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**Title:** Predictors of oropharyngeal cancer survival in Europe

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## **Abstract**

HPV16-positive oropharyngeal cancer (OPC) patients experience better outcomes compared to HPV16-negative patients. Currently, strategies for treatment de-escalation are based on HPV status, smoking history and disease stage. However, the appropriate cut-point for smoking and the role of other non-clinical factors in OPC survival remains uncertain. We examined factors associated with OPC outcome in 321 patients recruited in a large European multi-center study. Seropositivity for HPV16 E6 was used as a marker of HPV16 positive cancer. Hazard ratios (HR) and confidence intervals (CI) were estimated using Cox proportional models adjusted for potential confounders. Overall 5-year survival following OPC diagnosis was 50%. HPV16-positive OPC cases were at significantly lower risk of death (HR=0.49, 95%CI: 0.34- 0.70). A significant effect on OPC survival was apparent for female sex (HR 0.52; 95% CI: 0.3- 0.91) and being underweight at diagnosis (HR: 2.43, 95% CI: 1.4- 4.22). A 10 pack year smoking history was not associated with survival. This study confirms that HPV16 status is an independent prognostic factor for OPC survival while female sex lowers risk of death and being underweight at diagnosis increases the risk of death. Smoking was not an independent predictor of OPC survival.

## **Key message**

The role of patient-related factors in oropharyngeal cancer survival is unclear. Here we show that in addition to HPV16 status and disease stage, being female was associated with nearly 50% reduced risk and low BMI at diagnosis almost doubled the risk of death following oropharyngeal cancer.

**Key words:** Oropharynx cancer, HPV16, survival, tobacco, body mass index, gender



## Introduction

Cancers arising in the oral cavity and pharynx have an estimated global burden of 442,760 incident cases and 241,458 deaths each year[1]. Tobacco smoking and alcohol consumption explain nearly 70% of these cancers[2, 3]. Infection by human papillomavirus (HPV), particularly type 16 (HPV16) causes a subset of cancers, particularly those arising at the tonsil, oropharynx, soft palate and base of the tongue (collectively referred to as oropharyngeal cancers-OPC)[4]. Further, HPV16-positive (HPV16+) OPC is described to be epidemiologically, molecularly and clinically distinct from HPV16-negative (HPV16-) OPC[5]. The increasing incidence of OPC in several Western countries is attributed to the HPV16+ fraction[6-9].

Since HPV16+ OPC patients experience better survival outcomes compared to HPV16- patients, alternative staging has been recommended[10-12]. Recurrence remains a concern and it is unclear which patients may benefit from de-intensified treatment. Clinically, HPV status is ascertained based on HPV DNA and p16 expression or p16 expression alone. HPV status in combination with disease stage and patient smoking history (based on a 10 or 20 pack year cut off) is currently used to classify patients into prognostic groups and to identify candidates for de-escalation of treatment[10, 13]. This scheme has rarely been verified. Further, the appropriate cut-point for pack years of smoking remains uncertain. In addition, the role of other non-clinical risk factors in OPC survival is not fully understood.

To address these knowledge gaps, we tested 321 oropharyngeal tumors in a large series of well characterized European patients for HPV16 DNA, p16 expression and the corresponding sera for HPV16 serology. Applying rigorous protocols of sample processing; we aimed to evaluate the role of HPV16 and other risk factors in predicting OPC survival and recurrence.

## Methods

This analysis was based on cases from the European Alcohol Related Cancers and Genetic susceptibility in Europe (ARCAGE) study, conducted across 10 countries in Europe using a standardized protocol [14]. Briefly, over 2000 incident cases of the oral cavity, pharynx, larynx, esophagus and matched controls were recruited during 2002 to 2005. This analysis included squamous cell carcinoma of ICD-O diagnoses C01, C02.4, C05.1- C05.2, C09, C10. All participants underwent personal interviews to record lifestyle exposures. All cases were histologically or cytologically confirmed primary cancers, and cancer stage was ascertained based on the sixth edition of the staging atlas developed by the American Joint Committee on Cancer (AJCC). Tobacco use was broadly categorized as ever or never smokers, ever smokers were defined as individuals who smoked any tobacco product at least once a week for a year. Pack years were calculated for all types of tobacco smoking based on cigarette equivalents. Ever drinkers were those who reported ever consumption of any alcoholic beverage and the consumption of all types of alcoholic beverages was estimated and the total frequency was expressed in terms of drinks of alcohol per day [15]. A weighted composite score of oral hygiene and dental care was constructed as described previously [16] and included denture wear, age at start of denture-wearing and gingival bleeding. A weighted dental care score was also constructed by combining the frequency of tooth cleaning, use of toothpaste, toothbrush or dental floss and

frequency of dentist visits, where the maximum score of eight reflected poor dental care. Body mass index was calculated based on weight measured at the time of recruitment. BMI ranging from 18.5 to 25.0 was considered normal, below 18.5 underweight while >25.0 was considered overweight. Informed consent was obtained from all participants in the study and the study was approved by the ethical review boards at the participating centers.

Pretreatment serum samples were tested for HPV antibodies using the bead-based multiplex serology method [17, 18]. We have previously shown that HPV16 E6 antibody is a highly specific marker of HPV16+ OPC with false-positive rates less than 1% [17, 19-21]. For comparison, 198 paraffin-embedded OPC tumor blocks were qualitatively evaluated for p16 expression using the CINtec Histology P16<sup>INK4a</sup> Kit (9511, mtmlabs) following manufacturer's instructions. Expression was scored based on the percentage and intensity of nuclear or cytoplasmic staining. A combined score of 4 or greater was considered positive for p16<sup>INK4a</sup> overexpression. HPV genotyping was performed using the Type-Specific E7 PCR bead-based multiplex assay (TS-E7-MPG, IARC, France) [17, 22]. Given that HPV serology was available on all cases; our analysis defined a HPV+ tumor as HPV16 E6 antibody positive. We subsequently compared this to tumor results based on p16 expression and HPV DNA.

Participants underwent a one-time follow-up conducted between 2012 and 2015. Mortality data including cause and date of death were obtained from at least two information sources for 75% of cases, and one source for the remaining 25%. In Prague and Aviano follow-up was completed by review of medical charts alone. In all other centers, medical chart reviews together with information from population-based registries at the regional or national level were used. In Athens, Barcelona and Manchester physicians were contacted to obtain patient outcome information, while in Oslo, Zagreb and Glasgow cancer registries were consulted. In Bremen, Turin, Padova and Dublin mortality registries were examined. End of follow-up was defined as the date of last confirmed contact, vital status at censor, or date of death (if applicable). Over 96% of OPC patients' recruited in the study have complete follow-up. Overall survival (all-cause mortality) was evaluated using Cox proportional hazard models, predictors were explored for OPC overall and stratified by stage, sex and HPV16 status. Mortality was also explored using Kaplan Meier curves. The joint effects on survival were considered by combining cofactors in interaction models. We used chi-squared tests to examine heterogeneity in hazard estimates. All statistical analyses were performed using STATA statistical software, version 11 (StataCorp, College Station, TX), and all reported *P* values are two sided. Statistical significance was set at *P* less than 0.05.

## Results

Subjects were primarily male (77%), ever smokers (90%), alcohol drinkers (97%) and had a median age at diagnosis of 58. The vast majority of OPC were diagnosed at late stages; 49% in Stage IV, 25% in stage III and 26% in stages I and II. 31% of OPC cases were HPV16 E6 positive. OPC subjects were followed for 1257 person years during which 175 deaths occurred (mean follow-up of 3.92 years), of which 98 (56%) were due to head and neck cancer.

Overall 5-year survival following OPC diagnosis was 50% (95% CI: 43.9- 54.9). As described in table 1, in the univariate analyses there was significantly lower risk of death among 99 HPV16+ compared to 222 HPV- OPC



(HR=0.49, 95%CI: 0.34- 0.70). Five-year overall survival was 65% among HPV16+ compared to only 43% among HPV16- OPC ( $p<0.001$ , Figure 1). Other significant risk factors associated with OPC survival included female sex (HR 0.52, 95% CI: 0.35- 0.78), older age at diagnosis (HR: 1.24, 95% CI: 1.05- 1.45), smoking (HR 2.24, 95% CI: 1.39- 3.60), alcohol use (HR: 1.53, 95% CI: 1.13- 2.06), moderate dental care (HR: 1.58, 95% CI: 1.10- 2.29), being underweight at diagnosis (HR: 2.25, 95% CI: 1.38- 3.67) and late stage disease (HR: 2.84, 95% CI: 1.78- 4.55) (Table 1). When these covariates were combined in a single model, the risk of death remained significant lower among HPV16+ compared to HPV16- OPC (aHR: 0.51, 95%CI: 0.32- 0.80, Table 1). In addition, a significant effect on OPC survival was apparent for female sex (aHR 0.50: 95% CI: 0.29- 0.85), being underweight at diagnosis (aHR: 2.41, 95% CI: 1.38- 4.21) and higher disease stage (aHR: 2.63, 95% CI: 1.61- 4.03), but not for smoking (HR: 1.11, 95% CI: 0.59- 2.09), moderate dental care (aHR: 1.09, 95% CI: 0.72- 1.65), age (HR: 1.17, 95% CI: 0.95- 1.44) or alcohol use (HR: 1.02, 95% CI: 0.69- 1.51) (Table 1).

We further examined the robustness of the associations of being female or being underweight and OPC survival. No difference was observed in the association between being female and OPC survival among HPV16+ (HR: 0.30, 95% CI: 0.09- 0.95) or HPV16- OPC (HR: 0.67, 95% CI: 0.36- 1.37, ( $P$ -heterogeneity: 0.22). Conversely, the increased risk of death associated with being underweight at diagnosis was consistent for stage I and II (HR: 1.51, 95% CI: 0.17-13.30), stage III (HR: 4.94, 95% CI: 1.77-13.81) and stage IV disease (HR: 2.22, 95% CI: 1.08-4.55), with no heterogeneity in the estimates ( $p$  for heterogeneity: 0.39). We further examined these findings in the context of the recently proposed staging for HPV+ OPC that combines disease stage at diagnosis, age and smoking pack-years into four groups with differing prognosis; group I comprising of stage I-III OPC patients' with  $\leq 20$  pack years, group II of stage I-III patients' with  $>20$  pack years, group III were stage IV and  $\leq 70$  years old and group IV were stage IV with  $>70$  years of age [10]. Consistent with the initial report, overall survival decreased across these groups (data not shown). Notably, female sex (HR: 0.54, 95% CI: 0.33- 0.88) and being underweight at diagnosis (HR for: 2.59, 95% CI: 1.50- 4.46) remained consistently associated with OPC survival, independent of the suggested prognostic risk classification.

Unlike previously published studies, the lack of association of smoking with overall survival following OPC diagnosis was consistent (supplementary table 1). Although metrics of smoking exposure were associated with higher risk of death among OPC patients' in the univariate analysis (e.g. HR for 10 pack year cut-point: 2.24, 95% CI: 1.39-3.60), none of the associations remained significant in the multivariable models. Inclusion of stage and HPV16 status in the models significantly mitigated the effect for smoking (bHR for 10 pack year cut-point: 1.46, 95% CI: 0.80-2.69). Further, we observed no interaction between smoking and HPV16 status in the risk of death following OPC diagnosis ( $P$ -interaction: 0.50).

There were 84 recurrences among 260 cases with an average follow-up of 4.2 years. The risk of recurrence was lower in 82 HPV16+ than 178 HPV16- OPC (HR: 0.59; 95% CI: 0.36- 0.96, aHR: 0.55; 95% CI: 0.29- 1.06, Table 2). Three year recurrence-free survival was 77% in HPV16+ and 66% in HPV16- OPC ( $P=0.03$ , Figure 2). The only other factor associated with OPC recurrence was advanced stage disease (HR for stage IV OPC: 4.88; 95% CI: 2.12- 11.21). 27% (22/82) of patients with HPV16+ OPC had recurrence within the follow-up period. Other factors

associated with progression among HPV16+ OPC included moderate dental care (HR: 3.98, 95% CI: 1.19- 13.35) and advanced disease stage (HR for stage IV OPC: 7.54, 95% CI: 1.23- 46.13).

In this study, 198 corresponding tumors of 321 cases were tested for HPV DNA and p16 expression. Of these, 16 were excluded due to limited tissue. Of the remaining 156 tumors, 52 were HPV16 E6 seropositive of which 43 were positive for both HPV16 DNA and p16 expression while 44 were positive for p16 expression alone (Table 3). Among the 104 HPV16 E6 seronegative cases, 98 were HPV16 DNA or p16 negative while 84 were p16 negative. The agreement between HPV16 E6 serology and the combined marker (91%) was better compared to p16 alone (82%)(Table 3). In the univariate analysis, tumor HPV16 DNA positivity alone was marginally associated with OPC survival (HR: 0.66, 95% CI: 0.43- 1.02), while tumor p16-positivity alone (HR: 0.59, 95% CI: 0.38- 0.94) or dual positivity to HPV16 DNA and p16 (HR: 0.48, 95% CI: 0.29- 0.81) were significantly associated with OPC survival. The strongest association with survival however, was observed among OPC subjects who were HPV16 E6 serology positive (HR=0.44, 95% CI: 0.26- 0.74). In the fully adjusted model however, only the association between HPV16 E6 serology and OPC survival remained significant (HR: 0.39, 95% CI: 0.19- 0.71) (Table 4).

## Discussion

This study confirms HPV16 status as an independent prognostic factor for overall survival and recurrence-free survival among OPC patients'. We also report that women are at lower risk of death (both HPV+/HPV-), while being underweight at diagnosis increases the risk of death following OPC diagnosis.

The reduction in risk of death among HPV16+ OPC in this study was similar in magnitude to previous studies [13, 23]. Our results indicate that additional factors may have an important effect in predicting survival following OPC. In particular that women may have approximately 50% reduced risk of death compared to men following an OPC diagnosis. Importantly, this benefit was extended to late stages of disease and was consistent for both HPV16+ and HPV16- OPC suggesting the protective effect may be driven by inherent factors. Although an individual's gender has been long recognized as a key factor affecting cancer incidence, prognosis, and treatment response, the underlying mechanisms remain poorly understood. We also report that being underweight at diagnosis increases the risk of death following OPC by over two-fold. This association was robust across stage, sex and HPV16 status. Although it remains probable that low BMI at diagnosis reflects the disease itself, since cancer patients tend to lose weight rapidly following disease onset, it could also point towards the need for nutritional interventions prior to clinical treatment as a strategy towards potentially improved outcomes. The lack of association with low BMI at 30 years of age and OPC survival in this study (data not shown) supports this notion. It remains important to note that both low BMI at diagnosis as well as female sex remained independently associated even when considering the recently proposed prognostic risk classification for OPC. These findings warrant further validation in independent studies.

Previous studies have found tobacco smoking to be independently associated with overall survival as well as progression-free survival among OPC patients[5, 10, 13, 24]. Smoking, in particular, 10 or 20 pack year thresholds in conjunction with tumor stage and HPV status have therefore been proposed for risk stratification and [10, 13]to



identify patients who may benefit from treatment de-escalation. In this study, the lack of association between smoking and OPC survival was consistently observed irrespective of the smoking parameter examined (i.e., smoking status, or varying definitions for pack years including increments of 1 pack year, 10 pack years or thresholds of 10 and 20 pack years). Sequential adjustment of covariates indicates that HPV16 status and disease stage could potentially account for the survival differences due to smoking. Further, we found no interaction between smoking and HPV16 status in the risk of death following OPC. These results call for additional evidence in larger cases series in order to accurately establish the role of smoking and survival among HPV16+ OPC.

This study demonstrates a strong association between HPV16 status and OPC survival. Even though p16 and HPV16 DNA each showed utility in predicting mortality, they both appeared to be sub-optimal proxies for HPV16 status. While the utility of tumor HPV16 DNA and p16 as diagnostic markers of OPC is now well established [25-27], this study confirms the utility of HPV16 E6 serology as a robust prognostic marker of OPC.

Despite the advantage of the multi-centric design and the large number of participants enrolled in the parent study, the OPC subset was limited to 321 cases. Even so, to the best of our knowledge, this remains among the largest analysis of this rare cancer with detailed information on risk factors, patient outcome as well as HPV16 status. Important strengths of this study are the centralized testing for HPV16, uniform collection of survey and cancer outcome information in all centers. Some of the limitations include the absence of detailed treatment information that could potentially impact survival. However, the majority of the patients' were treated with surgery alone or in conjunction with chemotherapy or radiation therapy (68%). Further, disease stage was missing on 16% of OPC since the study was initially designed with a focus on risk factors for cancer occurrence, therefore comprehensive collection of clinical data was not emphasized.

In conclusion, this study confirms the markedly improved prognosis associated with HPV+ OPC in Europe. In addition, we report that being female and low BMI at diagnosis are important factors affecting overall OPC survival. Further large studies that also include comprehensive mutational profiling will be required to better understand the mechanisms underlying these prognostic subgroups.



**Conflict of interest statement:**

The authors declare that they have no conflict of interest.

**Table 1: Univariate and multivariate analyses of risk factors associated with death in 321 oropharyngeal cancer patients**

Characteristics	Dead/ Total	Univariate HR (95% CI)	Multivariate aHR (95% CI)
(N=321)			
<b>HPV16 E6 Serology</b>			
Negative	136/222	1.0	1.0
Positive	39/99	<b>0.49 (0.34- 0.70)</b>	<b>0.51 (0.32- 0.80)</b>
<b>Sex</b>			
Male	147/247	1.0	1.0
Female	28/74	<b>0.52 (0.35- 0.78)</b>	<b>0.50 (0.29- 0.85)</b>
Age (10 year increase)		<b>1.24 (1.05- 1.45)</b>	1.17 (0.95- 1.44)
<b>Smoking status<sup>a</sup></b>			
≤10 Pack-years	19/58	1.0	1.0
>10 Pack-years	155/262	<b>2.24 (1.39- 3.60)</b>	1.11 (0.59- 2.09)
<b>Alcohol use<sup>b</sup></b>			
≤2 drinks/ day	75/159	1.0	1.0
>2 drinks/ day	99/160	<b>1.53 (1.13- 2.06)</b>	1.02 (0.69- 1.51)
<b>Dental care<sup>c</sup></b>			
Good	36/84	1.0	1.0
Moderate	134/231	<b>1.58 (1.1- 2.29)</b>	1.09 (0.72- 1.65)
Poor	3/4	2.45 (0.76- 7.98)	0.46 (0.06- 3.44)
<b>BMI at diagnosis<sup>d</sup></b>			
Normal)	83/161	1.0	1.0
Underweight	20/25	<b>2.25 (1.38- 3.67)</b>	<b>2.41 (1.38- 4.21)</b>
Overweight	61/108	1.01 (0.73- 1.41)	1.12 (0.77- 1.63)
<b>Stage<sup>e</sup></b>			
I&II	22/71	1.0	1.0
III	35/67	<b>2.02 (1.18- 3.44)</b>	<b>1.78 (1.02- 3.10)</b>
IV	85/131	<b>2.84 (1.78- 4.55)</b>	<b>2.63 (1.61- 4.30)</b>

aHR: adjusted mutually for all covariates in the table, and additionally for center of recruitment

<sup>a</sup> 1 case missing smoking information

<sup>b</sup> 2 cases missing alcohol data

<sup>c</sup> missing information on 2 cases

<sup>d</sup> missing information on 27 cases

<sup>e</sup> missing stage on 52 cases

**Table 2: Univariate and multivariate analyses of risk factors associated with recurrence in 260 oropharynx cancer patients**

<b>Characteristics</b>	<b>HR (95% CI)</b>	<b>aHR (95% CI)</b>
<b>HPV16 E6 Serology</b>		
Negative	1	1
Positive	<b>0.59 (0.36-0.96)</b>	0.55 (0.29- 1.06)
<b>Sex</b>		
Male	1	1
Female	0.86 (0.52-1.40)	1.04 (0.52- 2.09)
<b>Age (10 year increase)</b>	1.13 (0.89- 1.43)	1.03 (0.77- 1.38)
<b>Smoking status</b>		
≤10 Pack-years	1	1
>10 Pack-years	1.38 (0.79- 2.43)	0.65 (0.30- 1.41)
<b>Alcohol use</b>		
≤2 drinks/ day	1	1
>2 drinks/ day	1.33 (0.87- 2.04)	0.99 (0.55- 1.81)
<b>Dental care</b>		
Good	1	1
Moderate	<b>2.25 (1.29- 3.95)</b>	1.82 (0.96- 3.45)
Poor	1.77 (0.23-13.42)	1.19 (0.15- 9. 69)
<b>BMI at diagnosis</b>		
Normal	1	1
Underweight	0.85 (0.30-2.36)	0.82 (0.27- 2.43)
Overweight	0.82 (0.51- 1.32)	0.74 (0.43- 1.27)
<b>Stage</b>		
I & II	1	1
III	2.12 (0.98-4.59)	<b>2.89 (1.18- 7.06)</b>
IV	<b>3.22 (1.61-6.44)</b>	<b>4.88 (2.12- 11.21)</b>

aHR: adjusted mutually for all covariates in the table, and additionally for center of recruitment



**Table 3: Concordance between serology and tumor markers of HPV16 infection in 153 oropharyngeal cancer patients**

<b>Tumor marker</b>	<b>HPV16 E6 serology</b>	
	Positive <i>N</i> =52	Negative <i>N</i> =104
<b>HPV16 DNA/ p16 status</b>	Agreement= 91%	
Positive	43	6
Negative	9	98
<b>p16 status</b>	Agreement=82%	
Positive	44	20
Negative	8	84

**Table 4: Multivariate analysis of the association between markers of HPV16 infection and risk of death in 235 oropharyngeal cancer patients**

<b>HPV marker</b>	<b>Dead/ Total</b>	<b>HR (95% CI)</b>	<b>aHR (95% CI)</b>
<b>HPV16 DNA</b>			
Positive	41/ 69	1.0	1.0
Negative	41/ 84	0.66 (0.43- 1.02)	0.77 (0.43- 1.35)
<b>p16</b>			
Positive	54/ 89	1.0	1.0
Negative	28/ 64	<b>0.59 (0.38- 0.94)</b>	0.72 (0.42- 1.24)
<b>HPV16 DNA/ p16</b>			
Positive	63/ 102	1.0	1.0
Negative	19/ 51	<b>0.48 (0.29- 0.81)</b>	0.63 (0.35- 1.15)
<b>HPV16 E6</b>			
Positive	63/ 104	1.0	1.0
Negative	19/ 49	<b>0.44 (0.26- 0.74)</b>	<b>0.36 (0.19- 0.71)</b>

aHR: adjusted for age, sex, smoking pack years, alcohol drink years, dental care, stage and center

**Figure 1(a): Overall survival among 321 oropharyngeal cancer patients' by HPV16 E6 status**

**Figure legend:**

Cumulative survival of all-cause mortality among patients diagnosed with oropharyngeal cancer by human papillomavirus type 16 (HPV16) E6 serology status. Patients who were seropositive (solid line;  $N=99$ ) and seronegative (broken line;  $N=222$ ) for HPV16 were compared for all-cause mortality.

**Figure 1(b): Recurrence-free survival of 260 oropharyngeal cancer patients by HPV16 E6 status**

**Figure legend:**

Recurrence-free survival among 260 patients diagnosed with oropharyngeal cancer by human papillomavirus type 16 (HPV16) E6 serology status. Patients who were seropositive (solid line;  $N=82$ ) and seronegative (broken line;  $N=178$ ) for HPV16 were compared for all-cause mortality.



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Figure 1a

$p=0.0001$





