **salivary glands**, malignant neoplasm of the **tonsils** and of the **oropharynx**, malignant neoplasm of the **nasopharynx**, malignant neoplasm of the **pyriform sinus** and of the **hypopharynx**, malignant neoplasm of the **other and ill-defined sites** in lip, oral cavity and pharynx, malignant neoplasm of the **nasal cavity**, malignant neoplasm of the **accessory sinuses**, and

[1] Considering cancer in both males and females together.

malignant neoplasm of the **larynx** (5). On the other hand, these subsets may be subsequently grouped into larger categories of cancer that share similar clinical characteristics as: cancer of the **lips**, cancer of the **oral cavity**, cancer of the **salivary glands**, **nasopharyngeal** cancer, **oropharyngeal** cancer (OPC)[2], **hypopharyngeal** cancer, cancer of the **nose**, **ear and paranasal sinuses**, and cancer of the **larynx**. The ICD-O-3 codes corresponding to each of these categories may be reviewed in *Table 3* in *Section 7.3*.

The etiology of head and neck cancer is multifactorial, a feature which directly contributes to the differences between the entities within the HNC group. Until the past decade, **alcohol** and **tobacco** were entitled as the principal etiological factors; historically, about 90% of patients with HNC had a history of tobacco use (2). The risk that this tobacco use confers in HNC is synergistically increased by alcohol overconsumption, yet alcohol consumption by itself independently contributes to HNC as well (2). However, in recent times, the decrease of smoking rates in developed countries due to public health efforts has resulted in the diminishment of tobacco related HNC. On a separate note, **Human Papillomavirus (HPV)**, a sexually transmitted infection which is recognized as one of the major causes of various infection-related cancers (6), has been rising in importance as the cause of a subset of HNCs that specially occur in the oropharynx -arising from lymphoid tissue located in the palatine tonsils and the base of the tongue-, and the oral cavity and larynx to a weaker extent (2). Despite most of HPV infections resolving spontaneously likewise not progressing to cancer, it has been proven that persistent infection with high-risk types of HPV (HR HPV) cause carcinogenic lesions (7). Aside from these two etiologies, exposure to other factors has been related to the development of HNC, being some of the most important: Epstein-Barr virus as a major etiology for nasopharyngeal cancer, betel quid and poor buccal hygiene for oral cavity cancer, occupational exposures like wood dust and asbestos for cancer of the nasopharynx, the paranasal sinuses and the nasal cavity, and radiation exposure for salivary glands cancer, among others (2).

[2] The oropharynx is limited by the anterior faucial pillars, the macroscopic taste buds, the soft palate, and the vallecula. Oropharyngeal cancer thereby includes those malignancies in the posterior third of the tongue or tongue base and the lingual tonsils, the tonsils and tonsillar pillars, the soft palate and the uvula, the vallecula, and the posterior wall of the pharynx in contact with the oral cavity (1).

The estimated annual burdens of head and neck cancer vary notably due to limited inconsistencies in categorization of topographical sites and differences among geographical regions, sex[3], and age groups. Therefore, these factors are taken into consideration when reviewing the epidemiological burden and trends in HNC. Data from the International Agency of Research on Cancer (IARC) from the GLOBOCAN 2020 statistical estimations report that, inside of HNC, oral cavity and lip cancer are the most common worldwide, followed by cancer of the larynx, nasopharynx, oropharynx, hypopharynx, and salivary glands, by order of incidence and mortality rates (8). Across Europe, the incidence of cancer at distinct HNC sites varies in the following manner: cancer of the oral cavity is predominant in the Nordic countries, while oropharyngeal cancer is most common in Western and Central countries (France, Germany, Switzerland, Austria, Slovenia, and Slovakia), and laryngeal cancer represents the main HNC in the remaining countries in Southern Europe (Bulgaria, Poland, Italy, Spain, Malta) (9). Looking into the prevalence and incidence rates for HNC in Spain, some differences can be noticed as well, compared to the worldwide trends. For instance, the most frequent location of HNC is the larynx, followed by oropharynx, oral cavity, and nasopharynx, in order of frequency (4). Specially in the region of Girona, in North-Eastern Spain, the HNC burden is not as substantial as it is in other geographical levels: in 2008-2012, the only HNC that was among the top 20 incident cancers was larynx cancer, and only in the male subgroup (10).

[3] Most of the literature that study HPV-related OPC take *gender* as a synonym for *sex* and use the term "women/females" for those people whose reproductive organs include ovaries, vagina, and uterus, and "men/males" for those with penis and testis. It is important to notice that the term *gender* should be considered with reference to social and cultural differences rather than biological ones (in which *sex* could be used, referring to the physical differences regarding reproductive organs, or *chromosomal sex*, defined as sexual chromosomes being XX or XY; in modern days, these do not always match). When studying HPV in OPC, both social/cultural and biological differences take part in the pathogenesis, thus *sex* and *gender* are involved. In this thesis, the words "male" and "female" have been used to define both gender and sex together, but the bias and lack of inclusivity in this terminology needs to be considered. In addition, the word "sex" is used for all definitions regarding disparities between "males" and "females". However, it needs to be considered that when these disparities are discussed according to risk behaviors, influence relies on *gender* differences, whilst when they are discussed according to inherent biology, it would be a matter of divergence between *sexes.*

3.2. CHANGE IN EPIDEMIOLOGICAL TRENDS AND HPV-RELATED OROPHARYNGEAL CANCER

Trends in the epidemiological burden of HNC have changed through the past decades. In the mid 20th-century, increasing numbers in the incidence rates of HNC were observed, as tobacco use prevalence peaked in-between the 1940 and 1960 in developed countries. Later, around the 1990s, incidence rates for overall HNC began to decline in concordance with the diminishment of cigarette consumption as a result of public health efforts (2). Nevertheless, the expected decrease for oropharyngeal cancer was not observed, but a significant increase otherwise from 1983 to 2002, predominantly in economically developed countries and at younger ages (<60 years) (11). Among men, OPC incidence significantly increased in the United States, Australia, Canada, Japan, and Slovakia, despite decreasing incidence of oral cavity carcinoma and lung cancer. In contrast, among women, in all countries with increasing OPC incidence there was a concomitant increase in incidence of oral cavity carcinoma and lung cancer (11). In recent years, the incidence of oropharyngeal squamous cell carcinoma has continued to increase in contrast to other subsites within the HNC region (12,13).

These changes in epidemiological trends have been attributed to a shift in HNC etiology, in which tobacco and alcohol use have been declining in importance at the expense of increasing oral HPV infection. Studies indicate that the most likely explanation for the origin of this change is a sexually acquired oral HPV infection that persists and evolves into a neoplastic lesion (14). Consequently, a different profile for oropharyngeal squamous cell carcinomas (OPSCCs), related to HPV, has been described, with a divergent epidemiological, molecular, and clinical-anatomopathological outline, out-setting new options of prevention and therapeutic approaches.

The epidemiology of the new HPV-related OPSCC subset has been reported differently among distinct geographical regions, as well as among age groups and sexes, outlining the characteristically high heterogeneity of the cancers in the head and neck area. In a worldwide level, 30% of the new OPC cases reported in 2012 were attributable to HPV.

These HPV-related OPSCC cases studied in this same year had a distribution that differed by sex, with 80% of the cases being in males and only a 20% in females, as well as by age group, finding only 20% of the cases below 50 year-olds, 20% above 70 year-olds, and 60% centered in-between 50-69 year-olds (15,16). In 2018, the incidence of OPC rose, but the proportion of cases attributable to HPV was still 30% and distribution by sex was still the same (17). Noticing this distribution, with higher substantial burden in males aged 50-69 years-old, the new subset of OPC was attributed initially to young (middle-aged) males (11), but this portrayal is recently changing as the entity is studied, as discussed further down.

Evidence centered into specific regions shows more noticeable incidence and mortality rates of overall OPC in countries from Eastern, Western and Northern Europe, North America, Oceania, and the Caribbean, compared to those in Southern Europe, Asia, South America, and Africa (18). More specifically, the number of HNC cases attributable to HPV (mainly OPCs) ranges greatly across continents in concordance, as it is depicted in *Annex 2*: the HPV-attributable fractions of HNC in North America (51%), Northern-Western Europe (42%), Eastern Europe (50%), Japan (46%), South Korea (60%) and Australia (41%), highly differ from those in Southern Europe (23%), China (23%), India (22%) and elsewhere (13%) (16). Many studies have reported the prevalence of HPV among cases of OPC crosswise in the world. The most relevant ones are summarized in *Annex 3.* Hence, it is concluded that contrary to other HPV-related cancer sites (cervix uteri, vagina, vulva, penis, and anus), HPV-related OPSCC shows higher burden in more economically developed regions compared to less developed regions, as portrayed in *Figure 1*. These findings likely reflect geographic differences in sexual behaviors relevant for oral HPV-infection as well as in traditional risk factors non-related to HPV like tobacco use and alcohol overconsumption, which are discussed later (9,11). Many countries from these mentioned regions, in addition to Africa and South America, lack of consistent evidence. Southern Europe is an example. Regarding prevalence of HPV in OPC in Southern Europe, few data have been presented. An international cross-sectional study identified fractions of HPV in OPSCC in Southern Europe to be around 7.6-9.4% (19). Two recent Spanish studies have backed up this evidence, locating these percentages at 6.1% (20) and 9.7% (21), but an Italian study has reported

them to be closer to 32.3% (22). As for the phenomenon on increasing incidence of overall OPC, pooled data from population-based Spanish cancer registries revealed in 2011 a reduction in the incidence of oral cavity cancer and an increase in overall oropharyngeal cancer (not distinguishing between HPV-related or HPV-unrelated), which was only significantly evident in males (23).

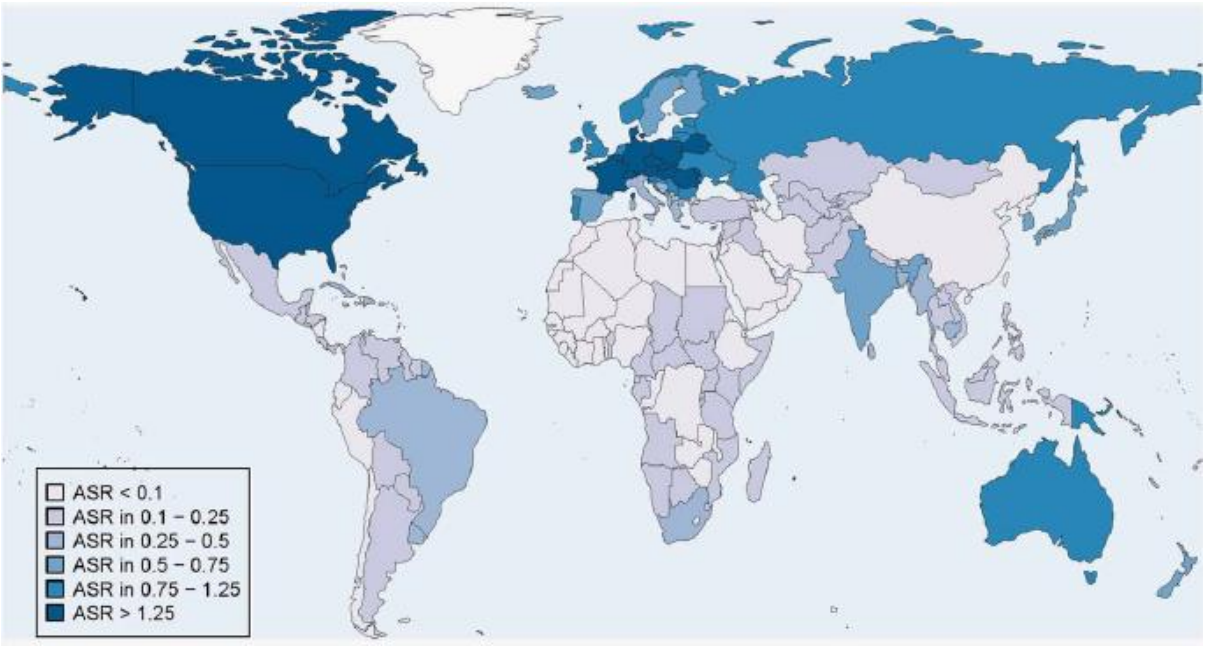


Figure 1. Age-standardized to the world population incidence rates (ASR), per 100.000 persons/year, of head and neck cancer cases (oropharynx, oral cavity, and larynx) attributable to HPV in 2012, both sexes (15).

3.3. MOLECULAR BIOLOGY AND NATURAL HISTORY OF HPV

3.3.1. STRUCTURAL CHARACTERISTICS OF HPV

HPVs are a type of non-encapsulated viruses characterized for being epitheliotropic. They infect mucosal and cutaneous epithelia, in which they induce cellular proliferation, a feature that etiologically links them to SCCs, as those particularly originate in the pluristratified squamous epithelium. In modern days, there are more than 200 genotypes of papillomaviridae identified (6), which are distinguished by their potential to induce oncogenic malignant transformation. In that matter, 12 types are considered high-risk (HR HPV): 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 (24). In OPSCC, the most involved genotype is **HPV16**, with a predominance of approximately 90-95%, a percentage that contrasts with that of HPV-related non-oropharyngeal head and neck SCCs (73%) and that of cervical cancer (61%). Other genotypes, like HPV33, HPV35, and HPV58 are detected in lower frequencies representing less than 10% of OPSCC, while HPV18 accounts for only 3% of them (3,25,26).

HPVs contain a single molecule of circular double-stranded DNA which is distinctively arranged in three main regions, consisting of:

i. an upstream regulatory region, which possesses transcription factor-binding sites and controls gene expression.
ii. an early region, which encodes for six genes (E1, E2, E4, E5, E6, and E7) that contribute to multiple functions such as viral replication and cell transformation.
iii. a late region, which encodes for the L1 and L2 capsid proteins that self-assemble to configure the virion (27).

Based on the cervical model, HPV's infectious cycle is well associated with the differentiation process of **keratinocytes**. In that matter, different viral genes are expressed in each state of keratinocyte differentiation, in concordance with their main functions, as it is illustrated in *Figure 2*. E1, E2, L1 and L2 are common proteins among all HPV genotypes that allow the virus to fulfill their life cycle. However, proteins E5, E6 and E7 are the ones found in HR-HPV serotypes that permit the virus to carry out carcinogenic action.

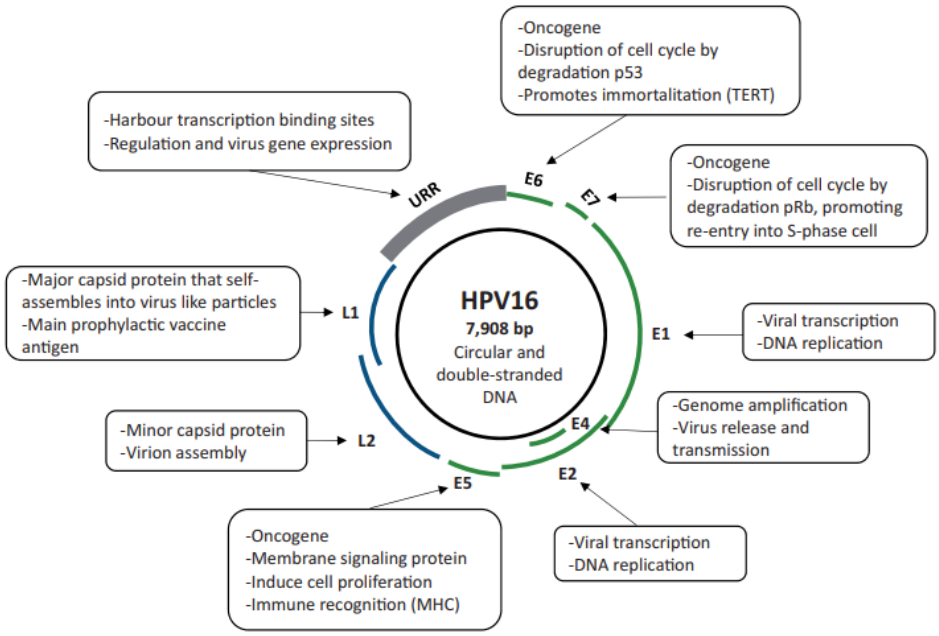


Figure 2. Function of HPV main genes. HPV genome, separated in three main regions: the upstream regulatory region (URR), an early region encoding six genes (E1-E7), and a late region encoding for the L1 and L2 capsid proteins (7).

3.3.2. HPV INFECTION: NATURAL HISTORY AND PREVALENCE

HPV infections are mainly sexually transmitted through direct skin or mucosa contact and represent the most common sexually transmitted infection worldwide. Most of these HPV infections (90%) clear spontaneously within two years, although this time depends on the HPV type and the immune status of the host. Hence, it is only in a minority of cases, when the virus evades immune detection and clearance, establishing a persistent infection, that it may cause clinical lesions (benign or malign) (7). The model for development of SCC involves the exposure to carcinogens (such as HPV infection) over time, leading to progressive genetic and epigenetic changes that accumulate and develop premalignant lesions, that may eventually progress to malignant lesions (3,25), as *Figure 3* portrays. However, to date, no oropharyngeal precancerous lesions have been described. Even so, it is believed that persistent infections may progress to invasive cancer even within 10 years from initial infection, a much shorter period than that in cervical cancer (7).

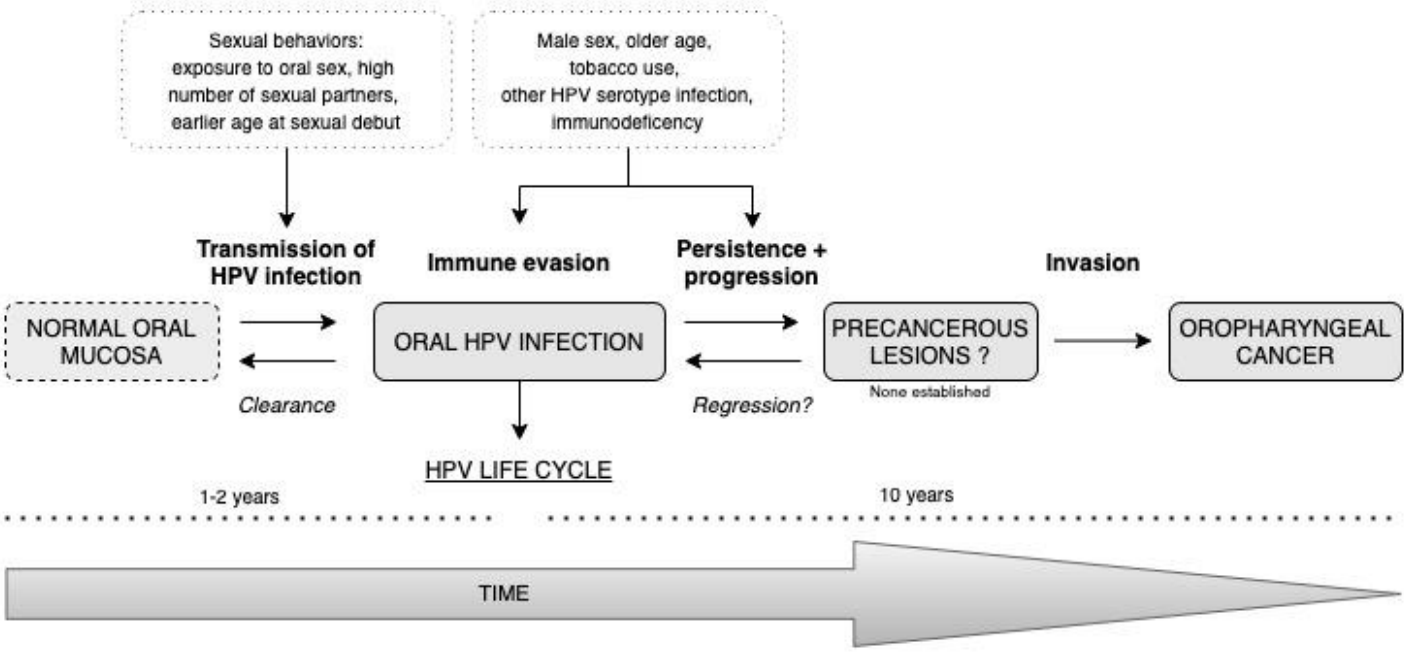


Figure 3. Natural history of Human Papillomavirus oral infection and its carcinogenesis. Normal oral mucosa is exposed to HPV, and when infection persists, it may lead to development of HPV-related precancerous lesions, which can then progress to invasive carcinoma. *HPV: human papillomavirus. Adapted from (7)*.

HPV-attribution to cancer is becoming well established, but in regard to understanding its epidemiological features and developing efficacious interventions to prevent HPV-related cancer it is essential to understand the patterns of HPV infection as well. Differences in this matter have been studied by anatomic site and by sex, but they can also be observed by age and geographical region.

On the one hand, lower prevalence of HPV infection is observed in the oral region in comparison to the anogenital region (28). Some explanations for this are that (i) HPV infection has a lower viral load in the oral cavity, (ii) transmission to the oral cavity on exposure is harder because of differences in local mucosal immunity, permissiveness of cells, epithelial resistance to microtrauma, and other properties of the oral cavity such as salivary flow, and (iii) HPV infection is cleared at a greater rate in the oral cavity (29). Overall, among healthy individuals, the absolute prevalence of oral HPV (any genotype) has been reported as 7-7.5% (30,31), but a recent metanalysis estimated the global oral HPV

prevalence to be 4.9% (32). The oral HPV16 prevalence has been observed as a much lower proportion, being 1% globally (28,30–32)**.** These global prevalences vary widely by geographical region, concordantly with those of HPV-related OPSCC. For instance, in Europe, overall oral HPV prevalence may range from 0 to 18%, and prevalence of all HR HPV types, from 0 to 14.5% (9), following a particular geographical pattern finding higher prevalence of oral HPV-infection in Northern Europe in comparison to Southern Europe. This pattern is outlined in *Annex 4*, which summarizes the most relevant studies of HPV oral infection in healthy population carried out to date in Europe, even though data are scarce, do not discriminate prevalence of HR HPV, and concern only regions from North and South Europe. Regarding the incidence rates for oral HPV infection, there is little evidence other than data worldwide and from non-European regions. A meta-regression analysis estimated the 12-month cumulative incidence to be 4.8% (31). The largest prospective study conducted to date reported it was 5.6 per 1.000 person-months, but it was in a cohort of only males who have sex with females (33); in concordance, another study conducted in a cohort of young adults reported it to be 5.7 per 1.000 person-months (29).

On the other hand, disparities by sex are based on both divergence in cultural and social behaviors and inherent biology differences between males and females. When the overall prevalence of oral-HPV is stratified by sex, there is a clear distinction of higher proportions in males compared to females, with a prevalence of 10.1% and 3.6% respectively (30). The reason why oral HPV infection has considerably higher prevalence in males than in females is not certainly known, but current hypotheses postulate that it may be because (i) males have more sexual partners and lifetime exposure to oral sex, therefore having more opportunities for HPV exposure, (ii) transmission of infection is more efficient when performing oral sex on infected female genitals due to mucosal surface in comparison to keratinized epithelium of penis, and because (iii) males may have some level of systemic immunity from possible cervical HPV infection or vaccination (28). Females demonstrate a higher HPV-16 seroprevalence than males (despite higher prevalence of genital and oral HPV DNA prevalence in males), and the incidence of transmission from females to males has been observed to be higher than the incidence from males to females, something that

suggests that epithelial cells of penile skin are more resistant to HPV infection than cervical epithelium (28). Overall, HPV causes oral cancer in both males and females. Nonetheless, the variation of oral-HPV infection prevalence may explain the differences of OPC incidence rates observed regarding sex. The increase of OPC incidence contributes to the diminishment of gender disparities in the HPV-related cancer burden, particularly in countries that have an effective prevention of cervical cancer. Therefore, enhanced understanding of HPV infection dynamics is key to promulgating more efficient strategies for prevention of HPV-related cancer in both sexes (28).

As it is established, HPV-related oropharyngeal cancer arises in specific topographical sites of the oropharynx, in the mucosa of the **tongue base and palatine tonsils**. In these sites, the mucosa is invaginated and forms crypts that are bordered with the reticulated epithelium and infiltrated by immune cells, as *Figure 4* depicts. It is within these crypts that HPV-related oropharyngeal tumors consistently emerge, contrary to HPV-unrelated malignancies, which mainly arise in the epithelium lining the tonsil surface. Unlike this superficial stratified non-keratinizing epithelium, the epithelium of the crypt lacks continuous basal lamina to separate it from the underlying lymphoid stroma. On the one hand, this allows intercellular spaces to be commonly filled with lymphoid cells, a feature that distinguishes HPV-related OPC, since favorable clinical outcome has been linked to a high frequency of tumor-infiltrating lymphocytes (TILs). On the other hand, this disrupted nature of lymphoepithelium is thought to enable the virus to readily gain access to exposed basal keratinocytes, making them the natural target of HPV infections (26).

HPV infects with predilection the lymphoid tissue in the oropharyngeal region, due to the microanatomy but other contributing factors as well, like biomarkers such as cytokeratin 7, which is characteristically expressed in the tonsil crypt and has been associated with higher vulnerability to HPV16 infection and transformation. The nature of these target cells is important as it is part of the explanation why epithelium at the oropharynx has different cancer development features compared to other anatomical sites. The most particular is that, as opposed to other HPV-infection sites, HPV-16 accounts for a

far greater proportion of OPSCC rather than other HPV genotypes. A given explanation is the fact that the immune system is much more active in the oropharynx than in the anogenital tract, a property likewise related to the microanatomy of the site. Therein, biological and epidemiological studies explain that HPV16 is more efficient at evading the host immune response, therefore being more likely to establish a persistent infection in the oropharynx compared to other serotypes (26).

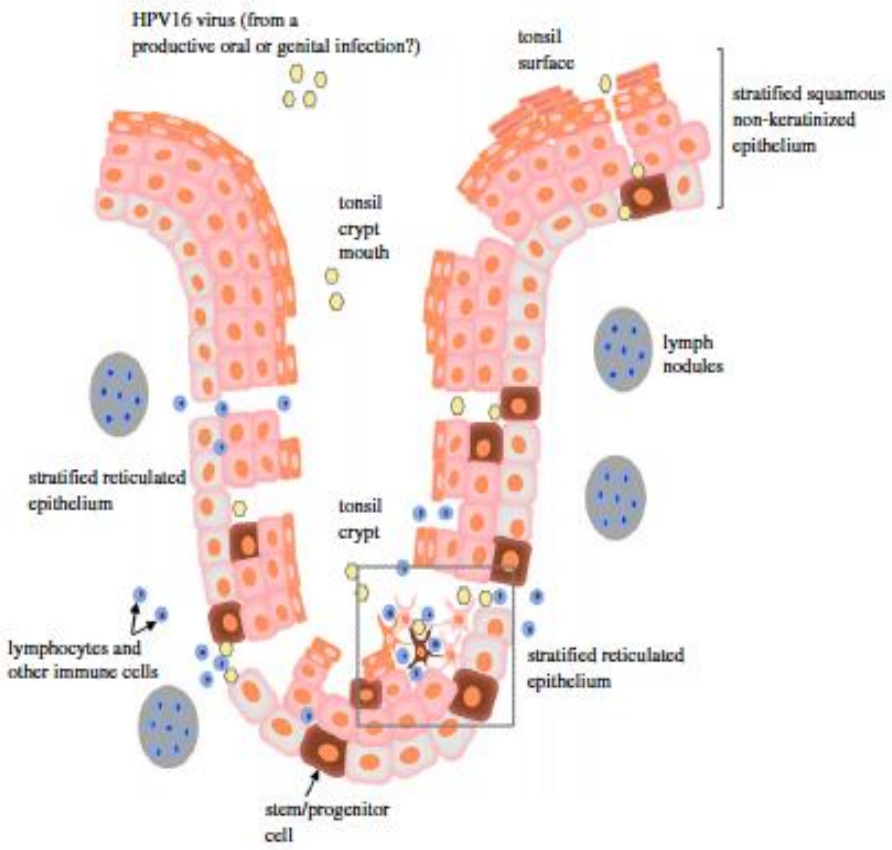


Figure 4. The tonsil epithelium. A schematic of the tonsil highlighting the difference in the epithelia lining the tonsil surface (stratified squamous non-keratinized epithelium) and the crypt (stratified reticulated). The boxed region depicts the typical keratinocytes and epithelium infiltration by lymphocytes (26).

3.3.3. MOLECULAR MECHANISMS OF HPV CARCINOGENESIS

It is currently unknown if HPV establishes a productive or abortive infection in the basal keratinocytes of the tonsil crypt, or if viral infection progresses to cancer via a neoplastic phase such as in cervical HPV infection. Unlike what it was firstly believed, HPV DNA is more commonly found unintegrated in oropharyngeal carcinomas in contrast with those that emerge in the cervix where chromosomal integration of HPV genomes is a common feature. Several forms of HPV DNA have been proposed to be present in OPSCC: episomal HPV DNA, integrated HPV DNA and HPV-human hybrid DNA. All in all, it is hypothesized that HPV-tonsil cancer may be either episome-driven or integrant-driven, finding a worse clinical outcome in the last case (26). These characteristics confer an important line when identifying future prognostic markers.

All things considered, factors allowing episomal HPV infection to transform into DNA integration or into cancerous lesions directly remain poorly understood. Theoretically, it is accepted that once immune evasion is established, integration of HPV DNA into cellular genome of basal keratinocytes allows the viral genome to be replicated in synchrony with cellular DNA (3,34), but transcription of E6 and E7 oncogenic proteins may occur when the virus is episomal as well.

As keratinocytes divide, daughter cells migrate upwards into the epithelium differentiation process, undergoing changes in lipid biochemistry, protein specializations and fusion into cornified sheets as they stop replicating. In HPV-infected cells, E6 and E7 proteins deregulate cell cycle checkpoint mechanisms, leading cells to enter synthesis-phase (S-phase) in an uncontrolled proliferation manner. Hence, viral genome amplification is allowed in cells that in normal conditions would have exited the cell cycle. Subsequently, cellular differentiation is followed as the expression of E6 and E7 is replaced by expression of E1, E2, E4 and E5. Afterwards, L1 and L2 proteins lead encapsulation of newly synthesized viral genomes and virions are yielded at the uppermost layers of the epithelium. Finally, viral release proceeds without cell lysis (3,27).

During this process, E5, E6 and E7 oncoproteins are the center of interest, as they hijack cell control mechanisms to induce carcinogenesis:

i. **Early oncoprotein 7 (E7)** binds to retinoblastoma family member proteins (pRb) and promotes their degradation, resulting in the release and activation of E2F transcription factor family, which induces an unscheduled re-entry into the S-phase of the cell cycle along with activation of viral replication (27). The cyclin-dependent kinase inhibitor p16INK4a (p16) prevents progression from growth 1-phase (G1-phase) to S-phase by inhibiting other cyclin-dependent kinases, which at their turn phosphorylate pRb. In normal conditions, pRb regulates p16 through negative feedback (35). Consequently, because of pRb degradation, p16 is overexpressed as it is released from the inhibitory activity of the pRb/E2F complex. This particularly takes place and is commonly detected in HPV-related tumors, which makes p16INK4a overexpression an acceptable surrogate marker for transcriptionally active HPV in oropharyngeal carcinogenesis (3).

ii. **Early oncoprotein 6 (E6)** is responsible for the inhibition of apoptosis in response to unscheduled S-phase entry, through several mechanisms: ubiquitination and proteasomal degradation of p53 and transcriptional activation by the human telomerase reverse transcriptase (hTERT) (27). In normal conditions, p53 controls the expression of genes that regulate cell cycle, DNA repair and apoptosis, triggering cell cycle arrest or cell death in case of DNA damage. E6 oncoprotein forms a complex with other proteins that culminate in the degradation of p53, and therefore loss of tumor suppression. In addition, E6 activates transcription of hTERT, leading to increased action of the telomerase complex and thereby unlimited proliferation (3,34).

iii. **Early oncoprotein 5 (E5)** promotes hyperproliferation, prevents apoptosis of infected cells and is likely to facilitate malignant progression (27). It is not always necessarily expressed, but when present, it influences immune recognition, contributing to immune evasion (34).

Summing up, as shown in *Figure 5*, aberrant proliferation is induced by E7, and facilitated by suppression of apoptosis by E6 mechanisms, with additional functions of both proteins to induce genomic instability by multiple mechanisms that lead to chromosomal mutations. A chronic HPV infection results in an expanded proliferation activity with depletion of quality control mechanisms, which leads to an accumulation of mutations in the cell predisposing to the development of HPV-associated cancers (27) additionally to genetic or epigenetic alterations (7). In that matter, some other factors have a role in adding genomic instability, like genetic polymorphisms and tobacco exposure (3). Some of these somatic genomic alterations in HPV-related OPSCC have been identified and are being studied to date, constituting potential therapeutic targets (7). Overall, immune response plays a key role in HPV carcinogenesis: if HPV-infected cells are rapidly eliminated by the immune system during early infection, despite the transforming properties of E6 and E7, there is not sufficient time to accumulate DNA damage and chromosomal abnormalities.

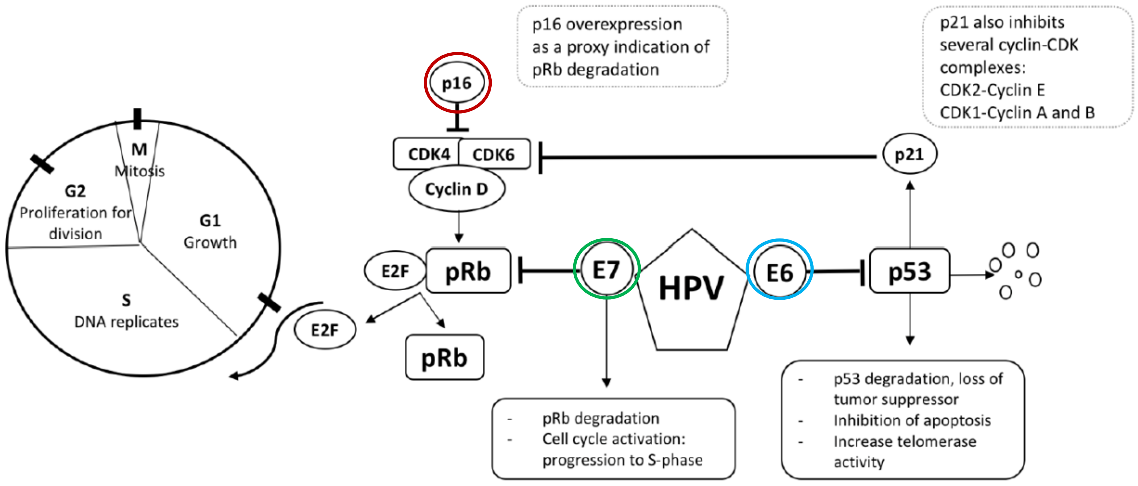


Figure 5: High risk HPV E6 and E7 oncoproteins and their role in cell cycle activation and loss of p53 tumor supresor. HPV infection leads to deregulation of the cell cycle and loss of p53 tumors suppressor. E7 HPV protein binds to pRb and promotes its degradation resulting in the release and activation of the E2F transcription, inducing an increase of cyclin-dependent kinase inhibitor p16INK4a and a reentry into S phase cell cycle. E6 HPV protein prevents the induction of apoptosis in response to the unscheduled S-phase entry through ubiquitination and proteosomal degradation of p53. p53, in normal conditions, induce p21 expression that inhibits cyclin-CDK complex and arrest cell cycle (7).

3.4. CLINICOPATHOLOGICAL DIFFERENCES OF HPV-RELATED OROPHARYNGEAL CANCER

As mentioned, oropharyngeal cancer's change in etiology has led to the differentiation of HPV-related OPSCC as a separate entity from HPV-unrelated OPSCC, with marked heterogeneity in various features, discussed herein and summarized in *Table 1.*

3.4.1. DEMOGRAPHICS

Firstly, it is noticeable that, despite age being related with increasing incidence in both groups, HPV-related OPSCC patients tend to be **younger**, up to 4-10 years (2,36). The median age of onset is about early mid 50s in HPV-related OPSCC compared to late 50-60s in HPV-unrelated OPSCC (2,37–39). This correlates with the trends observed lately, with a remarkable increase of incidence among younger individuals (40) and a higher magnitude of OPSCC increase at younger ages in countries from Northern Europe, North America, and Australia (11). Nonetheless, there is controversy in these findings as HPV-related OPSCC is also increasing in older patients (41). Evidence suggests trends are birth-cohort specific: for example, the increase of incidence has been more rapid in individuals born from 1939 to 1955 rather than that of those born from 1955 to 1969; thus, it is forecasted that in the future age burden will change as there will be a dramatic increase of incidence in older people 65 to 74 years of age, while that in people 45 to 54 years of age will be moderate (42). Studies in upcoming times will be necessary to determine the exact epidemiology of age in HPV-related OPSCC, as generational changes in sexual behavior, like the "sexual revolution" of 1960s for instance, contribute to the increased incidence observed later.

Secondly, the distribution of OPSCC by sex differs between the two entities as well. In both, a higher incidence is found in **males** than in females. However, the male-to-female ratio has been reported to be (i) much higher in HPV-related OPSCC in some series, being 4-5:1 versus 2-3:1 (2), or (ii) similar in both subtypes of OPSCC in other settings, being 3:1 (12). Even though the number of new cases is substantially higher in men, the percentage of OPSCC cases attributable to HPV is the same in both sexes (17). It is interesting to notice as well that the differences in this feature vary depending on the geographical region, as it

is reflexed in the trends of incidence; looking back, while the incidence of OPC in males significantly increased in Northern Europe, North America and Japan concomitant to the decrease of lung cancer and other HNC, the concordance was not seen among females in all countries with increasing OPC incidence (Denmark, Estonia, France, the Netherlands, Poland, Slovakia, Switzerland, and United Kingdom) (11). In Spain in particular, the statistically significant increase of oropharyngeal cancer cases has been reported in males only (23). The explanation of these patterns is believed to be due to differences in the HPV infection burden, previously discussed.

Lastly, patients that suffer from HPV-related OPSCC tend to have a **higher socioeconomic status** and education compared to those with HPV-unrelated OPC (2), which may probably be related to distinct smoking and sexual habits between high and low socioeconomic status groups.

3.4.2. RISK FACTORS

As HPV-related OPSCC does not have the same etiology as HPV-unrelated OPSCC, its risk factors differ as well.

As mentioned, classical risk factors associated with HPV-unrelated OPSCC are **tobacco** use and **alcohol** overconsumption. Individuals with HPV-related OPSCC are significantly less likely than their HPV non-related counterparts to be smokers, but tobacco exposure is still common among patients with HPV-related OPSCC (38). More than 90% of patients with HPV non-related OPSCC have a smoking history, compared to 50-60% of patients with HPV-related OPSCC (2). Despite tobacco and alcohol use being more associated with HPV-related OPSCC (43), tobacco still plays a role on HPV-related as HPV-related OPSCC patients who have smoking history have poorer outcome than those that do not (44), as discussed further down. Apart from that, alcohol overconsumption increases the risk of HPV non-related OPSCC, but its relationship with HPV-related OPSCC is still not clear.

To date, the risk factors for HPV-related OPSCC that have been studied are implicated with HPV infection history, but no factors on pre-carcinogenic and carcinogenic progression have been defined. Therefore, risk factors described at present are related to

(i) oral HPV infection and (ii) infection persistence. Indeed, demographic variations in oral infection explain part of the distinct epidemiology of HPV-related OPSCC previously mentioned, but not all of them, as tobacco use also plays a role.

Firstly, higher prevalence of **oral HPV infection** is associated with male gender, cigarette smoking, age (there is a stable prevalence with age, therefore finding higher prevalence in older individuals; with a bimodal distribution in some populations with higher prevalence in 30-34 year-olds and 60-64 year-olds), deficient immune status, history of anogenital warts and sexually-transmitted disorders, as well as specific sexual behaviors such as lifetime and recent number of oral sex partners, recent number of rimming partners, lifetime and recent deep kissing number of partners, lifetime number of any sexual partners and vaginal sex partners. Higher incidence of HPV infection is associated with current smoking and human immunodeficiency virus infection, recent oral sex, oral-anal contact, open-mouth kissing, and genital-oral autoinoculation. **Sexual behavior,** therefore, is a surrogate risk factor for oral infection; even though mentioned sexual behaviors have been associated to increased oral infection, only the following have specifically been determined as risk factors for HPV-related OPSCC: increased lifetime exposure to oral sex, vaginal sex, and oral-anal sex, higher number of overall sexual partners, and earlier age at sexual debut (45).

Secondly, **persistent** oral HPV infection appears to be increased with male gender, older age, current cigarette smoking, as well as seropositivity to low-risk HPV serotypes and history of genital warts (indicating a broader exposure and/or increased susceptibility to infection). It is important to notice that sexual behavior has not been associated with oral HPV persistence (45).

In conclusion, once oral HPV infection occurs, due to sexual contact, various host factors including smoking status, age and biological sex determine the risk of infection persistence. Further studies are needed to clearly define the weight of these risk factors in HPV-related OPSCC (30) and how progression to carcinogenic lesions occurs.

Table 1: *Main differences between HPV-related and HPV-unrelated OPSCC*

	HPV-related OPSCC	HPV-unrelated OPSCC
Age	Younger: early mid 50s	Older: late 50-60s
Sex	Males > Females (3-5:1)	Males > Females (2-3:1)
SES	Higher	Lower
Risk factors	Sexual behavior	Tobacco use Alcohol overconsumption
Incidence trends	Increasing	Mostly decreasing
Anatomical Site	Tonsil and base of the tongue (lymphoid tissue)	All sites Larynx and oral cavity most common
Clinical presentation (TNM classification)	Small T-size, large N involvement	Variable
Radiological image	Cystic nodal involvement Well-defined borders	Invasion of adjacent muscle Ill-defined borders
Histopathology	Basaloid or non-keratinizing	Keratinizing
Tumor differentiation	Poorly differentiated	Variable, moderately differentiated
Biological and genetic alterations	p16INK4a overexpression	Normal or low p16INK4a expression
	p53 degradation by E6	p53 mutation
	pRb degradation by E7	Normal levels of pRb; Rb pathway altered by mutations
	EGFR decrease	EGFR amplification
Response to treatment	Radiosensitivity	Radioresistance
Locoregional control	Better	Worse
Metastatic dissemination	Rarely	Yes
Secondary Primary Neoplasms	Less common	More common
Comorbidities	No	Yes
Survival: overall and disease-free	Better	Worse

HPV: human papillomavirus; SES: social economic status. Adapted from (2,7).

3.4.3. CLINICAL PRESENTATION, IMAGING AND HISTOPATHOLOGY

Typically, HPV-related OPSCC are presented with a lower T and a more **advanced N** according to the TNM classification compared to HPV unrelated tumors. Since the tumor size tends to be lower, they are less likely to be symptomatic and patients seek medical attention when nodal metastasis appears. Commonly, HPV-related OPSCC have larger nodal involvement in number and in various levels, characteristically as cystic metastases (46,47). This distinct behavior has led to an incorporation of a different classification of the TNM staging system (the 8th edition) into clinical practice. This classification is described and discussed further down in *Section 3.6.2*.

By imaging, HPV-related OPSCC are more characteristically found as enhancing and exophytic, small or even occult primary tumors with well-defined borders and lymph node involvement as cystic nodal metastases, in contrast with HPV non-related OPSCC, which are more likely to be found as isoattenuated masses with ill-defined borders and invading adjacent muscle (36).

In relation to histopathology, HPV-related tumors are usually **non-keratinizing** or **basaloid** squamous cell carcinomas, whereas HPV non-related tumors tend to be more keratinizing (47). Moreover, they are usually poorly differentiated, a feature that contradicts the general rule in which higher histological grade implicates decreased prognosis (47,48).

3.4.4. MOLECULAR BIOLOGY AND GENETIC ALTERATIONS

Molecular biology of HPV-related OPSCC is distinct from HPV-unrelated OPSCC, a feature which is thought to play an important role regarding the difference on the patients' outcomes and which widens the research frame to new therapeutic targets. In HPV-unrelated OPSCC, the p53 pathway is altered due to TP53 mutations, the Rb pathway due to 17p loss of heterozygosity and hypermethylation of p16INK4A promoter, and there is a decreased expression of p16 with normal levels of pRB and an amplification of the epidermal growth factor receptor (EGFR), along with other molecular changes (3). Contrarily, HPV-related OPSCC normally have an alteration of the p53 pathway due to E6-

mediated **degradation** of cellular p53 (inhibition instead of mutation), a deregulation of the Rb pathway with E7-mediated degradation of Rb, an **overexpression of p16INK4A**, and a tendency low expression of EGFR (48).

3.4.5. PROGNOSIS AND OUTCOMES

HPV status and p16 positivity separately have been reported as an independent prognostic factor of ameliorated overall and disease-free survival in OPSCC patients (38). Compared to HPV-unrelated OPSCC, HPV-related OPSCC has better rates of 3-year overall survival (82.4% vs. 57.1%) and 3-year progression-free survival (73.7% vs. 43.4%) (38), as well as 5-year overall survival (60-80% vs. 20-25%) (2,49). There are several factors contributing to better prognosis and outcomes of HPV-related to OPSCC, such as younger age, better performance status, fewer comorbidities, less tobacco use, and HPV inherent biology as the most important. Some of them are discussed below. However, even adjusted for confusing factors, HPV-positivity confers a 60-80% reduction risk of cancer death in comparison to similarly treated HPV-unrelated OPSCC (38,49,50).

A. RESPONSE TO TREATMENT

Patients with HPV-related OPSCC display an increased intrinsic sensitivity to radiotherapy in comparison to the HPV-unrelated forms of these malignancies. The mechanisms responsible for this are unclear. Firstly, it is believed that it may be due to the different genetic alterations HPV-related tumors harbor, which maintain an intact apoptotic response to radiation (51). Thus, residual (even though low) levels of normally functioning p53 in HPV-related OPSCC (which is not mutated as in HPV-unrelated OPSCC) are activated by radiation, leading to increased apoptotic signaling after radiation and cell death (52). Secondly, it is postulated that the higher radiosensitivity does not result from increased apoptosis but is rather associated with compromised DNA repair capacity in epithelial cells, as E6 and E7 oncoproteins inhibit regulation of cell cycle checkpoints and cells fail to resolve radiation-induced double strand breaks (53), a feature on study due to its association with the use of new target treatments to increase radiosensitivity in this type of cells (54). Thirdly, it is thought that p16INK4a impairs the DNA repair response independently of its

function in the cell cycle. However, findings suggest it is only the expression of nuclear p16INK4a that increases radiosensitivity of OPSCC cells, as well as better outcome, locoregional control and disease-free survival after chemoradiation. Hence, nuclear p16INK4a expression may be a potent marker to predict radiation response, but subcellular localization may need to be considered during stratification of patients, a consideration that is not included in clinical practice at the moment (55). In fourth place, differences in tumor microenvironment are considered to play an important role in radiation response. Tumor cells from these entities respond differently in terms of gene expression, as hypoxia signaling, which confers radioresistance in HPV non-related OPSCC, is lower in HPV-related malignancies (56). Lastly, even though oncoproteins in HPV-related tumors are associated with evasion of the immune response, immune infiltration is a predominant feature in this type of tumors; a high number of CD8 TILs and a low CD4/CD8 ratio is associated with an improved patient outcome (57).

On another note, it has been reported that cetuximab (an EGFR inhibitor) may not provide proper disease control in HPV-related OPSCC. The recently published results from the ARTSCAN III study, a phase III trial which compared treatment outcome and toxicity between radiotherapy with concomitant cisplatin versus concomitant cetuximab in a population of HNSCC (head and neck squamous cell carcinoma) patients (89% of which were p16-positive), showed an inferiority of locoregional control in the concomitant cetuximab treatment arm (58). It is thought that the low EGFR expression in HPV-related tumors may confer resistance to EGFR inhibitors, likewise, not responding to this treatment (44).

Lastly, as immunological response is more important in HPV-related OPSCC, stimulation of this immune response directed to viral specific tumor antigens may confer a better response to immunotherapy approaches in this type of tumors (51).

Features like so constitute a revelation of new concepts to be considered when managing OPSCC. They are relevant since they undertake the issue for selecting patients as candidates for new therapeutic strategies, with different staging approaches, intensification or de-escalation of radiation therapy and new targeted therapies.

B. LOCOREGIONAL CONTROL

Lower numbers of local and regional recurrences are noticed in HPV-related tumors compared to those that are HPV non-related. The explanation for this is the absence of "field cancerization effect", in which the tissue surrounding the tumor does not have carcinogenic alterations predisposing the development of locally recurrent cancer or other primary tumors. This is rooted by the absence of transcriptionally active HPV in the mucosa surrounding HPV-related OPSCC (59).

C. METASTATIC DISSEMINATION

The incidence of distant metastases appears to be lower in HPV-tumors. This is also due to the absence of the "field cancerization effect", in addition to the lower exposure to tobacco of HPV-related OPC patients (in HPV non-related OPC multiple sites and organs are exposed to the oncogenic effect of the primary risk factors), and the lower age of HPV-related OPC patients (47).

When metastatic dissemination takes place in HPV-related OPSCC, metastases present at a longer interval after completion of treatment (more than 2 years after initial treatment) and in nontraditional sites compared to those in HPV non-related OPSCC (47). In this matter, distant metastases from HPV-unrelated OPSCC mainly occur in the lung, followed by bone and liver, while HPV-related OPSCC metastases are observed with higher incidence of multiple organ dissemination and a higher frequency in the skin, intra-abdominal lymph nodes and brain (47).

D. SECOND PRIMARY NEOPLASMS

Lower secondary primary neoplasm (SPN) rates have been reported among HPV-related OPSCC patients compared HPV-unrelated OPSCC patients (5.6% vs. 14.6%) (38,47,60). Besides differences in index of risk, it has been reported that 70% of SPN among HPV-related OPSCC patients arise outside of typical tobacco-related sites (head and neck, lung and bronchus, esophagus, and bladder), some of them in the anogenital region (60). However, HPV-related OPSCC patients with a history of smoking have an SPN-free survival and risk profile more similar to those of HPV-unrelated patients rather than those of HPV-

related never-smokers (60). All in all, both HPV and tobacco influence the risk and location of SPN development among patients with OPSCC, so they need to be considered when stratifying the patients.

E. COMORBIDITY

The better outcome of HPV-related OPSCC patients is also due to the lack of other comorbidities (besides high rate of second primary, recurrent or synchronous tumors) that are found in HPV-unrelated OPSCC, which are attributable to the carcinogenic mechanisms of tobacco and alcohol (2), absent in most HPV-related OPSCC patients.

3.5. HPV DETECTION TECHNIQUES

Differently to cervix uteri cancer, HPV detection in OPSCC may reflect either a transitory infection or an infection with a defined carcinogenic role. Therefore, when considering a valid method to detect HPV in OPSCCs, a substantial issue to consider is its utility to recognize the presence of the virus and discern its potential as a driving force of tumorigenesis, an attribute limited to tumors that harbor transcriptionally active high-risk HPVs (61) which needs to be pondered in detection techniques.

HPV testing is currently mandatory for an accurate diagnosis and prognosis of patients with OPSCC, and a well-defined HPV status is relevant for future patient-handling implications as well as consistent epidemiological studies (7). Nonetheless, there is no clear consensus on which test, or combination of tests, is optimal for routine diagnostic use. Current standardized techniques, which appear summarized in *Table 2*, include the following explained below. For now, routine clinical practice includes microscopic evaluation of specimens and p16INK4a immunoassay as first-step evaluations; HPV-DNA tests are employed differently depending on the hospital, some as routine practice in multi-modality analyses and some as second-step assessments; and mRNA tests are restricted to research.

3.5.1. ROUTINE TECHNIQUES

The ROUTINE MICROSCOPIC EVALUATION consists in the detection of distinctive morphologic characteristics in hematoxylin and eosin-stained sections (61), some of them previously discussed, e.g., HPV-related tumors arise from the reticular epithelium in the tonsillar crypts, invading as rounded nests and sheets of non-keratinizing or basaloid cells that commonly appear surrounded by lymphoid cells. This evaluation is the first one on the HPV-detection algorithm and guides the interpretation of HPV testing when there are disparities with another detection assay result, prompting the consideration of repeating the assay or employing a different one (61).

The p16INK4A IMMUNOHISTOCHEMICAL ASSAY is, at the moment, the most widely applied technique, since it is conducted in the routine diagnosis of OPSCC and precedes any of the other techniques explained further down. It embraces the protein p16INK4a as a surrogate marker of HPV-involvement, as its overexpression accounts for the virus being transcriptionally active. Because of viral oncoprotein E7 binding to the retinoblastoma protein and its subsequent degradation, tumor cells are free to replicate in the presence of p16, which accumulates. This accumulation is detected by routine immunochemistry (IHC) (61). Compared to the Gold-Standard, p16 IHC assay presents a high sensitivity (of ≥ 90%) and a moderate specificity (of >80%) (62). Nevertheless, the discordance rates between these two tests are reported to be around 10% of the cases (63), and most of them are p16 false positive results. Thus, p16 overexpression is not specific for HPV-driven cancer and can be caused by some non-viral mechanisms such as dysregulation of epigenetic control or multiple transcription factors, some of which are associated with HPV-unrelated OPSCC carcinogenesis (3). This is the main reason why using p16 IHC as a standalone test is seen as questionable, as patients with p16 false positive results may have different clinicopathological characteristics from patients with true positive results (being more alike to HPV-unrelated OPC). In general, p16INK4a staining has a high predictive value to identify HPV-related cancers when the pattern shows a strong and diffuse nuclear and cytoplasmic staining in at least 70% or more of the tumor. Therefore, the result of the test is considered positive when these conditions are found. To avoid differences of interpretation, staining

of ≥50% but <70% of the tumor needs to be supported by other forms of HPV testing to consider it positive or negative. Nuclear and cytoplasmic staining of <50% is considered as a negative result (62). Recently, it has been demonstrated that it is nuclear, but not cytoplasmic, p16 overexpression that results in better outcome, locoregional control and disease-free survival, as it is the nuclear localization of p16 that is crucial for DNA repair (55). These findings depict the importance of p16 subcellular localization when stratifying patients according to HPV status and that nuclear p16 overexpression specifically may be the proper standalone marker for prediction of radiation sensitivity. Despite the lack of clarity on p16 expression and discrepancies in its interpretation, which have led to controversy surrounding its use as a surrogate biomarker (3), p16 IHC staining is usually used as a standalone test in current clinical practice, easy to perform and interpret, widely available and very strongly prognostic. The strengths lay on features regarding low cost and simplicity: it is relatively inexpensive, does not require specialized equipment or expertise, and therefore can be performed practically in any laboratory with immunohistochemical capabilities. As it is easy and clear to interpret, it presents a low interobserver variability (61). Prognosis for patients with p16-positive OPSCC has been shown to be significantly better than for patients with p16-negative OPSCC in multiple studies with large numbers of patients, including retrospective and prospective clinical studies, giving p16 positivity a value of independent prognostic indicator (38). Studies that compare several biomarkers related to HPV find p16INK4a to be the marker with the most consistent association with the Gold-Standard (19). Nonetheless, it has been concluded that the most accurate approximation to judge HPV carcinogenicity is to add to the p16 test an HPV-DNA test (64). Lastly, p16 should only be used as a surrogate HPV marker in the oropharynx, where it is so strongly proven to be correlated with transcriptionally active HPV and improved prognosis. In sites that are not preferentially targeted by HPV the likelihood that p16 overexpression accounts for transcriptionally active HPV is low, with possible involvement of non-viral mechanisms that must be considered (61).

3.5.2. HPV-DNA TECHNIQUES

The HPV-DNA POLYMERASE CHAIN REACTION (PCR) employs a wide variety of HPV type-specific primers that allow the amplification of HPV DNA and thus the detection of the presence of HPV. As a result, a high sensitivity is obtained at the expense of a low specificity, with a scarce capacity for discerning transitory infections from those that in fact cause transcendental oncogenic changes (61).

The HPV-DNA IN SITU HYBRIDIZATION (ISH) is a signal amplification technique that uses labeled DNA probes complementary to targeted viral DNA sequences, which allows the visualization of DNA within tumor cells, and therefore the identification of the typical punctate nuclear pattern caused by the integration of viral DNA in the host cells' genome. Since this pattern is specific for this process, this method has a high specificity, but as it is limited by the viral copy numbers, which in some cases may be low, its sensitivity is inferior. Even so, as it may be performed in formalin fixed and paraffin embedded tissues (FFPE), it is more integrated into clinical practice compared to DNA PCR detection. However, its results can be difficult to interpret, as signals are scant, non-uniformly distributed in a tumor. Overall, it correlates better with the presence of transcriptionally active HR HPV compared to HPV-DNA PCR (61).

3.5.3. E6/E7 HPV-mRNA TECHNIQUES

The E6/E7 HPV-mRNA PCR proves the presence of HPV and demonstrates it is transcriptionally active for E6 and E7 oncogenes (61). It is currently considered the *Gold-Standard* for clinically relevant HPV as it substantiates HPV causality (7,62). Nonetheless, it is a technically challenging method since it requires fresh frozen tissue samples, a significant hurdle to its routine diagnostic utility. Recent advances are transferring this technique to FFPE, but its use is mainly restricted to the research laboratory (61,62).

The E6/E7 HPV-mRNA ISH detects E6/E7 mRNA transcripts in FFPE tumor tissues. Thus, in comparison to HPV-DNA ISH it allows the identification of ongoing transcriptional activity of HR HPV infections, achieving a higher sensitivity. With early experience, its sensitivity and specificity appear to be superior to DNA ISH techniques. However, it is a

technique of recent development, with a technical difficulty and higher turnaround time, and has not yet been approved for clinical practice, but with expectations to become a practical option in the near future (61).

3.5.4. FURTHER APPROACHES

The MULTI-MODALITY HPV ANALYSIS is based on the performance of more than one HPV testing approach. The most common modality consists in a stepwise approach that uses p16 IHC as a screening test (due to its very high sensitivity), followed by an HPV-specific detection assay, such as HPV-DNA ISH or PCR-based methods, in those with a positive result. This step helps correct those false positive results included in the positive results of p16 immunostaining, while conserving the insight of the significance of HPV that p16 provides. Studies have concluded that this is the most accurate approximation to judge HPV carcinogenicity (64). However, since DNA ISH is not optimally sensitive, occasional patients that have false negative results are mischaracterized as HPV-unrelated. Plus, it is a methodology that has increased cost and increased turnaround times (61,62), two disadvantages that support its selective implementation in hospitals as routine use.

Many prognostic or predictive ALTERNATIVE BIOMARKERS in relation to HPV-related OPSCC carcinogenesis have been studied, such as MMP-7 and EGF, which are found to be predictive of systemic treatment response, survivin overexpression, which predicts improved response to radiotherapy, and programmed cell death 1 (PD-1): PD-L1 pathway, which facilitates HPV-related carcinogenesis in immune-privileged sites (like tonsils) (3). Overall, personalized diagnosis in HPV-related OPSCC may be possible with robust biomarker panels and studies of detailed DNA profiling, leading to an improved individualized management and an expansion of new potential therapeutic targets.

Table 2: *Main Human Papillomavirus detection techniques*

METHOD	DESCRIPTION	ADVANTAGES	DISADVANTAGES
ROUTINE MICROSCOPIC EVALUATION	Morphological characteristics on the microscope	-Inexpensive -Guide for other tests -When insufficient material	-Not sufficiently specific to be used alone
p16INK4A IHC ASSAY	Surrogate marker of E7 HPV activity	-High sensitivity -Inexpensive -Simple and accessible -Widely implemented -Prognostic predictor -Detects T-A HPV -For FFPE tissues	-Moderate specificity -Expression not exclusive from HPV infection -Only for oropharynx
HPV DNA PCR	Amplification of conserved regions of the HPV genome	-High sensitivity -HPV type specific -Availability in multiple hospitals -For FFPE tissues	-Low specificity -Unable distinguish T-A HPV -Risk of cross-contamination -Test characteristics vary depending on primers used
HPV DNA ISH	Detection and location of HPV in tissue or cells	-High specificity -Simple and accessible -Distinguishes integrated versus episomal HPV DNA -Feasible on FFPE material	-Low sensitivity at low viral load -Sometimes difficult to interpret
E6/E7 mRNA PCR	Detection of messenger RNA E6/E7 HPV	GS: -High sensitivity -High specificity -Detects T-A HPV	-Requires considerable technical expertise -Optimally performed on fresh frozen tissue -Difficult to reproduce on a clinical setting
E6/E7 mRNA ISH	Detection of messenger RNA E6/E7 HPV	-High sensitivity + High specificity (detects T-A HPV) despite quantity of viral load -Approaches the GS -For FFPE material	-Limited experience -Not yet optimized to run on most automated platforms nor approved for clinical use
MULTI-MODAL ANALYSIS	p16 + another method	-Combination of sensitivity of p16 with a more HPV-specific method -Gives insight into the biologic significance of detected HPV	-Increased cost -Increased turnaround time

FFPE: formalin-fixed paraffin-embedded; GS: gold-standard; HPV: human papillomavirus; IHC: immunohistochemistry; ISH: in situ hybridization; PCR: polymerase chain reaction; T-A: transcriptionally active. Adapted from (7,61,65).

3.6. MANAGEMENT: CURRENT INTERVENTION AND FUTURE TREATMENT IMPLICATIONS

The increase in the incidence of HPV-related OPSCC and the characterization of this particular entity has contributed to the necessity of reassessing the diagnostic and therapeutic management of oropharyngeal cancer. As management decisions for this type of cancer must consider not only disease control and survival outcomes but also toxicities and long-term functional consequences (66), this reassessment has been (and is still being) carried out regarding different aspects of the disease, which include the change in the staging system, the possibility of implementing de-intensification strategies and the research for targeted therapies.

3.6.1. INITIAL MANAGEMENT

At the moment, when an OPC is detected in a patient, a further study is performed, including the following complementary tests and examinations:

- A complete head and neck clinical examination with a fibreoptic endoscopy.
- A biopsy of the primary tumor site and/or a fine-needle aspiration of lymph node metastases.
- A p16 immunohistochemistry staining test.
- A head and neck computed tomography scan with contrast and/or a head and neck magnetic resonance imaging scan with contrast.
- Other tests, as clinically indicated, such as: preanesthetic studies, FDG- positron emission tomography, chest computed tomography (to rule out secondary neoplasms and advanced nodal involvement), and nutrition, speech and swallowing evaluations.

Depending on the tumor HPV status, assessed by the p16 IHC staining test, as well as the results of the clinical and imaging study, the patient is classified into the corresponding TNM staging.

3.6.2. THE TNM STAGE CLASSIFICATION

The TNM stage classification is a universal system used to provide an anatomic-based classification to adequately depict cancer prognosis. Thus, it addresses anatomic tumor extent using the "tumor" (T), "lymph node" (N), and "metastasis" (M) attributes, where T describes the extent of primary tumor, N refers to absence or presence and extent of overt regional lymph node(s), and M captures the absence or presence of distant metastasis. Accurate cancer staging is important for treatment selection and outcome prediction, research design, and cancer control activities (47). As it plays a pivotal role in clinical care, the evolution of treatment and advancing understanding disease behavior necessitate periodic updates to maintain relevance for contemporary cancer management. In response to the urgent need to properly depict the character and prognosis of HPV-related OPSCC, a new stage classification was introduced in the newly published **8th edition** of the TNM classification, which better reflects tumor biology and clinical behavior of this subset of OPC. Previously, several studies demonstrated the inadequacy of the 7th edition of the TNM for predicting outcomes of HPV-related disease, as it was discovered that most patients with high-risk HPV+ OPSCC had a stage IV disease but a prognosis rivaling the most curable cancers. In addition to the advantage in survival noted with unusually favorable outcome for stages III and IV disease, the lymph nodal involvement and extent of nodal disease seemed to not be associated with reliable prognostic ability either. From that point, surgical series have shown that traditional prognostic factors, such as higher N category and extranodal spread, are no longer prognostic. Herein, modifications have been added in the new staging system (47) which appear in *Annex 5.* In the new edition of TNM, OPCs are distinguished by their p16 IHC staining. The rationale for using p16 as a surrogate is because it is generally accepted as a reliable surrogate marker for HPV-driven carcinogenesis in oropharyngeal cancer, as commented before. Provided that stringent criteria for scoring and interpretation are followed, it is unlikely to be misrepresented if the proper context is considered addressing correct anatomic subsite (67). HPV status was initially seen as a stratification factor for prognosis algorithms, but its introduction to the staging system underlines the recognition of HPV-related OPSCC as a new disease with potentially different

treatments. However, this framework for clinical research and treatment decision-making must continue to acknowledge other important tumor factors, treatment factors and patient factors (like age, performance status, and smoking history), as this 8th edition of the TNM classification reflects prognosis under the current treatment paradigm.

3.6.3. CURRENT THERAPEUTIC INTERVENTION

The primary treatment for OPSCCs at present is mainly dependent on the stage of the disease at presentation, which correlates with the TNM staging explained, in addition to the patient's preferences and the opinion of a multidisciplinary team. To date, it remains independent of the HPV status. The standard treatment possibilities are based on the ideas:

- Patients with EARLY-STAGE DISEASE, which include those T1-T2 N0-N1 M0, are candidates to receive a **single**-treatment modality, in the form of surgery or radiotherapy. Radiotherapy allows organ preservation, and the recent use of altered fractionation schemes has improved outcomes in that matter compared to conventional fractionation. In regard to surgery, the advent of transoral robotic surgery, which is still in study, is a minimally invasive technique that has been associated with encouraging oncologic, functional and quality-of-life outcomes.
- In contrast, patients with LOCOREGIONALLY ADVANCED DISEASE, who are those diagnosed with T3-T4 tumors or lymph node involvement as N2/N3, are only tributary to **multi-**modality treatments, which incorporate a combination of them, in the form of (i) primary chemoradiation therapy with or without selective neck dissection or (ii) primary surgical resection (of the primary tumor and lymph node dissection as indicated) with or without reconstruction with postoperative chemoradiation (66). As for the combination of chemotherapy and radiotherapy, the widely accepted standard of care is high-dose cisplatin with concurrent radiotherapy although induction chemotherapy followed with concurrent chemoradiotherapy is also an option (68).

The aggressive treatment schemes mentioned expose patients to potential acute and long-term side effects derived from them. The **morbidity** associated includes xerostomia, mucositis, necrosis, osteoradionecrosis, pain, scarring, fibrosis, dysphagia, pulmonary aspiration, gastric tube dependence, hypothyroidism, tracheostomies, chronic fatigue, dental decay, carotid stenosis, stroke, nephrotoxicity, neurotoxicity, and ototoxicity.

3.6.4. DE-INTENSIFICATION STRATEGIES

Current therapeutic recommendations do not take HPV status into account. However, since patients with HPV-related OPSCC have a younger median age, the morbidity associated with current treatments will probably have a larger impact from the clinical, social, and economic point of view in this cohort in the next decades. This future impact of treatment comorbidities and the better prognosis this cohort has demonstrated has resulted in the interest of de-intensification trials seeking to find new treatment protocols that reduce acute and late treatment toxicity while maintaining the overall survival. At present, there are several clinical trials, some of them completed and some ongoing, with many options being evaluated in different OPSCC stages. To date, none of the trials have demonstrated significant evidence for treatment de-intensification, thus the standard of care has not changed. Some of the main strategies that have been described are (44):

(i) the use of cetuximab as an alternative to cisplatin given adjuvant to intensity-modulated radiation therapy, in which HPV-related OPSCC has proven resistance to cetuximab (69),
(ii) the reduction of radiation doses when given concurrently with chemotherapy as primary treatment after good response to induction chemotherapy,
(iii) the reduction of adjuvant chemotherapy or radiotherapy dose following primary treatment with surgery, guided by histopathological features in the resected specimen, such as TILs in HPV-related OPSCC,
(iv) the use of minimally invasive surgery followed by less intensive adjuvant protocols,
(v) the employment of adjuvant treatment with viral antigens (HPV E6 or E7) in HPV-related OPSCC, an opportunity to develop immune-based treatments with minimal toxicity, and
(vi) the use of biological targeted therapies, with therapeutic blockade of specific HPV carcinogenic pathways, such as PI3K or PD-1/PD-L1 inhibition (66).

The identification of the role of HPV in OPSCC presents an exciting opportunity to develop new therapeutic strategies to reduce morbidity associated with conventional treatments. Data from completed trials suggest a heterogeneous HPV-related OPSCC population, with non-smokers demonstrating improved survival compared to smokers. This underlines the importance for future trials to distinguish patients according to smoking history as well, to characterize unbiasedly groups for safe de-escalation (44).

3.7. PREVENTIVE AND SURVEILLANCE STRATEGIES

3.7.1. PRIMARY PREVENTION

The increase in incidence burden of HPV-related OPSCC underlines the crucial importance on the development of preventive strategies for this subtype of OPC. Primary prevention methods are still being assessed and developed, but foremost important is primary prevention of initial HPV infection. This includes HPV immunization targeting the L1 capsid proteins, with the current vaccinations available:

- Cervarix © vaccine, which includes serotypes 16/18 (bivalent)
- Gardasil © vaccine, which includes serotypes 6/11/16/18 (tetravalent)
- Gardasil 9 © vaccine, which includes serotypes 6/11/16/18/31/33/45/52/58 (nonavalent)

All these vaccinations include HPV16 genotype, but only Gardasil 9 has been approved for the prevention of HPV-related OPC and other HNC. In Spain, HPV systematic vaccination was introduced in 2008 with the aim of cervical cancer prevention, hence the nonavalent vaccine is currently administered to girls at the age of 11/12 as a part of the scholar vaccination program in 6th grade. Other recommendations allow free vaccination to patients in risk (with WHIM syndrome, women with a solid organ or hematopoietic precursors transplantation until 26 years of age, with HIV infection until 26 years of age, men who have sex with men until 26 years of age, sexual workers until 26 years of age and women who have received cervical excisional treatment) (70). In countries, such as the United Kingdom (UK), Ireland, and Denmark, there has been an entrance of males into national immunization programs. The efficacy of vaccination against oral HPV infection was assessed in a clinical trial in Costa Rica, and reduced prevalence of oral HPV was reported after 4 years of bivalent vaccination, as vaccine efficacy was found to be 93.3% (71). As for efficiency, vaccinated young adults from the United States of America (USA) had a lower oral prevalence of vaccine HPV-serotypes (6/11/16/18) compared to unvaccinated adults (0.11 vs 1.61%), while the prevalence of non-vaccine types was similar (3.98 and 4.74%) (72). All in all, it is suggested vaccination affords strong protection against oral HPV16

infection among males and females in general population, with potentially important implications for prevention of increasingly common HPV-related OPSCC.

3.7.2. SECONDARY PREVENTION

The prophylactic vaccine is not effective on established infections and cancer lesions, so strategies for secondary prevention are currently being studied. To detect and treat early disease, two main screening methods have been described. Firstly, HPV-based screening through gargle or brushing samples has proven good specificity (92%) and moderate sensitivity (72%) for oral HPV detection among patients diagnosed with OPSCC. Nonetheless, its utility is limited among the healthy population (as OPC is rare in general population, the test's positive predictive value is low). In addition, identifying only HPV oral infection is not useful as a screening test, since most oral HPV infections clear within 2 years, and identifying persistent HPV infection otherwise would improve the test's utility (73). Secondly, HPV16 E6-based antibody seropositivity has been presented as a marker for HPV-driven neoplastic process, as seropositivity was present in pre-diagnostic samples for 34.8% of OPSCC patients compared to 0.6% of controls, associating an increased risk of OPSCC among HPV16 E6 seropositive individuals that may be observed more than 10 years prior to diagnosis (74). The sensitivity of this assay, however, is currently unknown, as the capacity to detect OPSCC overall is higher in regions with higher number of cases. Thirdly and last, other methods such as visualization of early lesions with NBI or intraoral ultrasonography (OPSCC of the base of the tongue) and biomarkers based on mRNA are also being evaluated.

All things considered, there are not yet identifiable precancerous lesions of the OPSCC at which to intervene, and preventive screening tools might need to be considered in high-risk groups selectively to achieve higher test validity. New epidemiological and clinical data on HPV-related OPSCC provide better characterization of these high-risk groups.

3.7.3. TERTIARY PREVENTION

Besides primary and secondary prevention, other methods for tertiary prevention are being researched. To prevent cancer recurrence, some potential biomarkers have been assessed, but to date none have been validated. In this matter, the viral etiology of HPV-driven OPSCC may represent an opportunity to improve post-treatment disease surveillance, but to date, the following ones are described:

- detection of viral DNA, mRNA or p16 in tumor samples, in which HPV-DNA and p16 double positivity is the biomarker with best prognostic value (64).
- detection of HR HPV DNA in body fluids, in the form of oral rinses and blood samples, in which persistent oral infection is associated with worse survival.
- detection of change in E6 antibody levels, in which prediction results are controversial and no clear conclusion has been reported.

Further study and research are needed to find and implement valid preventive strategies for HPV-related OPSCC. The broadening of knowledge in relation to this subset of cancer, such as the epidemiological distribution of its burden and its trends, will contribute to developing efficacious interventions to prevent it.

4. JUSTIFICATION

Head and neck cancer covers a group of cancers with high variability of epidemiological and clinical features among the different types of neoplasms that it includes. The study of these features is essential to manage these diseases adequately. This remarkable heterogeneity of HNC has been observed as the shift in etiology in oropharyngeal cancer has caused a change in the epidemiological trends. Whilst HNC incidence has decreased in the past decades, OPC incidence has sharply increased, giving rise to an epidemic with growing burden of a new subset of OPC related to HPV.

The emergence of this subtype of OPC varies according to geographical region. It has predominantly been studied in regions of high burden of oral HPV infection, such as North America, Northern and Western Europe, Australia, and Japan, among others, but little is known about the onset of HPV-related OPC in Southern Europe. To our knowledge, there is no population-based literature on the trends in incidence and survival of HPV-related OPC in Southern Europe.

HPV-related OPC has a different natural history and molecular biology of carcinogenesis, which grant this subtype of OPC distinct demographic features and clinical presentation, and better survival. However, these characteristics have been described in regions of high burden of HPV-related OPC, thus it is still unclear how this disease behaves in Southern Europe. Further understanding of epidemiological and demographical distribution of increasing incidence of this subtype of OPC is crucial to develop and implement efficacious preventive strategies. Potential prevention methods such as vaccination and health promotion are accessible but not yet applied. The establishment of better prognosis in HPV-related OPC in comparison to HPV-unrelated OPC contributes to the need of reassessing the therapeutic management of the emerging subset of cancer, as the increase of life expectancy it confers sustains a higher impact of morbidity associated to current treatments.

This study will contribute to the knowledge of HPV-related OPC in an area of theoretical low prevalence of OPC and oral HPV infection, as the first population-based

study of trends in incidence and survival of HPV-related OPC in a region of Southern Europe. It will also give insight on demographical distribution according to sex and age. Our population-based design will be feasible using the database of the standardized cancer registry in the province of Girona, and the laboratory measurements we will apply are included in current clinical practice, featuring low cost, simplicity, and availability. Confidentiality will be kept throughout the process and laboratory determinations will be performed on tumorectomy/biopsy specimens, not causing any harm to patients.

5. HYPOTHESES

5.1. MAIN HYPOTHESIS

There has been an increase in the incidence of human papillomavirus-related oropharyngeal cancer and in the incidence of oropharyngeal cancer overall between 1994 and 2018 in the province of Girona.

5.2. SECONDARY HYPOTHESES

- HPV-related oropharyngeal cancer has a better prognosis in comparison to HPV-unrelated oropharyngeal cancer in the province of Girona.
- HPV-related oropharyngeal cancer has a different distribution in terms of age and sex compared to HPV-unrelated oropharyngeal cancer in the province of Girona.
- There has been a decrease in the incidence of head and neck cancer overall between 1994 and 2018 in the province of Girona.

6. OBJECTIVES

6.1. MAIN OBJECTIVE

To determine the trends in the incidence of oropharyngeal cancer, overall and in relation with p16 expression, between 1994 and 2018 in the population of the province of Girona.

6.2. SECONDARY OBJECTIVES

- To analyze the observed and the relative survival rates of patients diagnosed with p16-positive oropharyngeal cancer in comparison to those of patients with p16-negative oropharyngeal cancer.
- To define the epidemiological characteristics of patients with p16-positive oropharyngeal cancer in relation to age and sex compared to those of patients with p16-negative oropharyngeal cancer.
- To determine the trends in the incidence of head and neck cancer overall between 1994 and 2018 in the province of Girona.

7. METHODS

7.1. STUDY DESIGN

A population-based retrospective observational cohort study conducted from October 2020 to November 2021 by the "Grup d'Epidemiologia Descriptiva, Genètica i Prevenció del Càncer (GEDGPC) de l'Institut d'Investigació Biomèdica de Girona" (IdIBGi), in which we analyze the immunohistochemical expression of p16INK4a protein in tumor samples from patients diagnosed with primary oropharyngeal cancer in the period between 1994 and 2018 in the province of Girona, and assess the epidemiological trends and distribution of HPV-related oropharyngeal cancer in a population of Southern Europe.

7.2. STUDY SETTING

This study has been set in the Cancer Registry of the province of Girona (CRG) and the Pathology Departments of the hospitals that provide assistance in the health region of Girona, which include: the hospital of Palamós, the hospital of Figueres, the hospital of Blanes, Clínica Girona (which receives specimens from the hospital of Olot, Santa Caterina Hospital, and Clínica Girona itself), and the Hospital Universitari de Girona Doctor Josep Trueta (which receives specimens from hospital of Campdevànol and Trueta hospital itself).

7.3. CASE SELECTION

For the selection of the study cases, we retrieved data from the CRG (which served as our database) and selected them upon that compilation following a series of selection criteria.

7.3.1. DATABASE

The CRG is a population-based cancer registry located in the North-East of Catalonia which covers a defined population of 747,157 people residing in the province of Girona according to the census reported by the *Insitut d'Estadística de Catalunya (IDESCAT)* in 2018 (75). It belongs to the International Association of Cancer Registries (IACR) and to the Spanish Network of Cancer Registries [*Red Española de Registros del Cáncer* (REDECAN)] and publishes regularly in the International Agency for Research in Cancer (IARC) monograph "Cancer Incidence in Five Continents" (10). Its main aim is to identify and register, on an exhaustive and continuous basis, all new cases of cancer diagnosed each year to the residents of the province of Girona (regardless of the place of diagnosis), and to subsequently obtain epidemiological indicators of cancer in the region. Its activity, initiated in 1995, entails the systematic and exhaustive collection of data, the processing of this information, and the elaboration of results to determine the incidence, mortality, survival and prevalence of cancer, its distribution according to multiple variables, as well as the evolution over time and the future estimation of these indicators.

The CRG follows the international guidelines for the operation of population-based cancer registries (76), which cover all the process, from the definition of cases to the systems of operation and elaboration of results. These standards have been developed to ensure the reliability and comparability of data between different population-based cancer registries around the world.

7.3.1.1. Information sources

The information on new incident cases of cancer is obtained from public and private healthcare centers in the province of Girona, as well as from other public and private

remote centers (usually from Barcelona), to which some patients attend, either because those are the reference centers for their pathology or because patients themselves decide to go there. It is extracted from the following sources: hospital discharges, hematology and pathology laboratory, and death certificates[4]. These data are processed to obtain incidence rates, which are the basic information to calculate all other epidemiological indicators.

The information on mortality is obtained from the Mortality Registry of Catalonia, elaborated by the *Servei de Gestió i Anàlisi de la Informació* for the *Planificació Estratègica* of the *Direcció General de Recursos Sanitaris* of the Health Department of the *Generalitat de Catalunya,* and from the *Índice Nacional de Defunciones* of the Spanish *Ministerio de Sanidad, Consumo y Bienestar Social*. The original mortality data, which come from statistical death bulletins, also make it possible to obtain cases not identified in the other sources and to contrast the information already existing in the registry.

The information on the population of the province of Girona, stratified by sex and age group, is obtained from the estimates and projections made by the IDESCAT (75).

7.3.1.2. Definition and Registration of Cases

Any invasive malignant tumor, in situ carcinoma or tumor with uncertain behavior diagnosed in a patient resident in the province of Girona at the time of diagnosis or at the time of death (in DCO/DCN cases) is considered a case and is included in the database of the CRG. Therein, it is important to notice it is tumors that are recorded, not individuals, on the assumption that a single patient may have two or more tumors. Therefore, the incidence reflects the number of primary cancers, and not the number of patients with cancer.

The guidelines for registration and multiplicity criteria used are those defined in the *International Rules for Multiple Primary Cancers* of the IARC, the IACR and the European

[4] Those cases in which the only information available is the death certificate and cannot be identified after exhaustive research by the registry personnel are classified as *Death-Certificate-Only* (DCO) cases. Those cases that were initially notified by death bulletin but were posteriorly identified after revision are classified as *Death-Certificate Notification* (DCN). In DCO cases, the incidence date is considered as the date of death. Therefore, the percentage of DCO gives insight on quality data of the registry (it is important it remains below 5-10%).

Network of Cancer Registries (ENCR) (76). For the calculation and presentation of the incidence results, the international standard criteria defined in the joint recommendations of the ENCR, the IARC and the IACR are employed as well (76).

To define a case as a resident of the province of Girona, the CRG uses existing information from the databases of hospitals and other facilities, in addition to medical records and death certificates archived. In case of discordance between the different sources, the information is actively reviewed along with the files in the medical records and, in case the doubt remains, the place of residence is accepted according to the source of information considered to be the most reliable. A resident is recognized to be any person who resided in the province of Girona for the period of at least 2 years prior to their cancer diagnosis.

All things considered, the CRG records the following information of each case: demographical characteristics, pathology, localization and sub-localization according to the ICD-O-3 code (5), method and date of diagnosis, and date of death, if applicable.

7.3.1.3. Data processing

After the information is collected in the CRG, the data are processed prior to analysis. Quality control checks are performed to ensure uniformity, completeness of information, correctness of coding, lack of duplication, and validity and maximum accuracy, as well as other features regarding its overall quality.

Confidentiality is kept throughout the entire process, according to the provisions of the Data Protection European Union Directive 1995/46/EC and the *Ley Orgánica 3/2018 de 5 de diciembre* regarding the guarantee of data protection (77)**,** and in concordance with the ethical principles originated in the Declaration of Helsinki (78).

7.3.1.4. Time coverage

The CRG database has had a coverage since January 1994. As a result, our study does not include cases prior to that year. To complete the data on incidence, it is necessary to have all the information on new cases and mortality. In this matter, it should be borne in mind that there is always a time lag in obtaining this information, which affects our study

period as well. Thus, this is limited to the December 2018 as the latest month covered in the study.

It should be noted that the database is updated as the registry has more and better information and this may result in small variations between the results of different publications; for our study, the most recent information was considered as the best quality data available.

7.3.2. CASE SELECTION

As mentioned, our study cases were selected from the CRG database. Therefore, cases included any invasive malignant tumor, in situ carcinoma or tumor with uncertain behavior diagnosed in residents in the province of Girona. All tumors we selected were squamous cell carcinomas, included in the ICD-O-3 morphological codes 8070-8072 (5), as it is detailed below.

For each part of the study, we included cases fulfilling distinct criteria, as furtherly explained:

- Initially, we selected all head and neck primary tumors diagnosed from 1994 to 2018 included in the CRG with the ICD-O-3 codes (5) from topographical sites C00-C14 and C30-C32, and morphologies 8070-8072. The meaning of these codes is furtherly explained in *Table 3*. This cohort of cases was used to determine the overall HNC incidence and its trend.
- Following, we selected all oropharyngeal cancer primary tumors diagnosed from 1994 to 2018 recorded in the CRG with the ICD-O-3 codes (5) from topographical sites C01.9 (base of the tongue), C05.1-C05.2 (soft palate and uvula), C09.0-C09.9 (palatine tonsils), C10.0-C10.9 (oropharynx), and morphologies 8070-8072. This next cohort of cases was used to determine the overall OPC incidence and its trend.
- Successively, from this OPC cohort we selected all cases diagnosed only in the following periods of time: 1998-1999, 2003-2005, 2009-2011, and 2016-2018. This last cohort of cases was used to conduct the p16 expression-based analysis, in which we determined the incidence, the trend in the incidence and the survival of OPC cases according to their p16 expression. It was not considered feasible to carry out the immunohistochemical

study in the entire selection of cases in the chosen period (1994-2018); therefore, we assessed the p16 expression-based analysis using cases diagnosed within four time slices of the entire study period.

Table 3: *Topographical sites and morphologies of head and neck cancers and their corresponding ICD-O-3 codes*

TOPOGRAPHICAL SITE	***ICD-O-3 CODE***
Lip	C00.0-C00.6, C00.8-C00.9
Oral cavity *Tongue (excluding base),* *gum, floor of the mouth, hard palate,* *and other mouth locations*	C02.0-C02.4, C02.8-C02.9 C03.0-C03.1, C03.9-C04.1, C04.8-C05.0, C05.8-C06.2, C06.8-C06.9
Salivary glands	C07.9-C08.1, C08.8-C08.9
Oropharynx *Base of the tongue* *Soft palate + uvula* *Palatine tonsils + oropharynx*	 C01.9 C05.1, C05.2 C09.0-C09.1, C09.8-C10.4, C10.8-C10.9
Nasopharynx	C11.0-C11.3, C11.8-C11.9
Hypopharynx	C12.9-C13.2, C13.8-C13.9
NOS pharynx or overlapping*	C14.0, C14.2, C14.8
Nasal cavity + paranasal sinuses	C30.0, C31.0-C31.3, C31.8-C31.9
Larynx	C32.0-C32.3, C32.8-C32.9
MORPHOLOGY	***ICD-O-3 CODE***
SCC, NOS	8070
Keratinizing SCC	8071
Nonkeratinizing SCC	8072

*NOS: not otherwise specified; SCC: squamous cell carcinoma. *Overlapping lesion of lip, oral cavity, and pharynx.* Adapted from (5).

7.4. VARIABLES

7.4.1. INDEPENDENT VARIABLE

The involvement of human papillomavirus infection in OPC primary tumor samples, determined by the result of an immunohistochemical staining assay of p16INK4a protein expression, considered as:

- **p16-positive**:
  - nuclear and cytoplasmic staining in ≥ 70% of the tumor.

  or
  - nuclear and cytoplasmic staining in ≥50% but <70% of the tumor with a positive HPV-DNA-based test.
- **p16-negative**:
  - nuclear and cytoplasmic staining in <50% of the tumor.

7.4.2. OUTCOMES

In our study, we considered three main outcomes, which were dependent on the independent variable, and four other outcomes, which were recorded without having relation with the independent variable.

7.4.2.1. MAIN OUTCOMES (p16 expression-based analysis)

1. Incidence

The **incidence** has been defined as the number of oropharyngeal cancer cases that occurred during the calendar periods 1998-1999, 2003-2005, 2009-2011, and 2016-2018 in the province of Girona. It has been estimated as crude rate (CR) and age-standardized to the European standard population rate ($ASIR_E$).

2. Trends in incidence

The **trends in the incidence** have been defined as the behavior or behaviors (increase, decrease or neither) of the incidence rates of OPC over the period from 1998 to

2018 in the province of Girona. The parameter used to assess trends has been the Annual Percent Change (APC) of $ASIR_E$.

3. *Observed survival*

The **observed survival (OS)** has been defined as the percentage of patients alive in a period of 5 years from the diagnosis date, thus assessing the probability of survival of patients (as it considers mortality for both cancer and other causes). The last date of follow-up for all living patients has been estimated as of 31st December 2020 for all of them.

7.4.2.2. OTHER OUTCOMES

1. *Incidence of overall OPC*

The **incidence of overall OPC** has been defined as the number of cases of primary oropharyngeal cancer tumors that occurred during the period of 1994-2018 in the province of Girona. It has been estimated as CR, $ASIR_E$ and age-standardized to the WHO World standard population rate ($ASIR_W$).

2. *Trends in incidence of overall OPC*

The **trends in the incidence of overall OPC** have been defined as the behavior or behaviors (increase, decrease or neither) of the incidence rates of overall OPC over the period of 1994-2018 in the province of Girona. It has been assessed by APC of $ASIR_E$.

3. *Incidence of overall HNC*

The **incidence of overall HNC** has been defined as the number of cases of primary head and neck cancer tumors that occurred during the period of 1994-2018 in the province of Girona. It has estimated as CR, $ASIR_E$ and $ASIR_w$.

4. *Trends in incidence of overall HNC*

The **trends in the incidence of overall OPC** have been defined as the behavior or behaviors (increase, decrease or neither) of the incidence rates of overall OPC over the period of 1994-2018 in the province of Girona. It has been assessed by APC of $ASIR_E$.

7.4.3. COVARIATES

The following demographical variables which can contribute to the variation of the incidence and survival rates have been collected as covariates.

- **Age**: expressed in years at the time of diagnosis, measured in one-year intervals since the date of birth, as recorded in the CRG database. It has been used to adjust incidence and survival rates.
- **Sex**: defined as *male* or *female*, as recorded in the CRG database. Information from the CRG is detailed according to the chromosomal sex of the patient. Thus, patients with sex chromosome XY were defined as *male*, whilst patients with sex chromosome XX were defined as *female*.

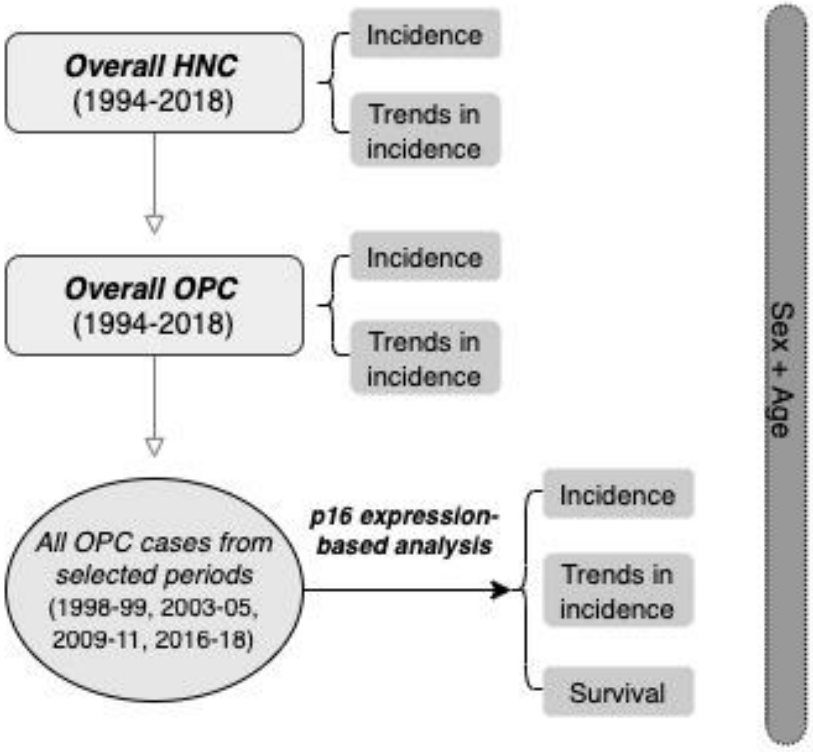


Figure 6. *Summary of the cohorts selected in the study (on the left) and the outcomes analyzed in each, as well as covariates considered in all of them (on the right).*

7.5. MEASUREMENTS AND DATA COLLECTION

7.5.1. MEASUREMENTS

7.5.1.1. Identification of tumor samples

We proceeded with the identification of formalin-fixed paraffin-embedded samples of the cohorts selected to undergo the p16 IHC assay, which consisted in primary oropharyngeal tumors from 1998-1999, 2003-2005, 2009-2011, and 2016-2018. FPPE samples were identified in the Pathology Departments of the hospitals that provide assistance in the health region of Girona, which include: the hospital of Palamós, the hospital of Figueres, the hospital of Blanes, Clínica Girona (which receives specimens from the hospital of Olot, Santa Caterina Hospital, and Clínica Girona itself), and the Hospital Universitari de Girona Doctor Josep Trueta (which receives specimens from hospital of Campdevànol and Trueta hospital itself). FFPE tissue sections had been obtained from pre-treatment biopsies and tumorectomies of the primary oropharyngeal tumors.

7.5.1.2. Determination of p16 expression

Determination of immunohistochemical expression of p16INK4a in paraffin-embedded specimens was carried out in the respective Pathology Departments, given that this procedure constitutes a routine examination preformed in current standard clinical practice by all the Pathology Departments mentioned. The determination of p16 was not effectuated in cases in which these data were already available.

Immunohistochemistry involves a technique that permits the visualization of the amount and the distribution of a certain molecule in a tissue (such as p16 protein in this case) through specific antigen-antibody reaction. IHC aims to detect, amplify, and confer visibility to a specific antigen or biomarker. The procedure is initiated with the detection of the molecule, for which a “primary antibody” is used. Following, to amplify this reaction, a “secondary antibody” tagged with several biotin molecules is needed, which binds to the “primary antibody”. After, the catalyzation of a color-producing reaction by a conjugated enzyme, peroxidase, takes place to allow the visualization of the whole complex. Peroxidase

enzymes are conjugated to streptavidin molecules, which have a high affinity to biotin molecules. It is specifically when streptavidin-peroxidase complexes bind to "secondary antibodies" that the peroxidases oxidase the 3,3'-diaminobenzidine (DAB) chromogen, turning it into a brown pigment. This pigment precipitates out of solution as a brown solid, locating the site of the studied molecule.

The main steps of the IHC process performed in the Pathology Laboratory during the study were the following:

- Fixation of the tissue in a 10% formaldehyde solution for material preservation.
- Fractioning of the tissue in sections of 3µ.
- Heating of the samples at 50ºC for 45 minutes.
- Insertion of the samples in the "VENTANA" System, which conducts the following automated proceedings:
    - Further heating of samples and removal of paraffin leftovers.
    - Buffer washing.
    - Antigenic recovery by breaking of formalin bridges, through heat and CC1 solution application.
    - Peroxidase inhibition to diminish false reactivity to endogen peroxidases.
    - Incubation of anti-p16INK4a antibodies ("primary antibodies").
    - Incubation of "secondary antibodies" and peroxidase enzymes.
    - Visualization of the reaction through the revealing substance DAB (chromogenic): when the substance reacts with oxygen, it acquires a characteristic brown color (see *Figure 7*).
- Contrasting of results with positive controls, made of non-pathological palatine tonsil samples, and microscopic evaluation with hematoxylin-eosin staining.

Once the procedure above was effectuated, the stained samples were evaluated. According to the grade of p16 expression they were categorized as *p16-positive* or *p16-negative* in the following manner:

- Nuclear and cytoplasmic staining in ≥ 70% of the tumor was categorized as *p-16 positive.*

- Nuclear and cytoplasmic staining in <50% of the tumor was categorized as *p16-negative*.

In our study, we did not have any samples with nuclear and cytoplasmic staining in ≥50% but <70% of the tumor. Therefore, no further evaluation by other forms of HPV detection (HPV DNA-based test) was needed in any of the cases.

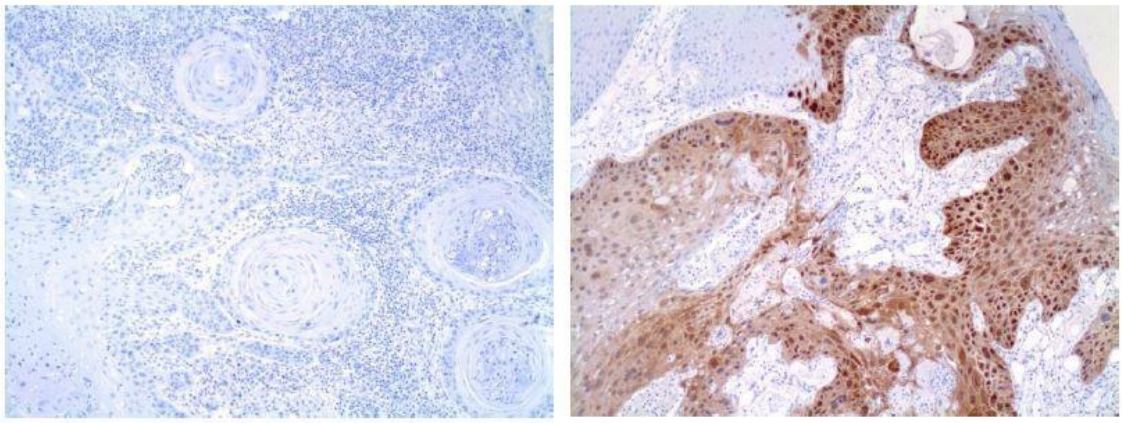


Figure 7. Example of p-16 IHC assay results: *p-16 negative* sample on the left (negative staining), and *p-16 positive* sample on the right (positive staining) (79).

7.5.2. DATA COLLECTION

An anonymized database was elaborated with the selected incident study cases identified (overall HNC cases, overall OPC cases, and p16 expression-based analysis cases). Clinical and pathological characteristics were obtained from the CRG database and collected for each case, mainly including demographical characteristics (covariates). Survival data of the p16 expression-based analysis cases were also collected and included in the anonymized database. Cases from the p16 expression-based analysis were classified as:

- *missing*, when the tumor sample was not found
- *non valuable*, when the tumor sample was found but the specimen was not of enough quality to perform the p16 expression IHC assay
- *p16-positive,* when the result of the p16 expression assay was considered as positive
- *p16-negative*, when the result of the p16 expression assay was considered as negative

The clinical data were handled in agreement with current confidentiality regulations inherent to the usual methodology of the CRG. The mutational results were also obtained anonymized to be incorporated into this database. All data were stored guaranteeing its confidentiality, security, and authenticity. Under no circumstances the information collected included data that would have allowed the patient's identity to be known, as each patient was only identified by a numerical code. In order to ensure the confidentiality of the study data, only the coordinating investigator and the team of investigators, the sponsor, the ethics committee, the competent health authorities, and the parties responsible for data analysis had access to the data. The documents generated during the study and the database was protected against unauthorized use by people outside the study and was therefore considered strictly confidential. The processing, communication, and transfer of the personal data of all participating study cases complied with the ethical aspects mentioned in *Section 11* and the provisions clarified in *Section 7.3.1.3*.

7.6. STATISTICAL ANALYSIS

We performed a descriptive analysis and estimated the incidence rates and trends in incidence for overall HNC and overall OPC. We then performed a p16 expression-based analysis including descriptive analysis and estimation of incidence rates, trends in incidence and survival rates of OPC in relation with p16 expression.

Descriptive statistics have been expressed as median and interquartile range (IQR) or mean and standard deviation (SD) for quantitative variables, and as absolute frequencies and percentages for qualitative variables.

Incidence has been estimated as Crude Rate (CR)[5], Age-Standardized to the European population Rate ($ASIR_E$)[6], and Age-Standardized to the WHO World population Rate ($ASIR_W$). The population of the province of Girona was provided by the IDESCAT (75) to

[5] Crude Rate: number of incident cases of cancer during the study period divided by the population studied. It is expressed as a number per 100.000 inhabitants per year.

[6] Adjusted Rate: a fictious summary rate statistically adjusted to remove the effect of age, to permit unbiased comparison between populations having different age structures. They should be understood as the rates that would occur in another population with an age structure equal to that used as the standard.

calculate the incidence rates. Age-standardized rates were calculated by stratifying the age into 18 groups of 5 years each using the 2013 European and World standard populations (10,80). Estimations were made with the direct method, as we had a large denominator (in our case the population of the province of Girona) and the frequency of events was not too reduced to get unstable rates. This method allowed to create comparable adjusted rates owing to the use of an identical standard population (in this case, the European and the World populations). We computed CR, $ASIR_E$, and $ASIR_W$ with the 95% Confidence Interval (CI) and results were expressed in 100.000 inhabitants per year.

The trends in the incidence have been assessed through the Annual Percent Change (APC) of $ASIR_E$, which indicates the average annual variation in rates expressed as a percentage increase or decrease. Joint-point statistical software was used to quantify the evolution of incidence with the APC and to evaluate changes in trends over time. The 95% confidence interval (95% CI) of the APC allowed us to evaluate the statistical significance or non-significance of the trend: if the CI contained 0, the trend was understood as statistically non-significant, whereas if the interval did not contain 0, it was considered that the trend increased (if the APC>0) or decreased (if the APC<0). In the study of trends over long periods of time such as in our study, the APC had a different behavior over time. In that case, turning points were detected and the APCs for separate time periods were assessed.

Observed survival (OS) estimates have been calculated using the Kaplan-Meier method. In all survival calculations, the estimate also had a CI of 95%. The long-rank test was used to determine statistical differences of OS curves according to p16 expression.

Statistical significance has been determined at p-value <0.05 for all analyses. Analyses have been performed using R version 4.0.3 (2020-10-10) and Joint-point Regression Program, Version 4.1.0 - April 2014, Statistical Research and Applications Branch, National Cancer Institute.

8. RESULTS

Figure 8 describes the workflow of the targeted study cases, samples collected, processed, tested, and included in the statistical analyses.

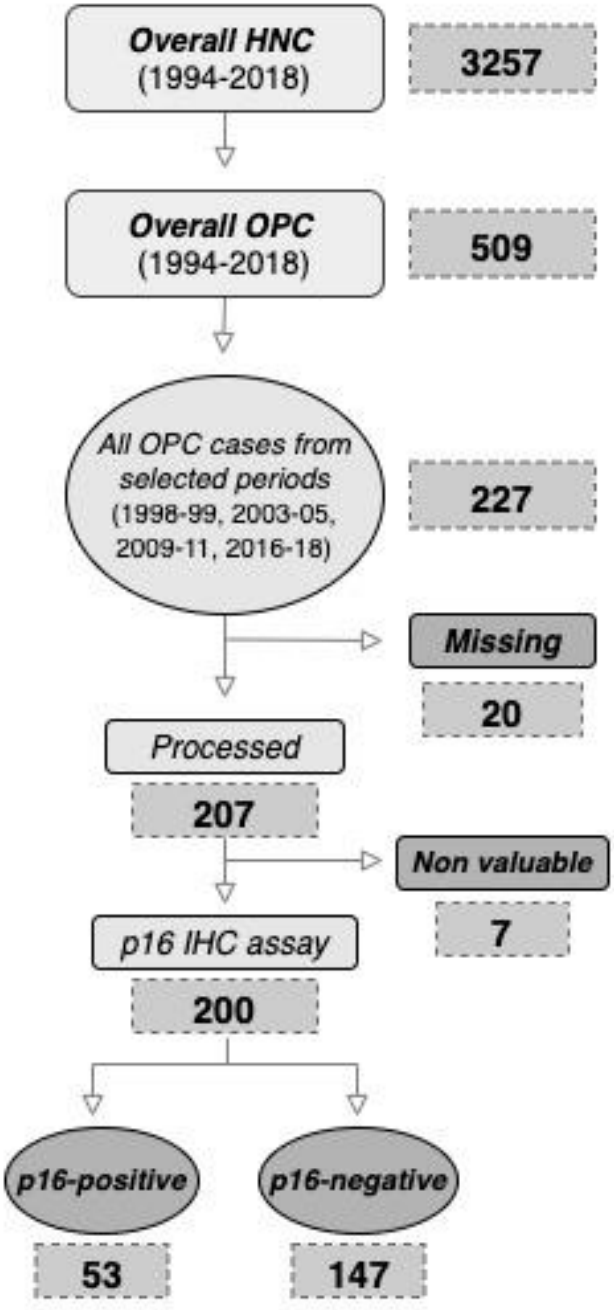


Figure 8. Study workflow. Hyphened boxes indicate the absolute number of cases belonging to each of the cohorts selected during the study circuit. *HNC: head and neck cancer; OPC: oropharyngeal cancer; IHC: immunohistochemistry.*

8.1. OVERALL HEAD AND NECK CANCER (1994-2018)

8.1.1. DESCRIPTIVE ANALYSIS

3257 cases of HNC were identified in the CRG database from the period 1994-2018. Demographical characteristics of this initial retrospective cohort have been summarized in *Table 4*. HNC was diagnosed at a mean age of 64 years and 5.8 times more in males than in females. Among the total, 85% of the cases were males while only 15% were females. Most part of the selected cases were laryngeal cancers (34.3%), followed by cancers of the oral cavity (21.6%) and oropharyngeal cancers (15.6%).

Table 4: *Demographical characteristics of head and neck cancer cases*

HEAD AND NECK CANCER (1994-2018)	**N=3257**
	n (%)
Sex	
Male ----------	2780 (85.4)
Female ----------	477 (14.6)
Age	
Median [IQR] ----------	64 [55-74]
Mean ± SD ----------	64.1 ± 12.7
Minimum, Maximum ----------	16, 102

IQR: interquartile range; SD: standard deviation; N/n: absolute number of cases

8.1.2. INCIDENCE RATES

Crude and adjusted incidence rates of HNC in the period 1994-2018 are presented in *Table 5*, stratified by sex. In our case series, the overall CR was 19.91 new cases per 100,000 inhabitants/year, and the $ASIR_E$, 22.37 new cases per 100,000 inhabitants/year. A substantial difference between males and females was observed, which was more notable in age-standardized rates. If $ASIR_E$ is considered, HNC was 6.7 times more incident in males than in females, and if $ASIR_W$ is looked upon, the rates were 7.3 times higher in males.

Table 5: *Incidence rates of overall head and neck cancer (1994-2018), stratified by sex*

	CR (95% CI)	**$ASIR_E$** (95% CI)	**$ASIR_W$** (95% CI)
Males	**33.94** (32.68 - 35.20)	**40.34** (38.84 - 41.91)	**21.32** (20.50 - 22.18)
Females	**5.84** (5.32 - 6.37)	**6.03** (5.50 - 6.61)	**2.94** (2.64 - 3.27)
Overall	**19.91** (19.23 - 20.6)	**22.37** (21.61 - 23.16)	**11.96** (11.53 - 12.42)

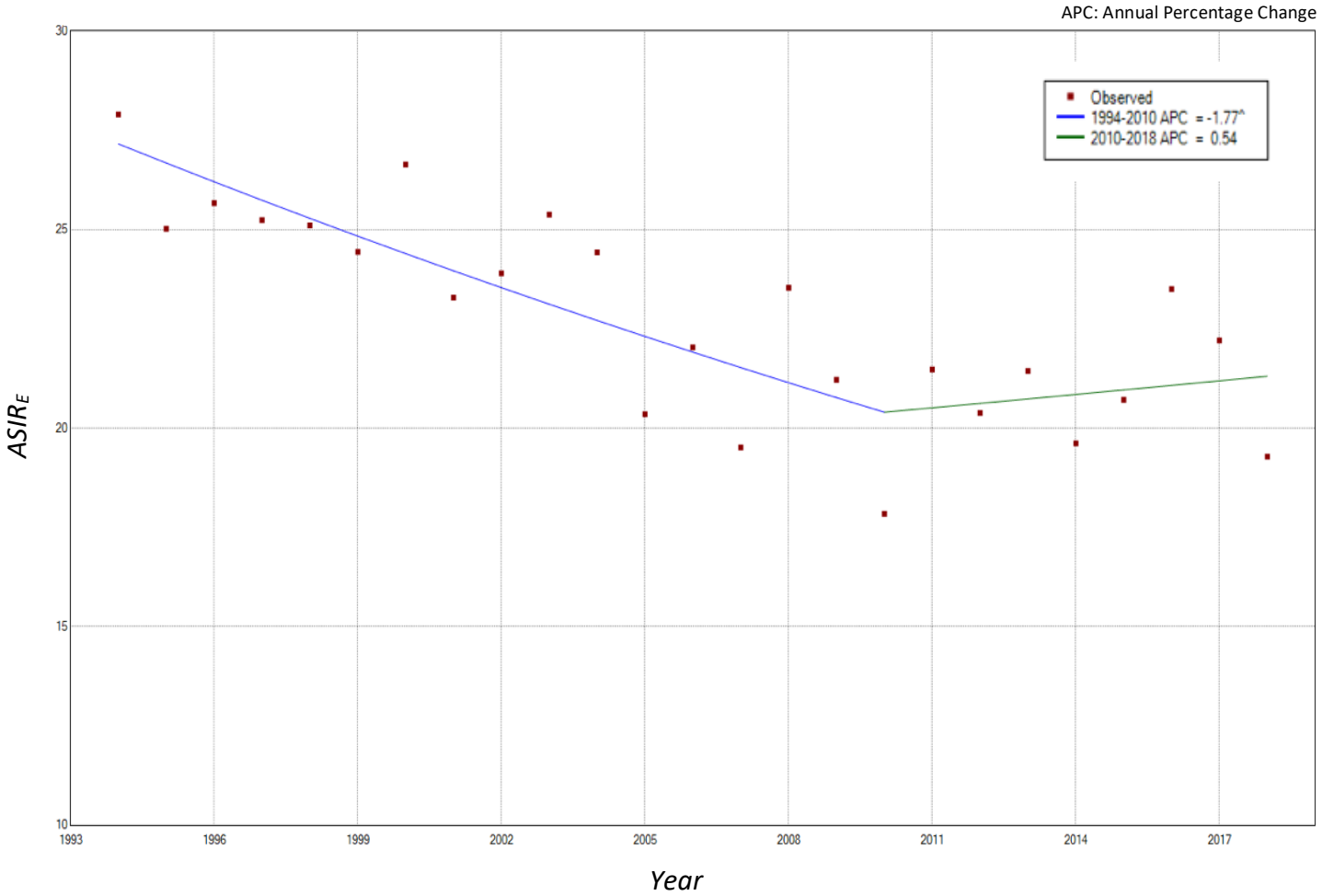
Rates per 100,000 inhabitants per year. CR: crude rate; CI: confidence interval; $ASIR_E$: age-standardized rate to the European population; $ASIR_W$: age-standardized rate to the World population.

8.1.3. TRENDS IN INCIDENCE

For the whole period 1994-2018, HNC incidence decreased significantly in both sexes. In the global Joint-point analysis of $ASIR_E$ trends, an overall APC of -1.2 (95% CI: -1.6; -0.7) was detected, as *Annex 6* depicts.

The Joint-point analysis of $ASIR_E$ computed to assess specific turning points in trends resulted in a significant decrease in incidence from 1994 to 2010 with an APC of -1.8 (95% CI: -2.5; -1.0) and a non-significant increase in incidence from 2010 to 2018 with an APC of 0.5 (95% CI: -1.6; 2.7). These results are portrayed in *Figure 9.*

Figure 9. *Trends in incidence of overall head and neck cancer, from 1994 to 2018. Joint-point analysis of age-standardized to the European population incidence rates ($ASIR_E$) assessing specific turning points.*



8.2. OVERALL OROPHARYNGEAL CANCER (1994-2018)

8.2.1. DESCRIPTIVE ANALYSIS

509 cases of OPC were identified in the CRG database from the period 1994-2018. Demographical characteristics of this retrospective cohort have been summarized in *Table 6*. OPC was diagnosed at a mean age of 61 years and 5.9 times more in males than in females. Among the total, 85% of the cases were males while only 15% were females, a distribution analogical to that observed in HNC. Most of the cases were diagnosed at the tonsil (41.4%). Tumors from the base of the tongue and other oropharyngeal locations accounted for a similar proportion of the sites of diagnosis (23.2% and 26.7% respectively). Meanwhile, a low share of the cases was diagnosed at the soft palate (8.6%).

Table 6: *Demographical and topographical characteristics of oropharyngeal cancer cases*

OROPHARYNGEAL CANCER (1994-2018)	**N=509**
Characteristics	n (%)
Sex	
Male	435 (85.5)
Female	74 (14.5)
Age	
Median [IQR]	60 [52-69]
Mean ± SD	60.9 ± 11.2
Minimum, Maximum	32, 93
Topographical site	
Base of the tongue	118 (23.2)
Soft Palate	44 (8.6)
Palatine tonsil	211 (41.4)
Oropharynx	136 (26.7)

IQR: interquartile range; SD: standard deviation; N/n: absolute number of cases

8.2.2. INCIDENCE RATES

Crude and adjusted incidence rates of OPC in the period 1994-2018 are presented in *Table 7*, stratified by sex. In our study cohort, the overall CR was 3.11 new cases per 100,000 inhabitants/year, and the $ASIR_E$, 3.48 new cases per 100,000 inhabitants/year. A substantial difference between males and females was noticed, which was more notable in age-standardized to the European population rates: according to $ASIR_E$, OPC was 6.1 times more incident in males than in females.

Table 7: *Incidence rates of overall oropharyngeal cancer (1994-2018), stratified by sex*

	CR (95% CI)	**$ASIR_E$** (95% CI)	**$ASIR_W$** (95% CI)
Males	**5.31** (4.81-5.81)	**6.13** (5.56–6.75)	**3.50** (3.17–3.88)
Females	**0.91** (0.7-1.11)	**1.00** (0.79-1.26)	**0.59** (0.46–0.78)
Overall	**3.11** (2.84–3.38)	**3.48** (3.19–3.80)	**2.04** (1.86-2.24)

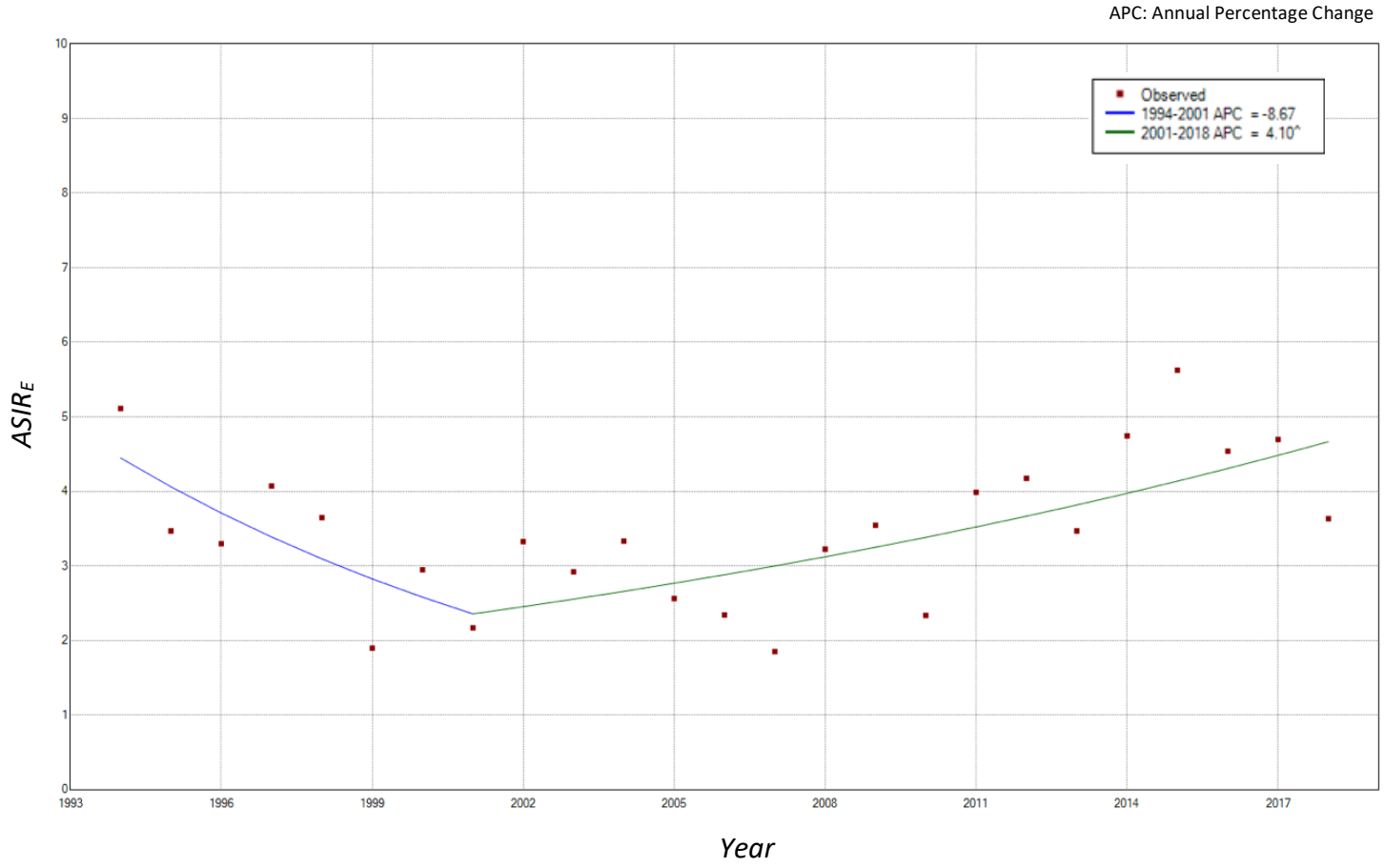
Rates per 100,000 inhabitants per year. CR: crude rate; CI: confidence interval; $ASIR_E$: age-standardized rate to the European population; $ASIR_W$: age-standardized rate to the World population.

8.2.3. TRENDS IN INCIDENCE

During the period 1994-2018, OPC incidence had a non-significant increase in both sexes. In the global Joint-point analysis of $ASIR_E$ trends, an overall APC of 1.2 (95% CI: -0.5; 2.7) was obtained, as *Annex 7* depicts.

Nonetheless, the Joint-point analysis of $ASIR_E$ computed to assess specific turning points in trends resulted in a significant increase in incidence from 2001 to 2018 with an APC of 4.1 (95% CI: 1.6; 6.7), but a non-significant decrease in incidence from 1994 to 2001 with an APC of -8.7 (95% CI: -16.8; 0.2). These results are portrayed in *Figure 10*.

Figure 10. *Trends in incidence of overall oropharyngeal cancer, from 1994 to 2018. Joint-point analysis of age-standardized to the European population incidence rates ($ASIR_E$) assessing specific turning points.*



8.3. P16 EXPRESSION-BASED ANALYSIS OF OROPHARYNGEAL CANCER

8.3.1. DESCRIPTIVE ANALYSIS

227 cases of OPC were identified in the CRG database from the selected periods of 1998-1999, 2003-2005, 2009-2011, and 2016-2018. They were classified according to the criteria described in *Section 7.5*. We obtained: 53 *p16-positive* cases (23.3%), 147 *p16-negative* cases (64.8%), 20 *missing* cases (8.8%), and 7 *non valuable* cases (3.1%).

We focused the analysis on the *missing* cases in comparison to those cases that were processed (with a valuable determination or not), and found no differences by age, sex, site, or histology. Interestingly, a significantly difference of distribution among the different periods was observed between the two groups, as depicted in *Table 8*. In our series, a high proportion of *missing* cases (30%) was obtained in the earliest period (1998-1999), which decreased (to 16.7%) in the following period (2003-2005) and was much lower in the most recent periods.

Table 8: *Missing versus non-missing cases included in the p16 expression-based analysis, stratified by calendar period*

	TOTAL	***MISSING***	***NON-MISSING***	*p value*
	N=227	**N=20**	**N=207**	
Period	**n (%)**	**n (%)**	**n (%)**	
1998-1999	26 (11.4)	8 (30.8)	18 (69.3)	<0.001
2003-2005	48 (21.1)	8 (16.7)	40 (83.3)	
2009-2011	65 (28.6)	3 (4.6)	62 (95.4)	
2016-2018	88 (38.8)	1 (1.1)	87 (98.9)	

N/n: absolute number of cases

The percentage of *p16-positive* cases among all selected cases was 23.3%, whilst the percentage of *p16-positive* cases among those that underwent the p16 IHC analysis was 26.5%.

Clinical and anatomopathological characteristics of the *p16-positive* and *p16-negative* cohorts have been summarized in *Table 9*. Significant differences between the two groups were observed regarding sex, topographical site, histology type, calendar period, and death. In contrast, age at diagnosis was not found to be significantly different in the two cohorts. In both cohorts there were more cases in males compared to females, but the ratio was higher in the p16-negative cohort. p16-negative cases were found to be 7.6 times more frequently in males than in females, whereas p16-positive cases were found only 2.5 times more in males. Interestingly, the percentage of p16-positive cases among the total of cases studied in each sex was higher in females than in males: 44% in females versus 20% in males. The mean age of diagnosis of the two groups was similar, finding no significant differences in that matter. Distribution of topographical site did significantly differ in both cohorts. Most of the p16-positive cases occurred in the palatine tonsil and base of the tongue (77.3%) while in p16-negative cases these two subsites accounted for only half of the cases (53%). Histology type was found to be significantly different between both groups as well, although this may be due to lack of specification when classifying specimens, as most of the cases in both groups were classified as *not otherwise specified (NOS) SCC* (see *Table 3*), and the proportion of these was higher in the p16-negative cohort. Aside from this *NOS* histological category, *keratinizing SCC* histology was more predominantly observed in p16-negative cases whereas *non-keratinizing SCC* histology was more associated to p16-positive cases. Of note, a difference of distribution regarding calendar periods was detected in-between the two cohorts. 65% of the p16-negative cases evaluated belonged to the first three period slices (1998-1999, 2003-2005, and 2009-2011), while the remaining 35% was from the last period (2016-2018). In contrast, the opposite distribution was observed in p16-positive cases, in which 60% belonged to the last period, whereas the remaining 40% was from the other three calendar periods. Despite this, in each period selection, there were always more p16-negative cases diagnosed. Lastly, a significant difference was

observed regarding death. Twice as many patients from the p16-positive cohort were alive rather than dead, while an inverse correlation was found in the p16-negative group, in which 3.3 times as many patients were found to be death rather than alive.

Table 9: *Description of cases included in the p16 expression-based analysis*

	TOTAL*	***P16 NEGATIVE***	***P16 POSITIVE***	*p-value*
	N=227	**N=147**	**N=53**	
	n (%)	n (%)	n (%)	
Sex				
Males	193 (85.0)	130 (88.4)	38 (71.7)	**0.004**
Females	34 (15.0)	17 (11.6)	15 (28.3)	
Age				
Median [IQR]	60 [54-70]	59 [54-70]	63 [55-70]	0.622
Mean ± SD	61.3 ±11.3	61.5 ± 11.4	62.4 ± 11.1	
Minimum, Maximum	33, 87	39, 87	33, 84	
Topographical site				
Base of Tongue	44 (19.4)	26 (16.3)	12 (22.6)	**0.010**
Soft Palate	26 (11.4)	22 (15.0)	1 (1.9)	
Palatine Tonsil	90 (39.6)	54 (36.7)	29 (54.7)	
Oropharynx	67 (29.5)	47 (32.0)	11 (20.7)	
Period				
1998-1999	26 (11.4)	16 (10.9)	2 (3.8)	**0.011**
2003-2005	48 (21.1)	32 (21.8)	7 (13.2)	
2009-2011	65 (28.6)	48 (32.6)	12 (22.6)	
2016-2018	88 (38.8)	51 (34.7)	32 (60.4)	

IQR: interquartile range; SD: standard deviation; N/n: absolute number of cases. (*): "Total" refers to all cases included in the p16 expression-based analysis, which involve *missing* and *non valuable* cases as well.

8.3.2. INCIDENCE RATES

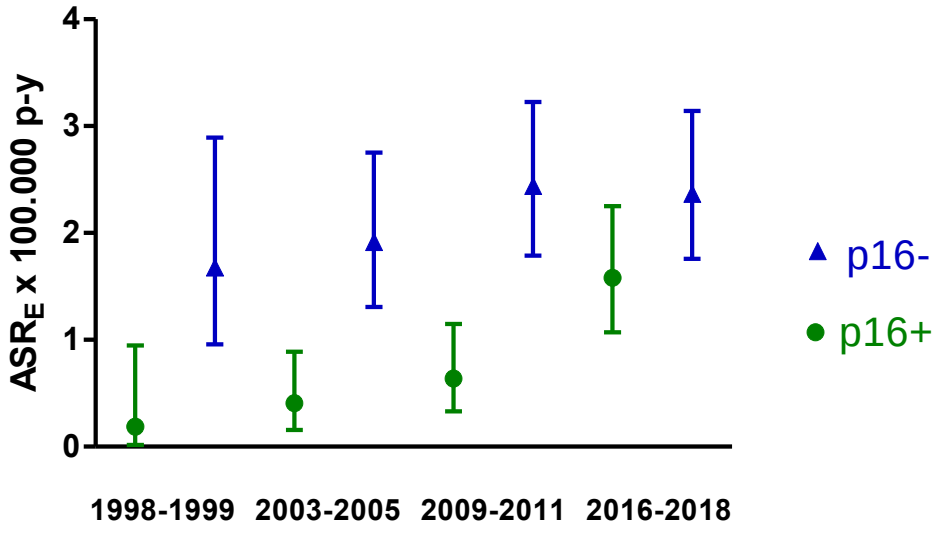
Crude and adjusted incidence rates of *p16-positive* and *p16-negative* cohorts in the periods 1998-1999, 2003-2005, 2009-2011, and 2016-2018 are presented in *Table 10*, stratified by calendar period. Incidence rates in the *p16-positive* cohort increased concordantly to the calendar periods. Of note, a substantial change in the $ASIR_E$ of this cohort was observed between the last two periods, in which the incidence almost tripled. The incidence rates from the *p16-negative* cohort increased along calendar periods as well, but not as sharply. Contrarily to the other cohort, the highest $ASIR_E$ of this group was found in the calendar period 2009-2011, finding a decreased rate in the most recent period 2016-2018. A visual display of these disparities in $ASIR_E$ in each of the calendar periods is given in *Figure 11*. Overall, higher incidence rates were found in the *p16-negative* cohort compared to the *p16-positive* cohort.

Table 10: *Incidence rates of p16-positive and p16-negative oropharyngeal cancer, stratified by calendar period*

	CR (95% CI)		**$ASIR_E$** (95% CI)	
Period	*P16+*	*P16-*	*P16+*	*P16-*
1998-1999	**0.18** (0.03-0.61)	**1.48** (0.88-2.35)	**0.19** (0.02-0.95)	**1.68** (0.96-2.89)
2003-2005	**0.37** (0.16-0.73)	**1.69** (1.18-2.36)	**0.41** (0.16-0.89)	**1.92** (1.31-2.75)
2009-2011	**0.53** (0.29-0.91)	**2.14** (1.60-2.82)	**0.64** (0.33-1.15)	**2.44** (1.79-3.26)
2016-2018	**1.43** (0.99-1.99)	**2.28** (1.71-2.97)	**1.58** (1.07-2.25)	**2.37** (1.76-3.14)

Rates per 100,000 inhabitants per year. CR: crude rate; CI: confidence interval; $ASIR_E$: age-standardized rate to the European population.

***Figure 11**. Age-standardized to the European population incidence rates ($ASIR_E$) in each of the calendar periods.*



P-y: person-year. ASR_E: age-standardized to the European population incidence rates.

8.3.3. TRENDS IN INCIDENCE

During the period 1998-2018, incidence of *p16-positive* OPC increased significantly, whereas a non-significant increase of incidence of *p16-negative* OPC was observed. Joint-point analysis of the $ASIR_E$ from the studied calendar periods of the *p16-positive* cohort detected an APC of 12.8 (CI 95%: 8.9; 16.8), as *Figure 12* illustrates. In contrast, the same analysis of the $ASIR_E$ of the *p16-negative* cohort resulted in an APC of 2.1 (CI 95%: -1.4; 5.8), as *Figure 13* portrays. Therefore, a difference in trends in incidence between the two cohorts was detected, in which a significant increase with substantial magnitude was observed in the incidence of the *p16-positive* cohort opposed to the *p16-negative* cohort, which experienced no significant changes in incidence.

***Figure 12**. Trends in incidence of p16-positive oropharyngeal cancer, from 1998 to 2018. Joint-point analysis of age-standardized to the European population incidence rates ($ASIR_E$).*

APC: Annual Percentage Change

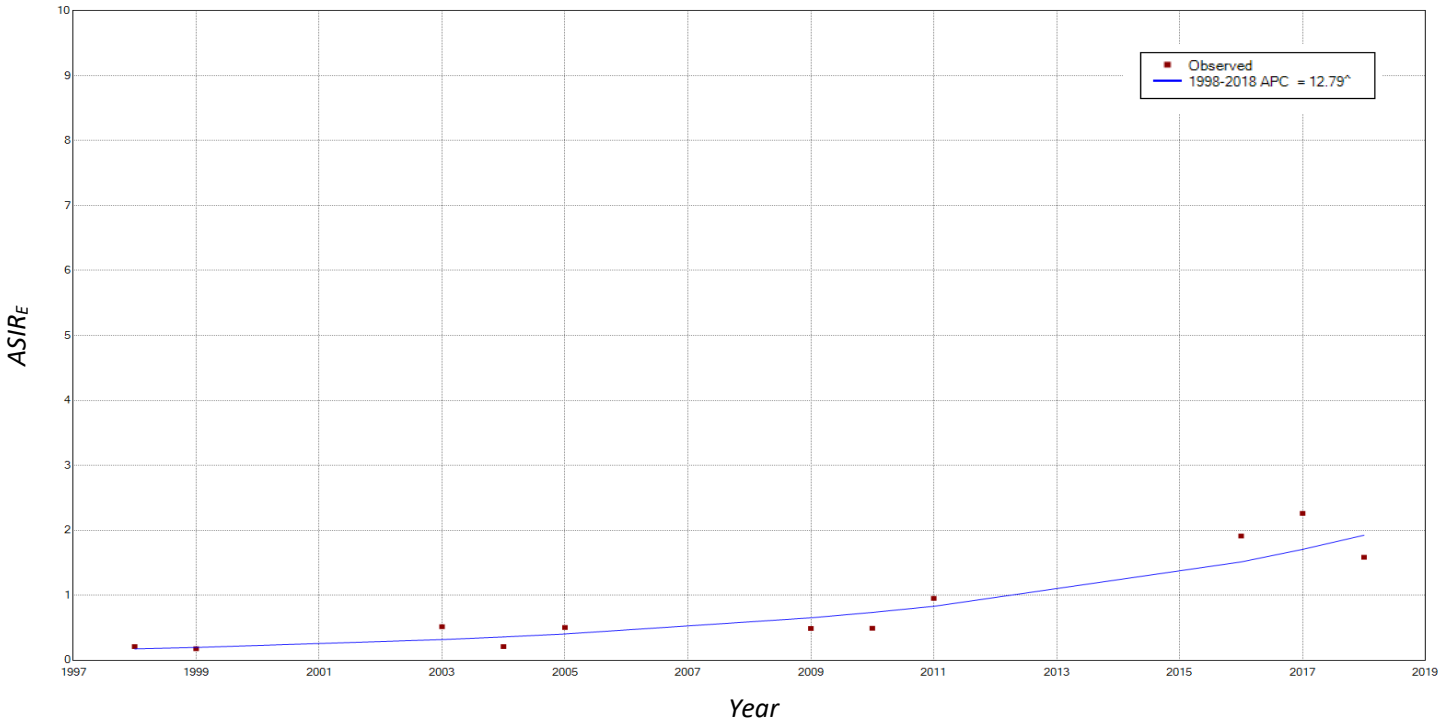
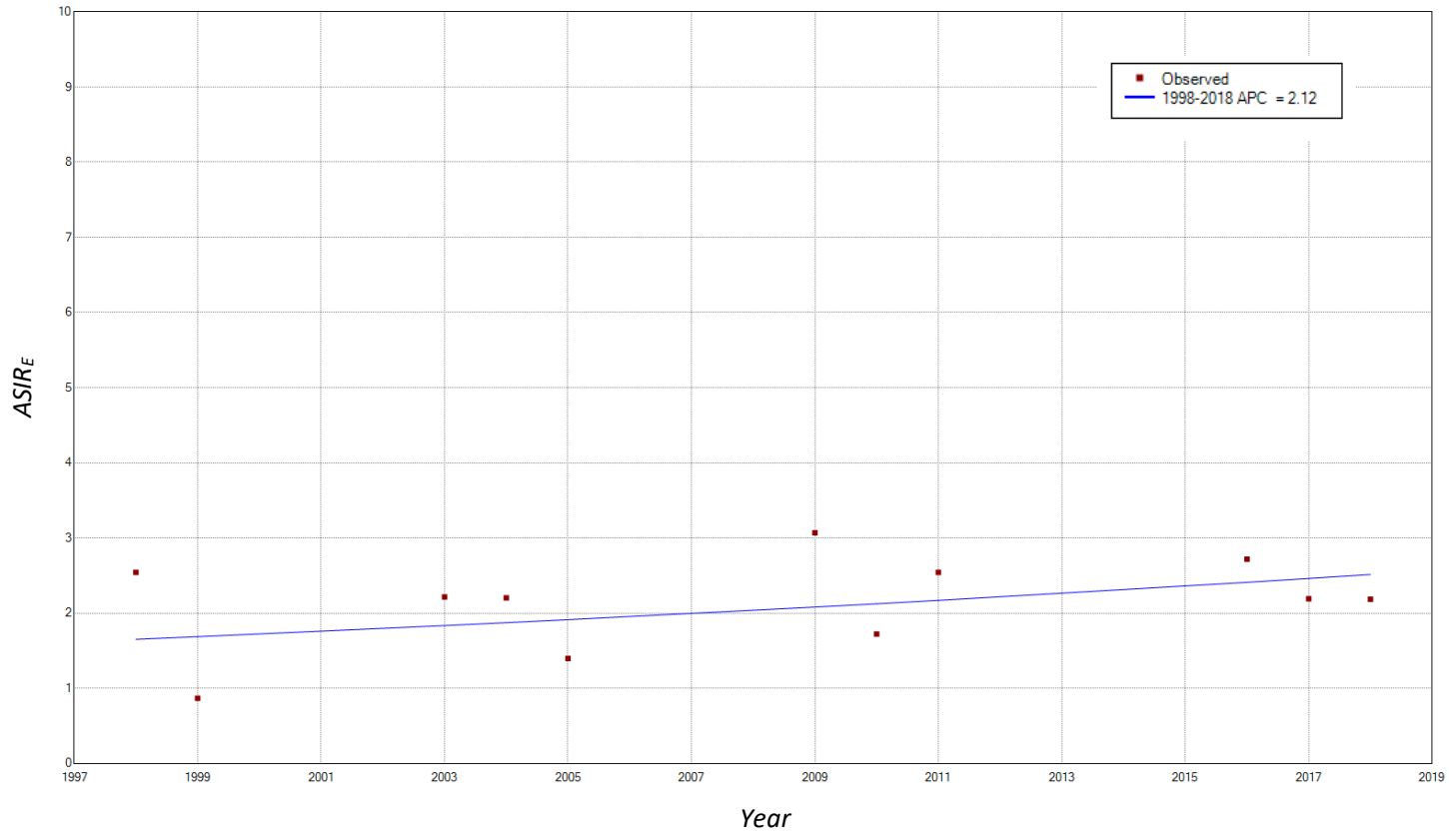


***Figure 13.** Trends in incidence of p16-negative oropharyngeal cancer, from 1998 to 2018. Joint-point analysis of age-standardized to the European population incidence rates ($ASIR_E$).*

APC: Annual Percentage Change



8.3.4. SURVIVAL

5-year observed survival (5-y OS) rates for *p16-positive* and *p16-negative* cohorts are presented in *Table 11*, stratified by sex. *Figure 14* gives a visual display of the overall 5-year OS in both sexes, while comparison between the 5-y OS of the *p16-positive* and *p16-negative* cohorts may be observed in *Figure 15*. Overall and in both sexes, half of the patients were still alive 5 years from diagnosis, as Kaplan-Meier curve depicts in *Figure 14*. The long-rank test showed a significant difference in 5-y OS between the two cohorts, as illustrated in *Figure 15*. In the *p16-positive* cohort, it was observed that 64.4% of the patients were still alive 5 years from diagnosis. Meanwhile, the probability of survival in the *p16-negative* cohort was much lower, being only 43.4% the proportion of patients alive 5 years from diagnosis.

5-y OS differed by sex in both cohorts. In the *p16-negative* cohort, higher survival rates were observed in males than in females, whereas the opposite was seen in the *p16-positive* cohort. This difference of 5-y OS between sexes was strongly marked in the *p16-negative* cohort (39.9% vs 14.7%, a difference of 25.2%), but it was of little magnitude in the *p16-positive* cohort (71.1% vs 67.0%, a difference of 4%).

Table 11: *5-year Observed Survival rates according to p16 expression, stratified by sex*

	MALES (95% CI)	***FEMALES*** (95% CI)	***BOTH SEXES*** (95% CI)
p16 positive	**67.0 %** (53.9-83.3)	**71.1 %** (50.5-100)	**64.8 %** (48.8-86.2)
p16 negative	**39.9 %** (32.5-42.0)	**14.7 %** (4.3-50.1)	**43.4 %** (35.5-53.0)
Overall	**47.8 %** (41.4-55.1)	**37.6 %** (23.1-61.2)	**49.5 %** (42.7-57.4)

CI: confidence interval.

Figure 14. *Overall 5-year Observed Survival of oropharyngeal cancer cases included in the p16 expression-based analysis (1998-2018).*

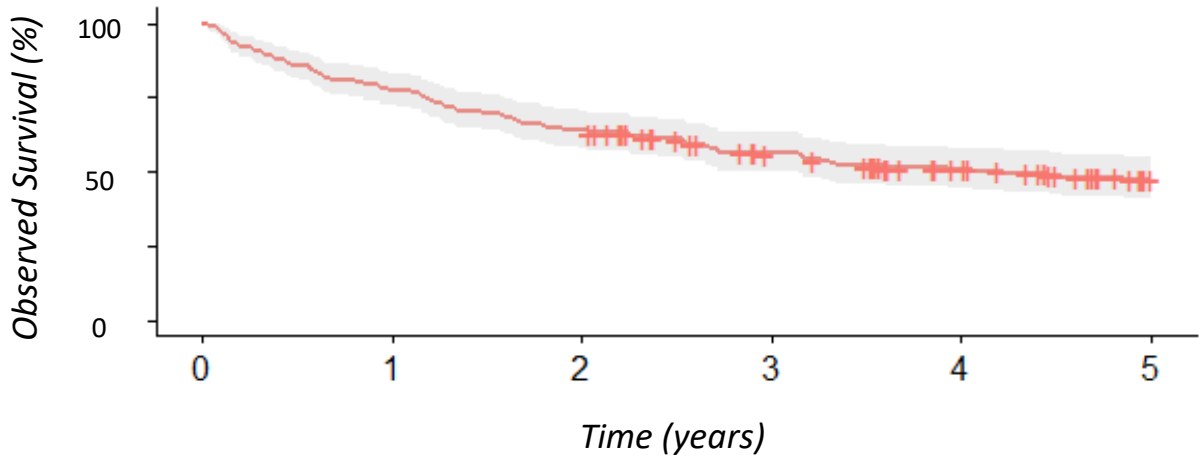
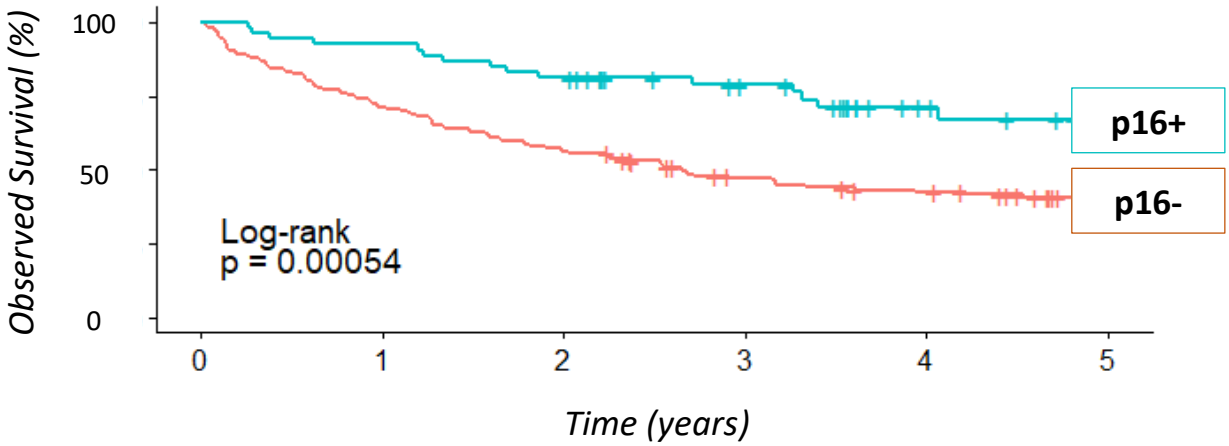


Figure 15*. 5-year Observed Survival according to p16 expression.*



9. DISCUSSION

We present the study of the trends in the incidence of overall HNC and overall OPC from 1994 to 2018 in the province of Girona through a Joint-point analysis assessing specific turning points as we consider it resulted in the best statistical model for result appraisal. We observed a statistically significant decrease in the incidence of overall HNC from 1994 to 2010, but a non-significant increase in the period 2010-2018. In parallel, we detected a significant increase in the incidence of overall OPC in the period 2001-2018 and a previous non-significant decrease from 1994 to 2001. These changes in epidemiological trends could be attributed to the shift in HNC and OPC etiology, in which tobacco and alcohol use have been declining in importance at the expense of oral HPV infection. A reduction in tobacco consumption has been noticed in Spain according to the Spanish National Health Survey, as the prevalence of daily smoking among Spaniards experienced a decline from 34% in 1995 to 24% in 2011 (81). This temporal variation in the traditional HNC risk factor could explain the diminishment of overall HNC cases observed in our study from 1994 to 2010. New regulations on tobacco were introduced that same year, 2010, and public health efforts became more intense, which caused a reduction of tobacco use to 20% at present. With the reported drastic drop of smoking, a further decrease in HNC would be expected from 2010-2018, but we did not observe it. Otherwise, we observed an increase in overall OPC from 2001 to 2018. Therefore, the absence of decrease in incidence observed in overall HNC from 2010 to 2018 seems to be attributable to the increase of OPC cases detected. Concordantly, the decrease in HNC is probably not ascribable to OPC, as a significant decrease in OPC was not seen.

A significant increase in incidence was observed as well in the HPV-related OPC cohort studied in the p16 expression-based analysis, while no changes were observed in the HPV-unrelated cohort. We hypothesized HPV-related incidence would increase as HPV-unrelated OPC incidence would decrease. However, when incidence trends of the two cohorts were reviewed, an increase in both was detected (from 1998 to 2018), although it was not significant in the HPV-unrelated group. On the one hand, this contrasts with the non-significant decrease in incidence of overall OPC observed from 1994 to 2001. Most

likely, the fact that the p16 expression-based analysis was carried out with cases from 1998 as the earliest year (instead of 1994) explains why a marked initial decrease in the incidence was not observed in any of the two cohorts. On the other hand, the detected non-significant increase in the HPV-unrelated cohort supports that there is still a dominant effect of smoking and that the impact of anti-tobacco measures is not seen yet. Nonetheless, a future decrease of incidence in HPV-unrelated OPC may be expected, as the $ASIR_E$ incidence obtained in the last calendar period insinuates a decreasing tendency.

We observed a difference of distribution of cases between the two cohorts regarding calendar periods, in which most of the HPV-unrelated cases belonged to earlier calendar periods and most of the HPV-related belonged mainly to the latest calendar period. Furthermore, the incidence rates for the HPV-related cohort experienced a 2.5-fold increase from the third (2009-2011) to the last (2016-2018) calendar periods. These findings, along with the magnitude of the increase in incidence in this cohort (APC of 12.8), justify how changes in recent times regarding sexual behaviors, likewise to those in tobacco habits, likely underlie the shift noticed in the obtained incidences. Sexual behavior has varied considerably for the past decades in Spain. An increase in number of sexual partners and a decrease in the median at first sexual intercourse has been reported across birth generations (82). Sexual behavior, such as higher number of lifetime sexual partners, younger age at first sexual intercourse and practice of oral sex, is a surrogate for HR HPV exposure. Thus, changes in sexual practices have potentially led to the higher incidence rates of HPV-related OPC observed in our study.

To our knowledge, this is the first population-based assessment of trends in incidence and survival of HPV-related OPC in a region of Southern Europe. Our results indicate that overall OPC incidence has started to sharply increase in the most recent years in our region, similarly to the observed increase in North America, Northern Europe, Japan, and Australia between the 1980s and the 2000s (11), and in concordance with the only population-based study of OPC incidence conducted in Spain to date (which did not discriminate between HPV-related and HPV-unrelated OPC) (23). We novelly report that

this increase observed, which has occurred concurrently to a decrease of overall HNC incidence, is most likely due to the increase in HPV-related OPC we detected in our setting.

HPV-related OPC is increasing in the province of Girona, about 20 years later than in other countries such as the United States (37) or Norway (83), equivalently to what has been observed in another region from Italy (22). This decalage is probably due to the difference in smoking drops and in the introduction of new sexual behaviors in between geographical regions across time. Tobacco consumption did experience a significant diminishment between the 1980s and the 2010s in countries from North America and Northern/Western Europe in contrast with Spain, where it did not experience a significant change (84). In addition, sexual revolution around the 1960s was not as prominent among Spaniards as it was in these mentioned regions, but a later change in social acceptance of sexual behaviors took place in the post-dictatorship era (1980s) otherwise.

$ASIR_W$ for OPC observed in our setting were higher than those that had been estimated in 2018 for Southern Europe (18). Even so, they were lower than the ones estimated for Northern/Eastern/Western Europe, as well as Northern America and Australia (82). Of notice, we found laryngeal and oral cavity cancers to still (4) be more frequent than OPC in our setting, in contrast with the distribution of proportion of HNC types that these other countries with higher burden of OPC have.

The observed percentage of HPV-related OPC cases among all OPC cases in our setting was 23.3%, considering all selected cases, and 26.5%, considering those cases that underwent the p16 IHC analysis. These percentages were similar to the overall worldwide fractions of HPV-driven OPC, 18.5%-22.4%, obtained in an international cross-sectional study including Spanish hospitals (19). Nonetheless, they were higher than the fractions observed for Southern Europe alone in that same study, which ranged from 7.6% to 9.4%, and those reported in other recent Spanish studies, in which the prevalence of HPV in OPC was 10% (21) and 6.1% (20). In contrast, they were lower than that newly reported in Italy, 32.3% (22). The higher percentage obtained in our cohort could be explained by the difference in the HPV detection method (as the other studies used double positivity to both HPV-DNA and p16 expression). The lower percentage in comparison to the Italian study

could be attributed to the higher number of patients we had, as well as the inclusion of cases from earlier calendar periods. Notwithstanding, results obtained are still inferior to those in other regions outside from Southern Europe, contrasting with those percentages of HPV in OPC from countries in Northern Europe and North America (as it may be observed in *Annex 3*). Indeed, great range of HPV-related OPC varies across geographical regions alike to sexual behavior, finding lower average age at first sexual intercourse, higher number of sexual partners (18) and higher proportion of ever having oral sex (85) in countries were HPV-related OPC is most common than HPV-unrelated OPC.

HPV-related OPC has been commonly depicted as an entity of higher burden in young males (11), and even though the increase in the incidence of this disease was initially reported as marked in this specific profile of patients, some differences were observed in our cohort.

Significant differences between the HPV-related and HPV-unrelated OPC cohorts were observed regarding sex. As most of the studies at present had reported in other regions, HPV-related OPC was more frequent in males in comparison to females, with alike percentages of distribution (49).

HPV-unrelated OPC was found to be 7.6 times more frequently diagnosed in males than in females, most likely due to the higher daily smoking prevalence observed in males compared to females in Spain (84). In contrast, HPV-related OPC had a 2.5-fold higher incidence in males. This greater impact of HPV-related OPC in males is likely explained by higher oral HR HPV infection rates in males versus females. Difference in sexual behaviors by sex has been reported among Spaniards, in which males practice oral sex in a higher proportion and a have higher average number of lifetime sexual partners in comparison to females (85,86). Yet, it is important to consider these features do not solely account for the disparities between males and females, as inherent biology plays a role as well. Our findings on the male-to-female ratio of the HPV-related OPC cohort being lower than that in the HPV-unrelated cohort contrast with those reported previously in the USA (2,12), in which there is higher sex disparity in HPV-related OPC in comparison to HPV-unrelated OPC.

Geographical differences regarding divergence between sexes may be mainly a consequence of the variation in male smoking habits. It is of interest to consider that the magnitude of sex disparities may be expected to change in the future, because of the universal HPV vaccination program being implemented only in females in our setting and transition to new norms of sexuality.

Interestingly, the percentage of HPV-related cases among the total of cases assessed in each sex was substantially higher in females (44%) than in males (20%), in concordance with reported data in Spain (21), Italy (22) and in an international setting (19), but in contrast with what was observed in the USA (37) or the UK (49). Of note, in the Spanish setting (21) these sex disparities disappeared after adjustment for cofounders, as females had lower smoking rates that biased the percentages obtained. Therefore, we cannot rule out the possibility of other cofactors being involved in these findings. Even though females had a higher attributable proportion than males in our study population, disease burden in females was still lower, as incidence rates were inferior.

Age at diagnosis was not found to be significantly different between HPV-related and HPV-unrelated OPC in our setting. The mean age of diagnosis of the two groups was similar, surrounding 61 years. We hypothesized incidence of HPV-related OPC would be most pronounced at younger individuals in comparison to those with HPV-unrelated OPC, a characteristic attributable to changes in sexual norms and fewer tobacco-associated cancers in younger birth generations. However, this effect was still not seen. We cannot rule out the possibility that our findings are related to birth-cohort-specific behaviors that would not be seen in individuals of older or younger generations. Generally, it is accepted that oral oncogenic HPV infection has a higher prevalence at 30-year-olds, increasing by age from there; estimations according to latency periods of approximately 10 to 30 years seem to contribute to an age at diagnosis surrounding 60 years, as we observed in our series.

These findings regarding age at diagnosis observed in our setting are in accordance with previous data reported in Spain (20) and Italy (22), and in contrast to the other recent reported data from Spain (21), in which HPV-related OPC was found to be higher in younger patients, as observed in high burden regions (2,37,49). Interestingly, the association found

between HPV-related OPC and younger age observed in the Spanish study was only present with cases that had double positivity for p16 expression and HPV-DNA, and was otherwise absent in cases that showed positivity for p16 expression alone. Accordingly, these age discrepancies could be explained by a higher accumulation of mutations with age in the case of p16 positive/HPV-DNA negative patients, which we did not detect in our study as we used p16 positivity alone. There is controversy on how the distribution of increase in HPV-related OPC incidence behaves regarding age, as some studies report predominant increase of this disease in younger ages (22) whilst other recent publications indicate a rising proportion of older patients (41). We did not stratify incidence according to age groups; therefore, we cannot conclude predominancy of increase in certain age cohorts in our setting.

Other significant differences between the two cohorts were observed regarding topographical site and histology type. As expected (21), the HPV-related cases occurred mainly in the palatine tonsil and base of the tongue, as opposed to the HPV-unrelated cohort. Likewise (47), keratinizing SCC histology was more predominantly observed in the HPV-unrelated cases whereas non-keratinizing SCC histology was more associated to HPV-related cases. These findings in topography and histology are concordant to the expected clinical and morphological differences between the two OPC entities, but are highly susceptible to bias, as the possibility of lack of specification and misclassification of subsites needs to be considered.

Lastly, as it has been known for a long time (38), our series confirm a better observed survival in HPV-related OPC in comparison to HPV-unrelated OPC (5-year OS: 64.8% versus 43.4%), like it has already been reported in other regions (22,37,49). This better outcome might be affected by several factors, such as tobacco use and alcohol consumption, sex, social economic status, performance status, comorbidities, HPV inherent biology, disease stage, and received treatment among others. However, in our study setting, it does not seem to be attributable to younger age. Survival also differed by sex in the two groups, as

higher survival rates were observed in females than in males in the HPV-related OPC cohort, whereas the opposite was seen in the HPV-unrelated OPC cohort. This difference of 5-y OS between sexes was strongly marked in the HPV-unrelated cohort, in contrast to the HPV-related cohort in which rates differed just slightly. We note our study is not a multivariant analysis of prognostic factors and, therefore, we do not assess the involvement of the mentioned possible cofactors in survival.

What's new?

A shift in etiology in oropharyngeal cancer has caused a change in the epidemiological trends of HNC: whilst overall HNC incidence has decreased, OPC incidence has sharply increased, giving rise to an epidemic of a new subtype of OPC related to HPV. The emergence of this entity has predominantly been studied in regions of high burden of oral HPV infection, such as North America and Northern/Western Europe, but little is known about the onset of HPV-related OPC in Southern Europe. To our knowledge, this is the first population-based assessment of trends in incidence and survival of HPV-related OPC in a region of Southern Europe. Our results indicate that epidemiological trends have begun to change similarly to those described in other geographical areas, but with a decalage of two decades. In addition, better survival has been observed in HPV-related OPC in comparison to HPV-unrelated OPC in our setting, as well as significant differences of distribution regarding sex. No differences have been found according to age at diagnosis between the two subtypes of OPC.

Limitations in our study are several. We decided to use p16 IHC expression as our only surrogate for HPV detection, for budgetary and practical reasons. It is important to consider that correlation between p16 positivity (when defined as nuclear and cytoplasmic staining in ≥ 70% of the tumor, as we did) and HPV presence is approximately of 90% (63). Therefore, we assume around 10% of the cases we classified as *p16 positive* are probably not HPV-driven (63). We are aware this percentage of false positive cases implies a substantial issue in the clinical setting, but we reckon that in the study of epidemiological data, like we provide, the magnitude of their impact on the results is more consequential in settings using lower numbers of cases.

We acknowledge our sample size is not as large as in other population-based studies, as the province of Girona is a region with lower burden of OPC and as we were not able to carry out the p16 IHC assay in all tumor samples from 1994-2018 for budgetary reasons. This has impacted our results. For instance, in the lack of significance in trends for overall

HNC and overall OPC, in which observed APC rates tended to be statistically significant; their analysis would have had more statistical power with a higher number of cases and a longer time span. Relatively small number of patients were included in the p16 expression-based analysis since, as mentioned, we selected all cases from specific calendar periods. 91.2% of the FFPE blocks of the identified cases were found, leaving an 8.8% of *missing* cases which we were not able to assess. Notwithstanding, there were no differences regarding sex, age, topography or histology between *missing* cases and *non-missing* cases, making both cohorts comparable. Significant differences were found according to calendar period, as in the earliest (1998-1999) the proportion of missing cases was of 30%, whilst in the last (2016-2018) it was of 1%. 3.1% of the obtained FFPE blocks were *non valuable* as specimen was not of enough quality for p16 determination. Distribution of sample distortion also correlated with antiquity of tumor blocks. These artifacts are probably due to implementation and development of p16 detection techniques in recent times and the inherent error of the retrospective design of our study. Withal, we consider our results have an extensive coverage (approximately 90%) of the population in our region.

Misclassification of cancer type by topography is another possible bias present in our study. It is inherent to the methodology of codification of tumors from the CRG and cannot be quantified. The anatomic proximity between the oropharynx and other surrounding topographical sites, added to the similarity of risk factors, often results in erroneous classification of this type of cancer. We reviewed all cases included in our study, and misclassified ones were reclassified accordingly, but we cannot have complete certainty all true oropharyngeal cases were included in our case selection, or some classified as OPC were primary tumors from other proximal sites extending to the oropharynx. Additionally, we decided not to include tumors of the lingual tonsil (ICD-O-3 code 2.04) as OPC cases as ICD-O-3 coding system defines them as part of the oral cavity (5). We reckon minor bias derives form that, as we did include tumors from the base of the tongue.

Having the CRG as our database and the retrospective nature of our cohort, we were not able to collect further clinical information from study cases besides age and sex, which would have been useful for cofounder adjustment of our results. Plus, the lack of

adjustment for cofounders regarding sex and age does not allow firm conclusions to be drawn regarding causal associations between HPV-relation and these demographical features. It is relevant to consider that the province of Girona is a region of higher social economic status compared to other regions in Spain and Southern Europe, which could cause different distribution of behaviors on the mentioned risk factors associated to both HPV-related and HPV-unrelated OPC.

The strengths of our study rely on the populational basis of our results, which can be extrapolated to the general population of the province of Girona. We provide the first population-based report of HPV infection as the underlying cause of increasing OPC incidence in Southern Europe. To date, no other studies have reported an analysis of trends in HPV-related OPC incidence in Southern Europe, as data were focused on prevalence of HPV in OPC alone (instead of incidence) and mainly in intrahospitalary settings. We also give a wide time coverage of incidence trends, from 1994 until most recent times, 2018. Lastly, we are the first study to assess better survival in HPV-related OPC versus HPV-unrelated OPC in Spain.

The increase in the incidence of HPV-related OPC underlines the crucial importance on the development of preventive strategies. The study of epidemiological distribution of its burden and its trends is critical for the implementation of efficacious public health interventions, as valid strategies may be inappropriate in places with lower disease burden and delayed increasing trends such as Southern Europe. On the one hand, health promotion and education of safe oral sex is essential to reverse the HPV-related OPC increasing epidemic, as youngsters do not regard oral sex as a threat and do not use protective measures to avoid sexually transmitted infections. On the other hand, HPV infection is potentially preventable by HPV vaccination, thus introduction of males into national HPV vaccination programs, likewise it has been done in Denmark or in the UK, upholds another horizon of effective prevention. Nonetheless, current incidence trends of OPC would not

reverse as a result of vaccination until after 2060 (87), so parallel development of secondary prevention strategies in high-risk individuals is of importance to stop the ongoing increase.

The better survival observed in HPV-related OPC in comparison to HPV-unrelated OPC sustains the need of reassessing therapeutic management of HPV-related OPC. As life expectancy increases, the morbidity associated to current treatments has a larger impact from the clinical, social, and economic point of view. Therefore, provision of a high-level quality of life and reduction of treatment toxicities and long-term functional consequences are important considerations in patients with this disease. Further research on de-intensification measures seeking to find new treatment protocols to reduce adverse side effects as well as development of targeted therapies to delineate individualized treatment to achieve more effectiveness are key to adapt to the change of paradigm of OPC.

10. CONCLUSIONS

- There has been a significant decrease in the incidence of overall head and neck cancer from 1994 to 2018 in the province of Girona.
- There has been a significant increase in the incidence of overall oropharyngeal cancer from 2001 to 2018 in the province of Girona.
- There has been a significant increase in the incidence of HPV-related oropharyngeal cancer between 1998 and 2018 in the province of Girona, sharply marked in the most recent years (2016-2018), whilst HPV-unrelated oropharyngeal cancer has not experienced any significant changes in incidence in this same period.
- HPV-related OPC has an increased observed survival, and therefore a better prognosis, in comparison to HPV-unrelated OPC in the province of Girona.
- There is a significant difference of distribution regarding sex between HPV-related OPC and HPV-unrelated OPC in the province of Girona. Both subtypes of OPC are more frequent in males, male-to-female ratio is higher in HPV-unrelated OPC, and the percentage of HPV-related OPC cases among all OPC cases in each sex is higher in females.
- Age at diagnosis does not significantly differ between HPV-related OPC and HPV-unrelated OPC in the province of Girona.

11. ETHICAL ASPECTS

The study had formal approval by the ethics committee of the Hospital Universitari Josep Trueta de Girona (*Comitè d'Ètica d'Investigació amb Medicaments de Girona (CEIm))* as reflected in *Annex 8*. Investigators strictly adhered to the provisions of the protocol submitted to the committee and to the standards of good clinical practice.

The study was performed in accordance with:

- The principles of the Declaration of Helsinki, adopted by the World Medical Association in Helsinki, in 1964, and last reviewed in 2013.
- The provision in the *Ley Española 14/2007 del 3 de julio*, on Biomedical Research.
- The standard of Good Clinical Practice issued by the Working Party on the Efficacy of Medicinal Products of the European Community in 1990 (CPMP/ICH/135/95) and the laws and regulations concerning these in force in Spain.
- The Oviedo Convention of April 4, 1997, on the protection of human rights and human dignity of the human being with regard to the application of biology and medicine, ratified in the Spanish Official Bulletin in October 1999.
- The provisions of the Data Protection European Union Directive 1995/46/EC and the *Ley Orgánica 3/2018 de 5 de diciembre* regarding the guarantee of data protection, and Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data.

Given that (i) the determination of p16 is included in the standard clinical practice, (ii) it is performed by immunohistochemical techniques in biopsy/tumorectomy specimens and (iii) this is a retrospective study with anonymized data from the CRG, informed consent from patients was not required.

As this was a retrospective observational study without drug intervention, no adverse effects, interference with physicians' prescribing habits or risk for the patients were contemplated. No risk/benefit assessment for patients was necessary.

12. WORK PLAN AND CHRONOGRAM

The activities developed during the study happened in the following sequence, in the timeline portrayed in *Table 12*.

STAGE 0: STUDY DESIGN

- **Activity 1:** bibliographic research about HPV-related OPC, HPV etiopathogenetic role in OPC, its impact in incidence and survival, previous research on epidemiological trends of HPV-related OPC, and its clinical implication. The research has been performed in databases such as Clinical Trials and PubMed.
- **Activity 2:** protocol elaboration (objectives, hypotheses, variables, methods, ethical considerations, budget, planification).

STAGE 1: ETHICAL EVALUATION AND STUDY APPROVAL

- **Activity 3:** presentation of the protocol to the ethics committees of the *Hospital Universitari Doctor Josep Trueta*.
- **Activity 4:** correction of the protocol according to suggestions by the ethics committees and approval.

STAGE 2: CASE SELECTION

- **Activity 5**: review of the CRG database, selection, and anonymization of cases.

STAGE 3: MEASUREMENTS AND DATA COLLECTION

- **Activity 6**: identification of FPPE tumor samples from the selected cases in the Pathology Departments.
- **Activity 7**: determination of p16 through IHC analysis in the Pathology Laboratories from each of the hospitals in the province of Girona.
- **Activity 8**: data collection of the results of the p16 assay, classification of cases in an anonymized database, and debugging of CRG database to include information on survival and demographics.

STAGE 4: DATA ANALYSIS AND INTERPRETATION

- **Activity 9**: statistical analyses: descriptive statistics, incidence, and trends in incidence of overall HNC and overall OPC, and descriptive statistics, incidence, trends in incidence, and observed survival of OPC cases from selected calendar periods according to p16 expression.
- **Activity 10**: statistical interpretation of the results and elaboration of the manuscript (discussion and extraction of conclusions).

STAGE 5: PRESENTATION AND PUBLICATION OF FINDINGS

- **Activity 11**: presentation of results.
- **Activity 12**: publication of results as a journal article.

Table 12: *Study chronogram*

Stage	***Task***	**2020**			**2021**												
		Oct	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Ago**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**
0	***A1:*** *bibliographic research*																
	A2: *protocol elaboration*																
1	***A3:*** *presentation to CEIm*																
	*A**4:*** *CEIm approval*																
2	***A5:*** *case selection*																
3	***A6:*** *identification of tumor samples*																
	A7*: p16 determination*																
	A8*: data collection*																
4	***A9****: statistical analyses*																
	A10*: statistical interpretation*																
5	***A11****: presentation of results*																
	A12*: publication*																

CEIm: ethics committee

13. BUDGET

The economic management of the project was carried out through the Institut de Investigació Biomèdica de Girona (IdIBGi), which has all the infrastructure to support such research and where different research groups in Girona converge. There was no financial support for this study, all expenses were allocated from the GEDGPC funds. The different expenses are summarized in *Table 13.*

PERSONNEL EXPENSES

The research team is composed by the GEDGPC staff and the pathologists from the participating hospitals, employed by their own institutions, supposing no additional expense to the project.

EXECUTIVE EXPENSES

Material for the bibliography research has not represented an additional expense. The work of the CRG technician, estimated to be 500€, has not had any cost for this project since they are employed by the *Institut Català d'Oncologia* (ICO). The determination of the p16 status is part of the clinical practice, so it had to be performed only in specimens prior to 2016. 2 sets for the determination of 100 samples costed 1.996€ plus 420€ of taxes. A pathology technician was subcontracted to carry out the p16 determinations, with a total salary of 500€. For the statistical analysis, an expert statistician was subcontracted; she was paid 500€ as well. No additional costs were sustained regarding tax application by the ethics committees or liability insurance.

PUBLICATION EXPENSES

Results will be published as a journal article. Considering the correction of language and the publication fees, the estimated subtotal of the publication costs is budgeted on 1.600€.

Table 13: *Summary of the study budget*

STUDY BUDGET		
ITEM	**COST**	**SUBTTOTAL**
PERSONNEL EXPENSES		
Research team	0 €	0 €
EXECUTIVE EXPENSES		
p16 determination	2.416 €	3.416€
Laboratory technician	500 €	
CRG technician	0€	
Statistician	500 €	
PUBLICATION EXPENSES		
Language proofreader	100 €	1.600€
Publication fee	1.500€	
TOTAL: 5.016€		

CRG: Cancer Registry of Girona

14. RESEARCH GROUP

The promoter of the study is the "Grup d'Epidemiologia Descriptiva, Genètica i Prevenció del Càncer" (GEDGPC) of "Institut d'Investigació Biomèdica de Girona" (IdIBGi), composed of epidemiologists, biologists, oncologists, and dermatologists from Girona. The research institute IdIBGi provides the necessary infrastructure for the management of the financial contributions of the projects and for their development (www.idibgi.org). This Institute was created in 2005 and is structured with research groups from the Hospital Universitari Doctor Josep Trueta Hospital in Girona, the University of Girona (UdG), the "Insitut de Diagnòstic per la Imatge" (IDI), the "Institut Català d'Oncologia" (ICO), the "Institut d'Assistència Sanitaria de Salt" (IAS), and the "Institut d'Atenció Primària" (EAP) / "Institut Català de Salut" (ICS) in Girona.

The members of the GEDGPC belong mostly to the ICO and to the ICS.

The members of the Group belong to different medical societies in their specialties and also take part in:

- RTICC RD12/0036/0056 *Red temática de Investigación en cáncer*. *Plan estratégico: registro de tumores. Epidemiológico, prevención y bioestadística* (Rafael Marcos-Gragera).
- EUROCARE Project, IARC (Rafael Marcos-Gragera).
- REDECAN. *Red de registros poblacionales de cáncer españoles* (Rafael Marcos-Gragera and Montse Puigdemont Guinart).
- GETTCC. *Grupo Español de Tratamiento de Tumores de Cabeza y Cuello* (Jordi Rubió).

The components of the research team that have participated in this project are:

Dr. Jordi Rubió Casadevall (PI)

Doctor of Medicine by the *Universitat Rovira i Virgili* (URV) in Tarragona. Attending Oncologist, Coordinator of the Medical Oncology Service of the ICO in Girona. Associate Professor of the Faculty of Medicine of the University of Girona (UdG).

Montserrat Puigdemont Guinart

Graduate in Nursing. Specialist in Epidemiology, technician at the Cancer Registry of Girona.

Arantza Sanvisens Bergé

Graduate in Statistics and in Biblioteconomy and Documentation. Specialist in Statistics, technician at the Cancer Registry of Girona.

Dra. Rosa Ortiz Duran

Doctor in Medicine. Specialist in Pathological Anatomy. Attending Pathologist of the Pathology Department of Dr. Josep Trueta Hospital (ICS). Associate Professor of the Faculty of Medicine of the University of Girona (UdG).

Dr. Rafael Marcos-Gragera

Doctor in Medicine by the *Universitat Autònoma de Barcelona* (UAB). Specialist in Epidemiology. Director of the Epidemiology Unit and Cancer Registry of Girona. Associate Professor of the Faculty of Medicine of the University of Girona (UdG).

15. BIBLIOGRAPHY

1. DAHANCA. Radiotherapy Guidelines 2020. Copenhagen: Danish Head and Neck Cancer Group; 2020.
2. Rettig EM, D'Souza G. Epidemiology of Head and Neck Cancer. Surg Oncol Clin N Am. 2015;24(3):379–96. Available from: http://dx.doi.org/10.1016/j.soc.2015.03.001
3. Woods RS, O'Regan EM, Kennedy S, Martin C, O'Leary JJ, Timon C. Role of human papillomavirus in oropharyngeal squamous cell carcinoma: A review. World J Clin Cases. 2014;2(6):172.
4. Seom.org. Tumores cabeza y cuello. [Internet]. Madrid: Sociedad Española de Oncología Médica; 2021 [cited 2021 Oct 3]. Available from: https://seom.org/info-sobre-el-cancer/orl
5. World Health Organization. International classification of diseases for oncology (ICD-O). 3rd ed. Geneva: World Health Organization; 2013.
6. International Agency for Research on Cancer. Monographs on the Evaluation of Carcinogenic Risks to Humans. Human Papillomaviruses. Vol. 90, Iarc Monographs On The Evaluation Of Carcinogenic Risks To Humans. World Health Organization; 2007. Available from: http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Monographs+on+the+Evaluation+of+Carcinogenic+Risks+to+Humans+VOLUME+90+Human+Papillomaviruses#0
7. Taberna M, Mena M, Pavón MA, Alemany L, Gillison ML, Mesía R. Human papillomavirus-related oropharyngeal cancer. Ann Oncol. 2017;28(10):2386–98.
8. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209–49.
9. Hartwig S, Syrjänen S, Dominiak-Felden G, Brotons M, Castellsagué X. Estimation of the epidemiological burden of human papillomavirus-related cancers and non-malignant diseases in men in Europe: A review. BMC Cancer. 2012;12.

10. Bray F, Colombet M, Mery L, Piñeros M, Znaor A, Zanetti R, et al. Cancer Incidence in Five Continents Vol. XI. Lyon: International Agency for Research on Cancer; 2021.
11. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Paula Curado M, Ferlay J, Franceschi S, et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J Clin Oncol. 2013;31(36):4550–9.
12. Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: A virus-related cancer epidemic. Lancet Oncol. 2010;11(8):781–9. Available from: http://dx.doi.org/10.1016/S1470-2045(10)70017-6
13. De Camargo Cancela M, De Souza DLB, Curado MP. International incidence of oropharyngeal cancer: A population-based study. Oral Oncol. 2012;48(6):484–90. Available from: http://dx.doi.org/10.1016/j.oraloncology.2011.12.013
14. Serrano B, Brotons M, Bosch FX, Bruni L. Epidemiology and burden of HPV-related disease. Best Pract Res Clin Obstet Gynaecol. 2018;47(2018):14–26.
15. de Martel C, Plummer M, Vignat J, Franceschi S. Worldwide burden of cancer attributable to HPV by site, country and HPV type. Int J Cancer. 2017;141(4):664–70.
16. Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. Lancet Glob Heal. 2016;4(9):e609–16. Available from: http://dx.doi.org/10.1016/S2214-109X(16)30143-7
17. de Martel C, Georges D, Bray F, Ferlay J, Clifford GM. Global burden of cancer attributable to infections in 2018: a worldwide incidence analysis. Lancet Glob Heal. 2020;8(2):e180–90. Available from: http://dx.doi.org/10.1016/S2214-109X(19)30488-7
18. Bruni L, Albero G, Serrano B, Mena M, Gómez D, Muñoz J. Human Papillomavirus and Related Diseases Report in the World. Barcelona: ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre).; 2019.
19. Castellsagué X, Alemany L, Quer M, Halec G, Quirós B, Tous S, et al. HPV Involvement in Head and Neck Cancers: Comprehensive Assessment of Biomarkers

in 3680 Patients. J Natl Cancer Inst. 2016;108(6):1–12.

20. Rodrigo JP, Heideman DAM, García-Pedrero JM, Fresno MF, Brakenhoff RH, Díaz Molina JP, et al. Time trends in the prevalence of HPV in oropharyngeal squamous cell carcinomas in northern Spain (1990-2009). Int J Cancer. 2014;134(2):487–92.
21. Mena M, Frias-Gomez J, Taberna M, Quirós B, Marquez S, Clavero O, et al. Epidemiology of human papillomavirus-related oropharyngeal cancer in a classically low-burden region of southern Europe. Sci Rep. 2020;10(1):1–11. Available from: https://doi.org/10.1038/s41598-020-70118-7
22. Del Mistro A, Frayle H, Menegaldo A, Favaretto N, Gori S, Nicolai P, et al. Age-independent increasing prevalence of Human Papillomavirus-driven oropharyngeal carcinomas in North-East Italy. Sci Rep. 2020;10(1):1–10.
23. De Souza DLB, De Camargo Cancela M, Bernal Perez M, Curado MP. Trends in the incidence of oral cavity and oropharyngeal cancers in Spain. Head Neck. 2011;34:649–54.
24. Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, et al. A review of human carcinogens--Part B: biological agents. Lancet Oncol. 2009;10(4):321–2. Available from: http://dx.doi.org/10.1016/S1470-2045(09)70096-8
25. Zaravinos A. An updated overview of HPV-associated head and neck carcinomas. Oncotarget. 2014;5(12):3956–69.
26. Roberts S, Evans D, Mehanna H, Parish JL. Modelling human papillomavirus biology in oropharyngeal keratinocytes. Philos Trans R Soc B Biol Sci. 2019;374(1773).
27. Bravo IG, Felez-Sanchez M. Papillomaviruses: Viral evolution, cancer and evolutionary medicine. Evol Med Public Heal. 2015;2015(1):32–51.
28. Giuliano AR, Nyitray AG, Kreimer AR, Pierce Campbell CM, Goodman MT, Sudenga SL, et al. EUROGIN 2014 roadmap: Differences in human papillomavirus infection natural history, transmission and human papillomavirus-related cancer incidence by gender and anatomic site of infection. Int J Cancer. 2015;136(12):2752–60.
29. Pickard RKL, Xiao W, Broutian TR, He X, Gillison ML. The prevalence and incidence of oral human papillomavirus infection among young men and women, aged 18-30

years. Sex Transm Dis. 2012;39(7):559–66.

30. Gillison ML, Broutian T, Pickard RKL, Tong ZY, Xiao W, Kahle L, et al. Prevalence of oral HPV infection in the United States, 2009-2010. JAMA - J Am Med Assoc. 2012;307(7):693–703.
31. Wood ZC, Bain CJ, Smith DD, Whiteman DC, Antonsson A. Oral human papillomavirus infection incidence and clearance: A systematic review of the literature. J Gen Virol. 2017;98(4):519–26.
32. Mena M, Taberna M, Monfil L, Arbyn M, De Sanjose S, Bosch FX, et al. Might oral human papillomavirus (HPV) infection in healthy individuals explain differences in HPV-attributable fractions in oropharyngeal cancer? A Systematic Review and Meta-analysis. J Infect Dis. 2019;219(10):1574–85.
33. Kreimer AR, Bhatia RK, Messeguer AL, González P, Herrero R, Giuliano AR. Oral Human papillomavirus in healthy individuals: A systematic review of the literature. Sex Transm Dis. 2010;37(6):386–91.
34. Tommasino M. The human papillomavirus family and its role in carcinogenesis. Semin Cancer Biol. 2014;26:13–21. Available from: http://dx.doi.org/10.1016/j.semcancer.2013.11.002
35. Khleif SN, Degregori J, Yee CL, Otterson GA, Kaye FJ, Nevins JR, et al. Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity. Proc Natl Acad Sci U S A. 1996;93(9):4350–4.
36. Cantrell SC, Peck BW, Li G, Wei Q, Sturgis EM, Ginsberg LE. Differences in imaging characteristics of HPV-positive and HPV-negative oropharyngeal cancers: A blinded matched-pair analysis. Am J Neuroradiol. 2013;34(10):2005–9.
37. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol. 2011;29(32):4294–301.
38. Ang K, Harris J, Wheeler R, Weber R, Rosenthal D, Nguyen-Tân F, et al. Human Papillomavirus and Survival of Patients with Oropharyngeal Cancer. N Engl J Med. 2010;363:24–35.

39. Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, Begum S, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst. 2008;100(6):407–20.
40. Gayar OH, Ruterbusch JJ, Elshaikh M, Cote M, Ghanem T, Hall F, et al. Oropharyngeal carcinoma in young adults: An alarming national trend. Otolaryngol - Head Neck Surg (United States). 2014;150(4):594–601.
41. Windon MJ, D'Souza G, Rettig EM, Westra WH, van Zante A, Wang SJ, et al. Increasing prevalence of human papillomavirus–positive oropharyngeal cancers among older adults. Cancer. 2018;124(14):2993–9.
42. Tota JE, Best AF, Zumsteg ZS, Gillison ML, Rosenberg PS, Chaturvedi AK. Evolution of the oropharynx cancer epidemic in the United States: Moderation of increasing incidence in younger individuals and shift in the burden to older individuals. J Clin Oncol. 2019;37(18):1538–46.
43. D'Souza G, Wentz A, Kluz N, Zhang Y, Sugar E, Youngfellow RM, et al. Sex Differences in Risk Factors and Natural History of Oral Human Papillomavirus Infection. J Infect Dis. 2016;213(12):1893–6.
44. Masterson L, Moualed D, Liu ZW, Howard JEF, Dwivedi RC, Tysome JR, et al. De-escalation treatment protocols for human papillomavirus-associated oropharyngeal squamous cell carcinoma: A systematic review and meta-analysis of current clinical trials. Eur J Cancer. 2014;50(15):2636–48. Available from: http://dx.doi.org/10.1016/j.ejca.2014.07.001
45. Rettig E, Kiess AP, Fakhry C. The role of sexual behavior in head and neck cancer: Implications for prevention and therapy. Expert Rev Anticancer Ther. 2014;15(1):35–49.
46. Goldenberg D, Begum S, Westra WH, Khan Z, Sciubba J, Pai S, et al. Cystic lymph node metastasis in patients with head and neck cancer: an HPV-associated phenomenon. Head Neck. 2008;30(7):898–903.
47. Huang SH, Perez-Ordonez B, Liu FF, Waldron J, Ringash J, Irish J, et al. Atypical

clinical behavior of p16-confirmed HPV-related oropharyngeal squamous cell carcinoma treated with radical radiotherapy. Int J Radiat Oncol Biol Phys. 2012;82(1):276–83.

48. Reimers N, Kasper HU, Weissenborn SJ, Stützer H, Preuss SF, Hoffmann TK, et al. Combined analysis of HPV-DNA, p16 and EGFR expression to predict prognosis in oropharyngeal cancer. Int J Cancer. 2007;120(8):1731–8.

49. Evans M, Newcombe R, Fiander A, Powell J, Rolles M, Thavaraj S, et al. Human Papillomavirus-associated oropharyngeal cancer: An observational study of diagnosis, prevalence and prognosis in a UK population. BMC Cancer. 2013;13.

50. Psyrri A, Rampias T, Vermorken JB. The current and future impact of human papillomavirus on treatment of squamous cell carcinoma of the head and neck. Ann Oncol. 2014;25(11):2101–15. Available from: https://doi.org/10.1093/annonc/mdu265

51. Vu HL, Sikora AG, Fu S, Kao J. HPV-induced oropharyngeal cancer, immune response and response to therapy. Cancer Lett. 2010;288(2):149–55. Available from: http://dx.doi.org/10.1016/j.canlet.2009.06.026

52. Kimple RJ, Smith MA, Blitzer GC, Alexandra D, Martin JA, Yang RZ, et al. Enhanced radiation sensitivity in HPV-positive head and neck cancer. 2014;73(15):4791–800.

53. Rieckmann T, Tribius S, Grob TJ, Meyer F, Busch CJ, Petersen C, et al. HNSCC cell lines positive for HPV and p16 possess higher cellular radiosensitivity due to an impaired DSB repair capacity. Radiother Oncol. 2013;107(2):242–6. Available from: http://dx.doi.org/10.1016/j.radonc.2013.03.013

54. Nickson CM, Moori P, Carter RJ, Rubbi CP, Parsons JL. Misregulation of DNA damage repair pathways in HPV-positive head and neck squamous cell carcinoma contributes to cellular radiosensitivity. Oncotarget. 2017;8(18):29963–75.

55. Dok R, Asbagh LA, Van Limbergen EJ, Sablina A, Nuyts S. Nuclear p16INK4a expression predicts enhanced radiation response in head and neck cancers. Oncotarget. 2016;7(25):38785–95.

56. Hanns E, Job S, Coliat P, Wasylyk C, Ramolu L, Pencreach E, et al. Human

Papillomavirus-related tumours of the oropharynx display a lower tumour hypoxia signature. Oral Oncol. 2015;51(9):848–56. Available from: http://dx.doi.org/10.1016/j.oraloncology.2015.06.003

57. Saber CN, Grønhøj Larsen C, Dalianis T, Von Buchwald C. Immune cells and prognosis in HPV-associated oropharyngeal squamous cell carcinomas: Review of the literature. Oral Oncol. 2016;58(2016):8–13. Available from: http://dx.doi.org/10.1016/j.oraloncology.2016.04.004
58. Gebre-Medhin M, Brun E, Engström P, Cange HH, Hammarstedt-Nordenvall L, Reizenstein J, et al. ARTSCAN III: A randomized phase III study comparing chemoradiotherapy with cisplatin versus cetuximab in patients with locoregionally advanced head and neck squamous cell cancer. J Clin Oncol. 2021;39(1):38–47.
59. Rietbergen MM, Braakhuis BJM, Moukhtari N, Bloemena E, Brink A, Sie D, et al. No evidence for active human papillomavirus (HPV) in fields surrounding HPV-positive oropharyngeal tumors. J Oral Pathol Med. 2014;43(2):137–42.
60. Peck BW, Dahlstrom KR, Gan SJ, Caywood W, Li G, Wei Q, et al. Low risk of second primary malignancies among never smokers with human papillomavirus-associated index oropharyngeal cacners. Head Neck. 2013;35(6):794–9.
61. Bishop JA, Lewis JS, Rocco JW, Faquin WC. HPV-related squamous cell carcinoma of the head and neck: An update on testing in routine pathology practice. Semin Diagn Pathol. 2015;32(5):344–51. Available from: http://dx.doi.org/10.1053/j.semdp.2015.02.013
62. Westra WH. Detection of human papillomavirus (HPV) in clinical samples: Evolving methods and strategies for the accurate determination of HPV status of head and neck carcinomas. Oral Oncol. 2014;50(9):771–9. Available from: http://dx.doi.org/10.1016/j.oraloncology.2014.05.004
63. Larsen G, Gyldenløve M, Jensen DH, Therkildsen MH, Kiss K, Norrild B, et al. Correlation between human papillomavirus and p16 overexpression in oropharyngeal tumours: A systematic review. Br J Cancer. 2014;110(6):1587–94.
64. Mena M, Taberna M, Tous S, Marquez S, Clavero O, Quiros B, et al. Double

positivity for HPV-DNA/p16ink4a is the biomarker with strongest diagnostic accuracy and prognostic value for human papillomavirus related oropharyngeal cancer patients. Oral Oncol. 2018;78(October 2017):137–44.

65. Mirghani H, Amen F, Moreau F, Guigay J, Ferchiou M, Melkane AE, et al. Human papilloma virus testing in oropharyngeal squamous cell carcinoma: What the clinician should know. Oral Oncol. 2014;50(1):1–9.

66. Urban D, Corry J, Rischin D. What is the best treatment for patients with human papillomavirus-positive and -negative oropharyngeal cancer? Cancer. 2014;120(10):1462–70.

67. Lydiatt W, O'Sullivan B, Patel S. Major Changes in Head and Neck Staging for 2018. Am Soc Clin Oncol Educ B. 2018;(38):505–14.

68. Petit C, Lacas B, Pignon JP, Le QT, Grégoire V, Grau C, et al. Chemotherapy and radiotherapy in locally advanced head and neck cancer: an individual patient data network meta-analysis. Lancet Oncol. 2021;22(5):727–36.

69. Mehanna H, Robinson M, Hartley A, Kong A, Foran B, Fulton-Lieuw T, et al. Radiotherapy plus cisplatin or cetuximab in low-risk human papillomavirus-positive oropharyngeal cancer (De-ESCALaTE HPV): an open-label randomised controlled phase 3 trial. Lancet. 2019;393(10166):51–60.

70. Boix Martínez R, Portela Moreira A, Pérez Gonzáles A, Navarro Alonso JA, Salmerón García F, Rego Romero E, et al. Revisión del programa de vacunación frente a virus del papiloma humano en España. Ministerio de Sanidad. 2013;1–61. Available from: https://www.mscbs.gob.es/profesionales/saludPublica/prevPromocion/vacunaciones/docs/PapilomaVPH.pdf

71. Herrero R, Quint W, Hildesheim A, González P, Strujik L. Reduced Prevalence of Oral Human Papillomavirus (HPV) 4 Years after Bivalent HPV Vaccination in a Randomized Clinical Trial in Costa Rica. PLoS One. 2013;8(7):1–9.

72. Chaturvedi AK, Graubard BI, Broutian T, Pickard RKL, Tong ZY, Xiao W, et al. Effect of prophylactic human papillomavirus (HPV) vaccination on oral HPV infections among young adults in the United States. J Clin Oncol. 2018;36(3):262–7.

73. Gipson B, Robbins H, Fakhry C, D'Souza G. Sensitivity and Specificity of Oral HPV Detection for HPV-Positive Head and Neck Cancer. Oral Oncol. 2017;77:52–6.
74. Kreimer AR, Johansson M, Waterboer T, Kaaks R, Chang-Claude J, Drogen D, et al. Evaluation of Human Papillomavirus Antibodies and Risk of Subsequent Head and Neck Cancer. J Clin Oncol. 2013;31(21):2708–15.
75. Idescat.cat. Población a 1 de enero. Provincias. [Internet]. Barcelona: Generalitat de Catalunya; 2021 [cited 2021 Nov 3]. Available from: https://www.idescat.cat/pub/?id=aec&n=245&lang=es
76. Izquierdo Font Á, Marcos-Gragera R, Vilardell Gil ML, Puigdemont Guinart M, Vidal Vila A, Fuentes Fernández J, et al. El càncer a Girona. Incidència, Mortalitat i Supervivència. Girona: Institut Català d'Oncologia; 2021.
77. Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales. Boletín Oficial del Estado, núm. 294, (6-12-2018).
78. Declaration of Helsinki. Helsinki: World Medical Association; 2013.
79. Aguilar Lizarralde Y. Utilidad de la Determinación Inmunohistoquímica de la Proteína p16 en el Carcinoma de Lengua. Correlación con la Infección por el Virus del Virus del Papiloma Humano. Málaga; 2014.
80. Revision of the European Standard Population. Brussels: Eurostat, European Union; 2013.
81. Mscbs.gob.es. Encuestas Nacionales de Salud de España de años anteriores. [Internet]. Madrid: Ministerio de Sanidad, Consumo y Bienestar Social; 2021 [cited 2021 Nov 3]. Available from: https://www.mscbs.gob.es/estadEstudios/estadisticas/encuestaNacional/aniosAnteriores.htm
82. Bruni L, Albero G, Serrano B, Mena M, Gómez D, Muñoz J. Human Papillomavirus and Related Diseases in Europe. Barcelona: ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre); 2019.
83. Hansen BT, Campbell S, Nygård M. Long-term incidence trends of HPV-related cancers, and cases preventable by HPV vaccination: A registry-based study in

Norway. BMJ Open. 2018;8(2):1–9.

84. Ng M, Freeman MK, Fleming TD, Robinson M, Dwyer-Lindgren L, Thomson B, et al. Smoking prevalence and cigarette consumption in 187 countries, 1980-2012. JAMA - J Am Med Assoc. 2014;311(2):183–92.

85. Heck JE, Berthiller J, Vaccarella S, Winn DM, Smith EM, Shan'gina O, et al. Sexual behaviours and the risk of head and neck cancers: A pooled analysis in the International Head and Neck Cancer Epidemiology (INHANCE) consortium. Int J Epidemiol. 2009;39(1):166–81.

86. Palacios-Ceña D, Carrasco-Garrido P, Hernández-Barrera V, Alonso-Blanco C, Jiménez-García R, Fernández-de-las-Peñas C. Sexual behaviors among older adults in Spain: Results from a population-based national sexual health survey. J Sex Med. 2012;9(1):121–9.

87. Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C. Epidemiology of human papillomavirus-positive head and neck squamous cell carcinoma. J Clin Oncol. 2015;33(29):3235–42.

88. Zamani M, Grønhøj C, Jensen DH, Carlander AF, Agander T, Kiss K, et al. The current epidemic of HPV-associated oropharyngeal cancer: An 18-year Danish population-based study with 2,169 patients. Eur J Cancer. 2020;134:52–9.

89. DAHANCA. DAHANCA 30: a randomized non-inferiority trial of hypoxia-profile guided hypoxic modification of radiotherapy of HNSCC. Copenhagen: Danish Head and Neck Cancer Group; 2014. p. 1–51. Available from: www.dahanca.dk

16. LIST OF TABLES AND FIGURES

17. ANNEXES

17.1. ANNEX 1: *International Classification of Diseases for Oncology Edition 3*

HEAD AND NECK CANCER TOPOGRAPHICAL CODES (5)

C00 LIP (*excludes skin of lip C44.0)*

C00.0 External upper lip
Vermilion border o upper lip
Upper lip, NOS (*excludes skin of upper lip C44.0)*

C00.1 External lower lip
Vermilion border of lower lip
Lower lip, NOS (*excludes skin of lower lip C44.0)*

C00.2 External lip, NOs
Vermilion border of lip, NOS

C00.3 Mucosa of upper lip
Frenulum of upper lip
Inner aspect of lower lip

C00.4 Mucosa of lower lip
Inner aspect of lower lip
Frenulum of lower lip

C00.5 Mucosa of lip, NOS
Inner aspect of lip, NOS
Internal lip, NOS
Frenulum of lip, NOS
Frenulum labia, NOS

C00.6 Commissure of lip
Labial commissure

C00.8 Overlapping lesion of lip

C00.9 Lip, NOS *(excludes skin of lip C44.0)*

C01 BASE OF TONGUE

C01.9 Base of tongue, NOS
Dorsal surface of base of tongue
Posterior third of tongue
Posterior tongue, NOS
Root of tongue

C02 OTHER AND UNSPECIFIED PARTS OF TONGUE

C02.0 Dorsal surface of tongue, NOS
Anterior 2/3 of tongue, dorsal surface
Midline of tongue
Dorsal surface of anterior tongue

C02.1 Border of tongue
Tip of tongue

C02.2 Ventral surface of tongue, NOS
Anterior 2/3 of tongue, ventral surface
Frenulum linguae
Ventral surface of anterior tongue, NOS

C02.3 Anterior 2/3 of tongue, NOS
Anterior tongue, NOS

C02.4 Lingual tonsil

C02.8 Overlapping lesion of tongue
Junctional zone of tongue

C02.9 Tongue, NOS
Lingual, NOS

C03 GUM

C03.0 Upper gum
Maxillary gingiva
Upper alveolar mucosa
Upper alveolar ridge mucosa
Upper alveolus
Upper gingiva

C03.1 Lower gum
Mandibular gingiva
Lower alveolar mucosa
Lower alveolar ridge mucosa
Lower alveolus
Lower gingiva

C03.9 Gum, NOS
Gingiva, NOS
Alveolar mucosa, NOS
Alveolar ridge mucosa, NOS
Alveolus, NOS
Periodontal tissue
Tooth socket

C04 FLOOR OF MOUTH

C04.0 Anterior floor of mouth

C04.1 Lateral floor of mouth

C04.8 Overlapping lesion of floor of mouth

C04.9 Floor of mouth, NOS

C05 PALATE

C05.0 Hard palate

C05.1 Soft palate, NOS *(excludes nasopharyngeal surface of soft palate C11.3)*

C05.2 Uvula

C05.8 Overlapping lesion of palate
Junction of hard and soft palate

C05.9 Palate, NOS
Roof of mouth

C06 OTHER AND USPECIFIED PARTS OF MOUTH

C06.0 Cheek mucosa
Buccal mucosa
Internal cheek

C06.1 Vestibule of mouth
Alveolar sulcus
Buccal sulcus
Labial sulcus

C06.2 Retromolar area
Retromolar triangle
Retromolar trigone

C06.8 Overlapping lesion of other and unspecified parts of mouth

C06.9 Mouth, NOS
Buccal cavity
Oral cavity
Oral mucosa
Minor salivary gland, NOS

C07 PAROTID GLAND

C07.9 Parotid gland
Parotid, NOS
Stensen duct
Parotid gland duct

C08 OTHER AND UNSPECIFIED MAJOR SALIVARY GLANDS

Note: *Neoplasms of minor salivary glands should be classified according to their anatomical site; if location is not specified, classify to C06.9*

C08.0 Submandibular gland
Submaxillary gland
Wharton duct
Submaxillary gland duct

C08.1 Sublingual gland
Sublingual gland duct

C08.8 Overlapping lesion of major salivary glands

C08.9 Major salivary gland, NOS
Salivary gland, NOS
(excludes minor salivary glands, NOS C06.9)

C09 TONSIL

C09.0 Tonsillar fossa

C09.1 Tonsillar pillar
Faucial pillar
Glossopalatine fold

C09.8 Overlapping lesion of tonsil

C09.9 Tonsil, NOS *(excludes lingual tonsil C02.4 and pharyngeal tonsil C11.1)*
Faucial tonsil
Palatine tonsil

C10 OROPHARYNX

C10.0 Vallecula

C10.1 Anterior surface of epiglottis

C10.2 Lateral wall of oropharynx
Lateral wall of mesopharynx

C10.3 Posterior wall of oropharynx
Posterior wall of mesopharynx

C10.4 Branchial cleft *(site of neoplasm)*

C10.8 Overlapping lesion of oropharynx
Junctional region of oropharynx

C10.9 Oropharynx, NOS
Mesopharynx, NOS
Fauces, NOS

C11 NASOPHARYNX

C11.0 Superior wall of nasopharynx
Roof of nasopharynx

C11.1 Posterior wall of nasopharynx
Adenoid
Pharyngeal tonsil

C11.2 Lateral wall of nasopharynx
Fossa of Rosenmuller

C11.3 Anterior wall of nasopharynx
Nasopharyngeal surface of soft palate
Pharyngeal fornix
Choana
Posterior margin of nasal septum

C11.8 Overlapping lesion of nasopharynx

C11.9 Nasopharynx, NOS
Nasopharyngeal wall

C12 PYRIFORM SINUS

C12.9 Pyriform sinus
Piriform sinus
Pyriform fossa
Piriform fossa

C13 HYPOPHARYNX

C13.0 Postcricoid region
Cricopharynx
Cricoid, NOS

C13.1 Hypopharyngeal aspect of aryepiglottic fold
Aryepiglottic fold, NOS *(excludes laryngeal aspect of aryepiglottic fold C32.1)*
Arytenoid fold

C13.2 Posterior wall of hypopharynx

C13.8 Overlapping lesion of hypopharynx

C13.9 Hypopharynx, NOS
Hypopharyngeal wall
Laryngopharynx

C14 OTHER AND ILL-DEFINED SITES IN LIP, ORAL CAVITY AND PHARYNX

C14.0 Pharynx, NOS
Pharyngeal wall, NOS
Wall of pharynx, NOS
Lateral wall of pharynx, NOS
Posterior wall of pharynx, NOS
Retropharynx
Throat

C14.2 Waldeyer ring

C14.8 Overlapping lesion of lip, oral cavity and pharynx
Note: *Neoplasms of lip, oral cavity and pharynx whose point of origin cannot be assigned to any one of the categories C00 to C14.2*

C30 NASAL CAVITY AND MIDDLE EAR

C30.0 Nasal cavity *(excludes nose, NOS C76.0)*
- Internal nose
- Naris
- Nasal cartilage
- Nasal mucosa
- Nasal septum, NOS *(excludes posterior margin of nasal septum C11.3)*
- Nasal turbinate
- Nostril
- Vestibule of nose

C30.1 Middle ear
- Inner ear
- Auditory tube
- Eustachian tube
- Mastoid antrum
- Tympanic cavity

C31 ACCESORY SINUSES

C31.0 Maxillary sinus
- Maxillary antrum
- Antrum, NOS

C31.1 Ethmoid sinus

C31.2 Frontal sinus

C31.3 Sphenoid sinus

C31.8 Overlapping lesion of accessory sinuses

C31.9 Accessory sinus, NOS
- Accessory nasal sinus
- Paranasal sinus

C32 LARYNX

C32.0 Glottis
- Intrinsic larynx
- Laryngeal commissure
- Vocal cord, NOS
  - True vocal cord
  - True cord

C32.1 Supraglottis
- Epiglottis, NOS *(excludes anterior surface of epiglottis C10.1)*
- Extrinsic larynx
- Laryngeal aspect of aryepiglottic fold
- Posterior surface of epiglottis
- Ventricular band of larynx
  - False vocal cord
  - False cord

C32.2 Subglottis

C32.3 Laryngeal cartilage
- Arytenoid cartilage
- Cricoid cartilage
- Cuneiform cartilage
- Thyroid cartilage

C32.8 Overlapping lesion of larynx

C32.9 Larynx, NOS

17.2. ANNEX 2

Table 14: *Number of all Head and Neck Cancer cases attributable to Human Papillomavirus (oropharynx, oral cavity, and larynx) in world regions*

Region		Males	Females
AFRICA	Sub-Saharan Africa	**360**	**150**
	Northern Africa/Western Asia	**240**	**80**
ASIA	India	**5,600**	**1,000**
	Other Central Asia	**760**	**300**
	China	**950**	**270**
	Japan/Republic of Korea	**1,500**	**350**
	Other Eastern Asia	**1,000**	**280**
AMERICA	Latin America	**980**	**280**
	Northern America	**7,000**	**1,900**
EUROPE	Europe	**11,000**	**2,800**
OCEANIA	Australia/New Zealand	**290**	**80**
	Other Oceania	**30**	**10**
Less developed countries		**8,600**	**2,100**
More developed countries		**22,000**	**5,500**
WORLD		**30,000**	**7,500**

Adapted from (15).

17.3. ANNEX 3

Table 15: *HPV prevalence in cases of oropharyngeal cancer from worldwide studies*

Region	Country	Study: Author / Year		HPV prevalence in OPC	Nº Tested
NORTHERN EUROPE	Norway	*Hannisdal*	2010	51.8%	137
	Sweden	*Attner*	2010	74.7%	95
	United Kingdom	*Thavaraj*	2011	70.4%	142
		Evans	2013	55%	83
SOUTHERN EUROPE	Italy	*Licitra*	2006	18.9%	90
		Del Mistro	2020	32.3%	130
EASTERN EUROPE	Czech Republic	*Rotnáglová*	2011	65.1%	109
WESTERN EUROPE	France	*Fonmarty*	2015	31.0%	71
	Germany	*Holzinger*	2012	50.3%	199
	Netherlands	*Henneman*	2015	34.9%	146
OCEANIA	Australia	*Hong*	2013	57.3%	647
NORTH AMERICA	Canada	*Nichols*	2013	52.6%	95
	United States of America	*Chaturvedi*	2011	44.1%	263
		Jordan	2012	79.0%	233
		Walline	2013	88.0%	208
		D'Souza	2014	64.6%	164
		Isayeva	2014	62.7%	102
		Steinau	2014	72.4%	557
ASIA	India	*Bahl*	2014	22.9%	105
	Taiwan	*Al-Swiahb*	2010	16.4%	274
	Japan	*Hama*	2014	50.3%	157
	Turkey	*Tural*	2013	51.9%	81

HPV: human papillomavirus; OPC: oropharyngeal cancer. Adapted from (18).

17.4. ANNEX 4

Table 16: *Oral HPV prevalence among healthy populations in Europe*

Region	Country	Study: Author / Year		HPV prevalence	Nº Tested	Study population
NORTHERN Europe	**Finland**	*Kero*	2011 2012	**17.2%** **18.3%**	137	Pregnant women + their spouses
	United Kingdom	*Kujan*	2006	**8.0%**	50	Healthy volunteers from university dental hospital
SOUTHERN Europe	**Italy**	*Migaldi*	2012	**1.2%**	81	Patients undergoing to routine oral examination
	Greece	*Lambropoulous*	1997	**9.5%**	169	Healthy population receiving routine oral examination

HPV: human papillomavirus. Adapted from (82).

17.5. ANNEX 5

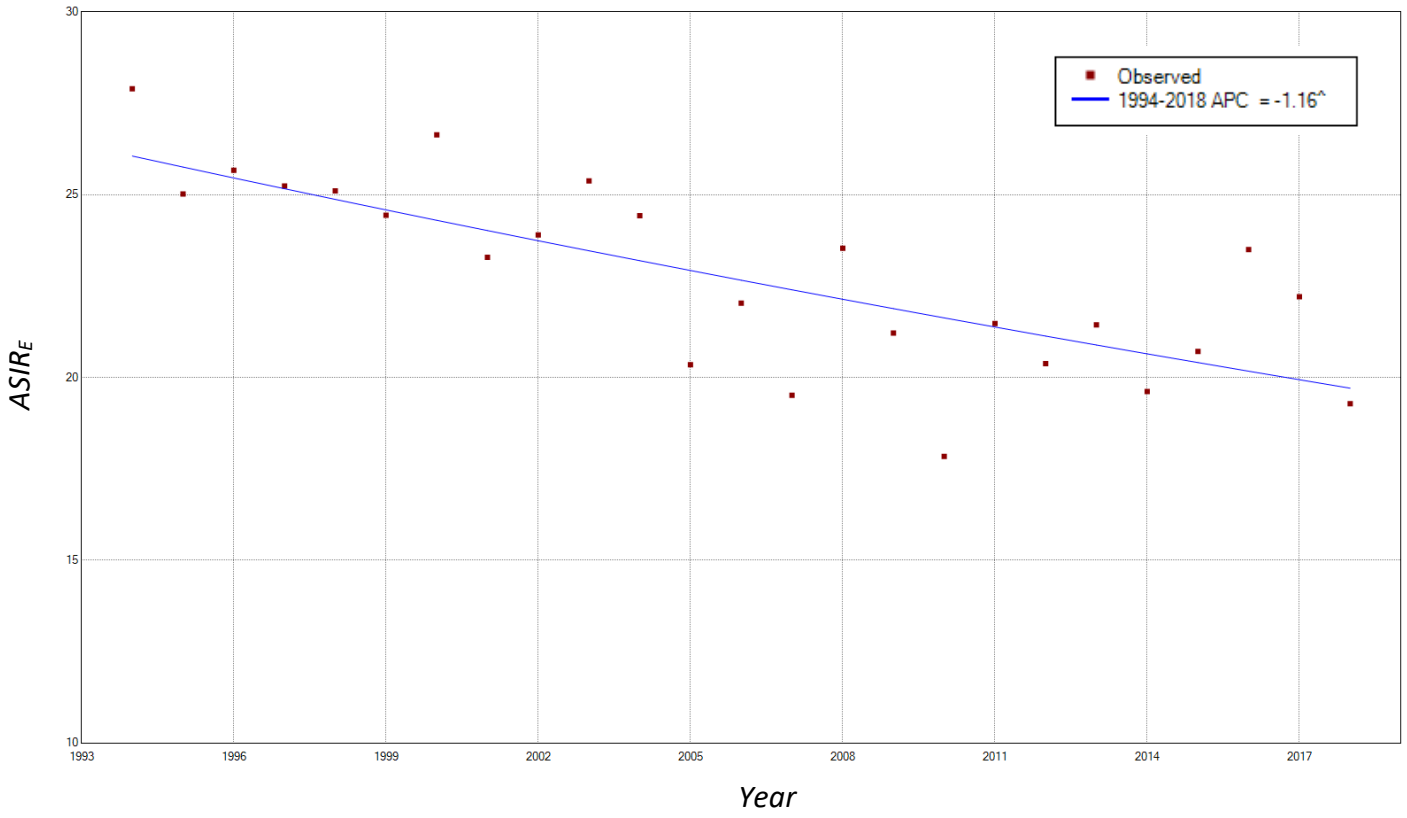
Table 17: *Main differences between the 7th and the new 8th edition of the TNM staging system classification for HPV-related OPSCC developed by the UICC*

	7th EDITION TNM	8TH EDITION TNM
STAGE CLASSIFICATIONS	**Stage I** (T1N0) **Stage II** (T2N0) **Stage III** (T3N0 or T1-T3N1) **Stage IVa** (T4aN0-1 or T1-T4N2) **Stage IVb** (T4b or T1-T4N3) **Stage IVc** (M1)	**Stage I** (T1-T2 N0-N1) **Stage II** (T1-T2N2 or T3N0-N2) **Stage III** (T4 or N3) **Stage IV** (M1)
MAIN N DIFFERENCES (lymph nodes)	**N1**: metastasis in a single ipsilateral lymph node, <3cm **N2a**: metastasis in a single ipsilateral lymph node >3cm but <6cm **N2b:** metastasis in multiple ipsilateral lymph nodes, <6cm **N2c:** metastasis in bilateral or contralateral lymph nodes <6cm	**N1:** ipsilateral metastasis in lymph node(s), <6cm **N2**: bilateral or contralateral metastasis in lymph node(s), <6cm
MAIN T DIFFERENCES (tumor)	**T4a:** tumor invades larynx, extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible **T4b:** tumor invades lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base or encases carotid artery	**T4:** tumor invades any of the following: larynx, deep/extrinsic muscle of tongue, medial pterygoid, hard palate, mandible, lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base or encases carotid artery

Extracted from (7).

17.6. ANNEX 6

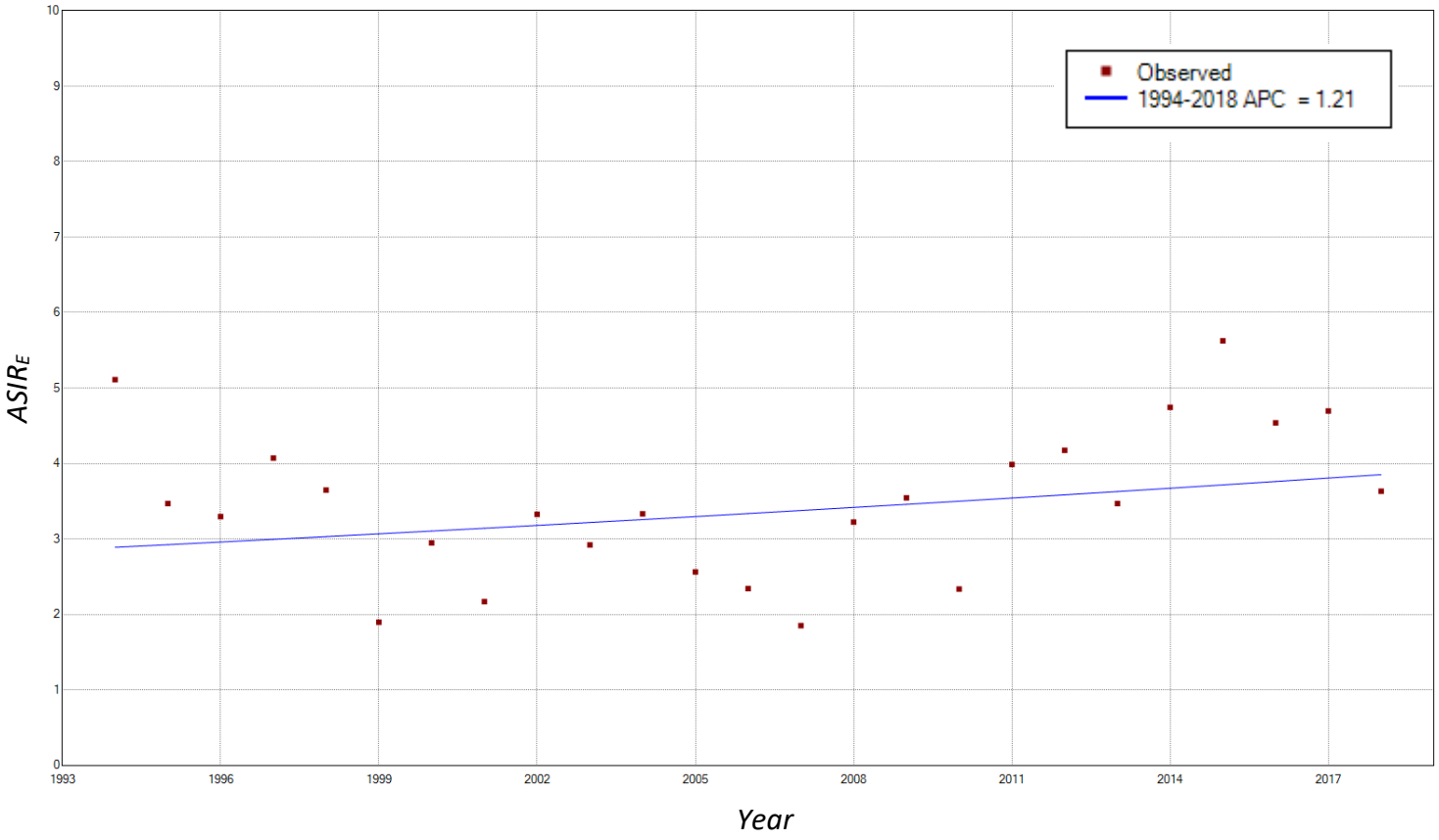
Figure 16. Trends in incidence of overall head and neck cancer, from 1994 to 2018. Global joint-point analysis.



APC = -1.16 (95% CI: -1.6; -0.7)

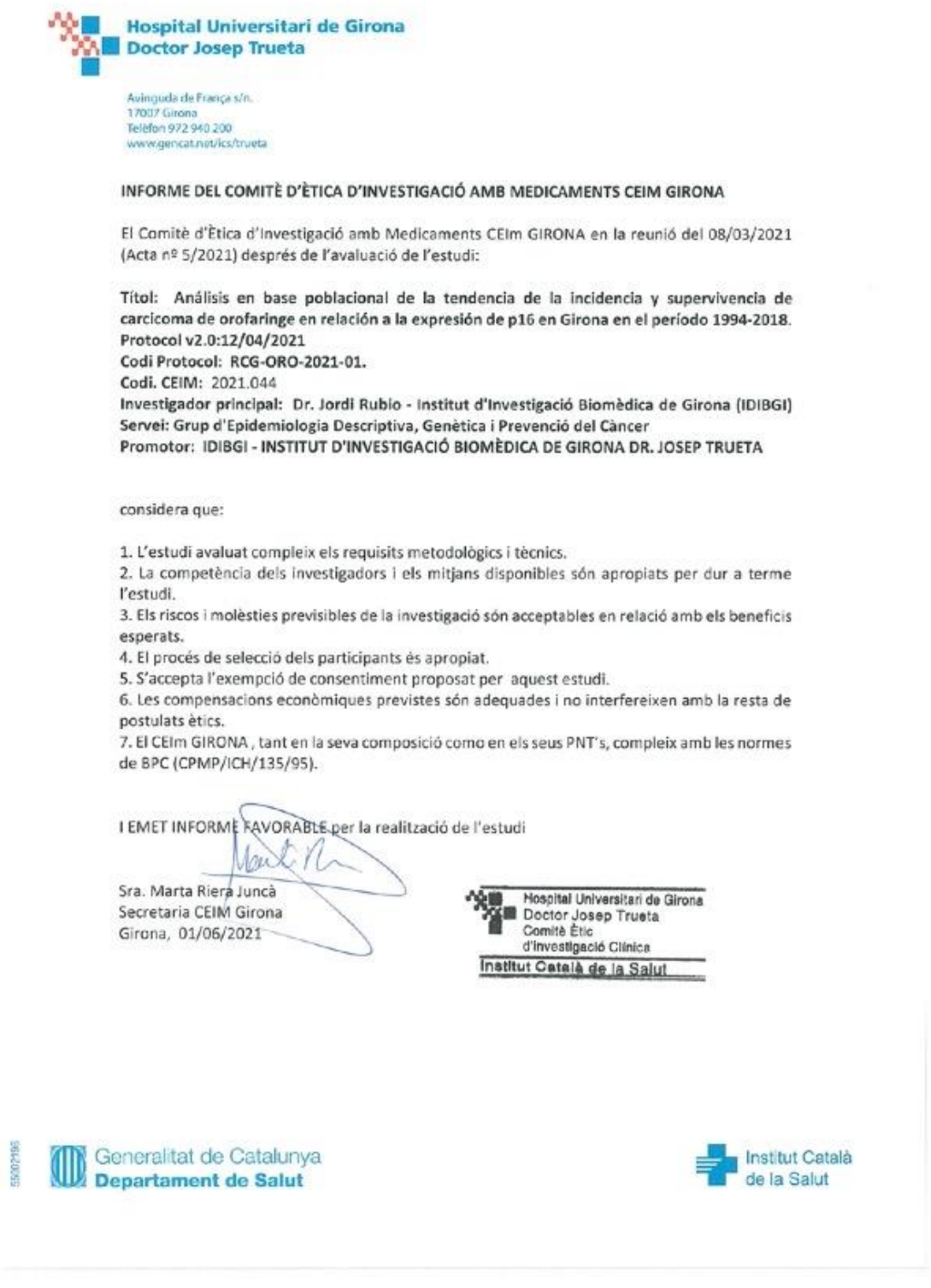
17.7. ANNEX 7

Figure 17. Trends in incidence of overall oropharyngeal cancer, from 1994 to 2018. Global joint-point analysis.



APC = 1.21 (95% CI: -0.5; 2.7)

17.8. ANNEX 8



Hospital Universitari de Girona
Doctor Josep Trueta

Avinguda de França s/n.
17007 Girona
Telèfon 972 940 200
www.gencat.net/ics/trueta

INFORME DEL COMITÈ D'ÈTICA D'INVESTIGACIÓ AMB MEDICAMENTS CEIM GIRONA

El Comitè d'Ètica d'investigació amb Medicaments CEIm GIRONA en la reunió del 08/03/2021 (Acta nº 5/2021) després de l'avaluació de l'estudi:

Títol: Análisis en base poblacional de la tendencia de la incidencia y supervivencia de carcicoma de orofaringe en relación a la expresión de p16 en Girona en el período 1994-2018.
Protocol v2.0:12/04/2021
Codi Protocol: RCG-ORO-2021-01.
Codi. CEIM: 2021.044
Investigador principal: Dr. Jordi Rubio - Institut d'Investigació Biomèdica de Girona (IDIBGI)
Servei: Grup d'Epidemiologia Descriptiva, Genètica i Prevenció del Càncer
Promotor: IDIBGI - INSTITUT D'INVESTIGACIÓ BIOMÈDICA DE GIRONA DR. JOSEP TRUETA

considera que:

1. L'estudi avaluat compleix els requisits metodològics i tècnics.
2. La competència dels investigadors i els mitjans disponibles són apropiats per dur a terme l'estudi.
3. Els riscos i molèsties previsibles de la investigació són acceptables en relació amb els beneficis esperats.
4. El procés de selecció dels participants és apropiat.
5. S'accepta l'exempció de consentiment proposat per aquest estudi.
6. Les compensacions econòmiques previstes són adequades i no interfereixen amb la resta de postulats ètics.
7. El CEIm GIRONA , tant en la seva composició como en els seus PNT's, compleix amb les normes de BPC (CPMP/ICH/135/95).

I EMET INFORME FAVORABLE per la realització de l'estudi

Sra. Marta Riera Juncà
Secretaria CEIM Girona
Girona, 01/06/2021

Hospital Universitari de Girona
Doctor Josep Trueta
Comitè Ètic
d'Investigació Clínica
Institut Català de la Salut

Generalitat de Catalunya
Departament de Salut

Institut Català
de la Salut

Figure 18. Assessment report emitted by the ethics committee approving the study.

17.9. ANNEX 9: *CLINICAL TRAINEESHIP IN DENMARK*

As opposed to Spain, OPC is now the most common HNC diagnosed in Denmark (1). A recent populational-based study from Denmark has reported that there has been a threefold increase in the incidence of HPV-related OPC from 2000 to 2017, but a twofold increase in that of HPV-unrelated OPC (88).

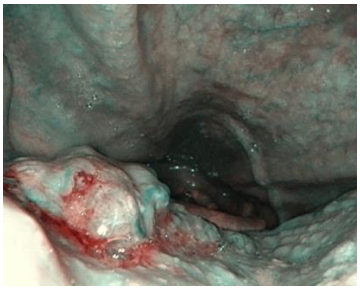
The Danish Head and Neck Cancer Group (DAHANCA) has several ongoing and already finished clinical trials assessing de-intensification strategies and the development of new target therapies to give response to these increasing trends. The DAHANCA 30 trial, "A randomized non-inferiority trial of hypoxia-profile guided hypoxic modification of radiotherapy of HNSCC", is an example (89). Hypoxic modification of radiotherapy with Nimorazole proved to increase radiosensitivity in hypoxic HNSCC in a previous DAHANCA trial (DAHANCA 5). With that, in Denmark, Nimorazole is added in radiotherapy schemes in most of HNSCC. Recently, a hypoxia gene profile has been developed to discriminate between more and less hypoxic tumors, which may help define responders and non-responders to Nimorazole. The basis of the discrimination is the cumulated expression of 15 hypoxia responsive genes, quantified from the tumor biopsy. In the DAHANCA 30 trial, expected hypoxia profile guided non-responders are randomized to +/- Nimorazole during radiotherapy, in order to verify clinical use of gene profiling in selecting relevant patients for hypoxic modification of radiotherapy with Nimorazole.

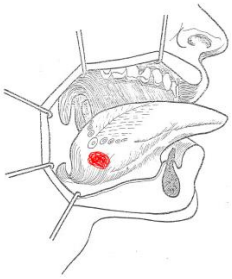
As mentioned previously, differences in tumor microenvironment between HPV-related HNSCC and HPV-unrelated HNSCC are considered to play an important role in radiation response. Tumor cells from these entities respond distinctly in terms of gene expression and cell survival. Therefore, hypoxia signaling, which confers radioresistance in HPV non-related HNSCC, is lower in HPV-related malignancies, which display more radiosensitivity (56).

During my clinical traineeship at the Oncology Department in the Zealand University Hospital, Denmark, I got the chance to be in touch with HPV-related OPC patients included

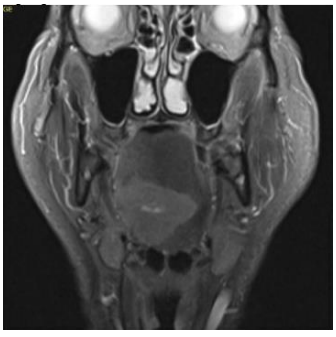
in some of the DAHANCA clinical trials. Herein I present a case report of one of them, who was included in the DAHANCA 30 trial, and suffered from a p16-postive OPC.

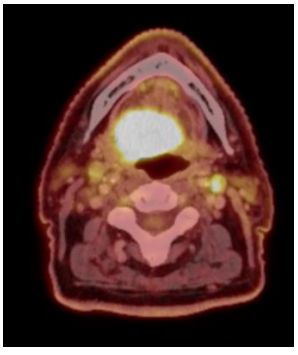
The clinical case presented addresses a 73-year-old male patient who consulted for a 6-month course of pain in the right jaw. He referred no tobacco use or alcohol overconsumption. The patient consulted for pain in the right jaw that had started in late December 2020. He had been examined several times before, and recurrently consulted with diffuse pain in the jaw which irradiated up to the right ear and the right side of the neck and intensified with chewing. The clinical exploration was mostly anodyne except for a solid tumorous process in the right side of the tongue base, estimated to be 4x3x3cm in size, with hard induration and infiltrative growth on palpation (see images below). During the neck exploration, a larger solid lymph node conglomerate was palpated below the sternocleidomastoid muscle in ipsilateral lymphatic levels 2 and 3.



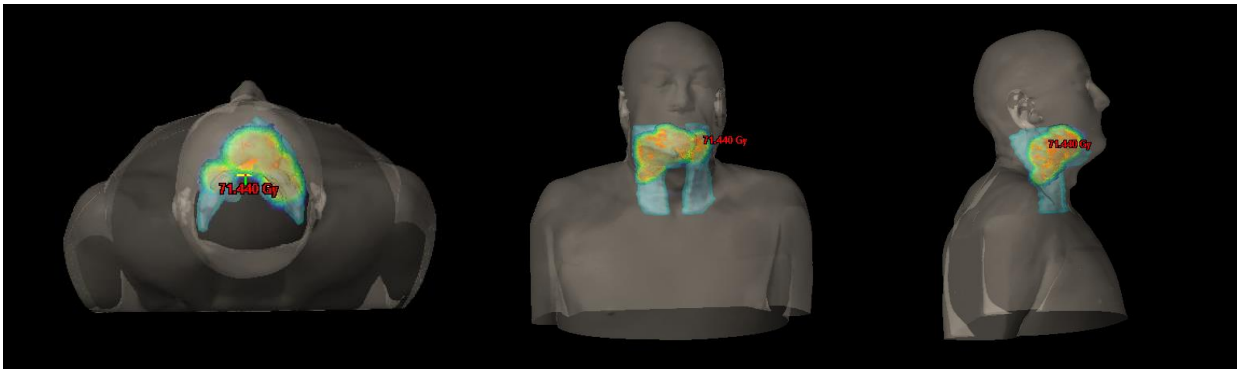


A biopsy of the primary tumor was conducted through fibroscopy, and the histology results determined a squamous cell carcinoma with overexpression of p16 protein. An Ultrasound-guided Fine Needle Aspiration was taken from one of the lymph nodes in level 2 and cyst fluid was collected for cytology analysis, which resulted in metastasis of squamous cell carcinoma. After the imaging study with head and neck MRI and PET-CT (see below) and thorax CT, the patient was diagnosed with a T3N2bM0 squamous cell carcinoma of the tongue base with p16 overexpression.





The case was discussed within a multidisciplinary team, who considered the patient non-eligible for surgery due to the localization of the tumor. Thus, the therapeutic approach decided was curative-intended adjuvance with chemotherapy and radiotherapy, which the patient accepted after being adequately informed. The established radiation treatment was 2Gy x 34, 6F/W with concomitant chemotherapy consisting in 6 series of weekly Cisplatin. As mentioned, the patient fulfilled inclusion criteria and was included in the DAHANCA30 trial, for which he had to take 2500mg of Nimorazole an hour and a half before the radiation session. Besides assessing the patient, one of the tasks I was able to work in was the radiotherapy plan. Here below is the final portrayal of that plan:



Joining the Head and Neck Cancer Unit at Zealand University Hospital allowed me to be in contact with a different population group from the one in Girona. Therefore, during my clinical stay I was able to access information of a different population sample of OPC, and to make a comparative of the two cohorts (from the region of Girona and from Zealand Region) in terms of oropharyngeal carcinoma and p16 protein expression. Overall, this experience helped me to deeply understand how HPV-related OPC does vary according to geographical region, as disease burden and distribution of this regarding sex and age was characteristically distinct in Denmark to what we have observed in the region of Girona.