



FACULTAD DE MEDICINA  
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## GRADO EN MEDICINA

### TRABAJO FIN DE GRADO

**Implicaciones del virus del papiloma humano y la  
ciclina D1 en el cáncer de orofaringe**

**Cyclin D1 and human papillomavirus implications in  
oropharyngeal cancer**

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**ABSTRACT:**

Oropharyngeal cancers (OPC) are subdivided into two different entities based on HPV infections, which are mainly by p16 subtype alone, due to better overall survival (OS) rates. However, differences in the techniques used and the few cohorts looking for other HPV subtypes suggest the necessity to approach this further. Moreover, there is still a group of HPV+ patients that present a lethal and aggressive disease. Cyclin D1 overexpression has been proposed as a possible marker to better stratify these patients although results differed among studies. Due to that, this study aimed to assess HPV prevalence and subtypes in a cohort of 54 OPC patients from HUMV as well as Cyclin D1 expression among them to determine its effect on prognosis. Our results show a high HPV prevalence (38% of patients) and confirm HPV patients increased OS ( $p=0,035$ ). p16 was the most common HPV-subtype (50%), followed by 59, 58 and 53. 38% of patients had multiple infections strongly contrasting with other cohorts (5%). Moreover, not significant differences were observed when looking at HPV+/cyclin D1 positive patients OS ( $p=0,933$ ). However, due to low sample number and different cut-offs in other studies we can't affirm this forcefully so further analysis should be done.

**Key words:** Oropharyngeal cancer, Cyclin D1, human papillomavirus

## **RESUMEN:**

Los cánceres de orofaringe (CO) se pueden dividir en dos entidades diferentes en función de las infecciones por VPH, principalmente causadas por el subtipo 16, debido a una mejor supervivencia. Sin embargo, las diferencias en las técnicas usadas y las pocas cohortes mirando otros subtipos del VPH sugieren la necesidad profundizar más en este campo. Además, hay un grupo de pacientes VPH+ que presentan una enfermedad más letal y agresiva. La sobreexpresión de Ciclina D1 ha sido propuesta como un posible marcador para mejorar la estratificación de estos pacientes aunque los resultados difieren entre diferentes estudios. Debido a esto, este estudio tuvo el objetivo de determinar la prevalencia de VPH y sus subtipos en una cohorte de 54 pacientes de CO del HUMV así como la expresión de Ciclina D1 entre ellos para determinar los efectos en el pronóstico. Nuestros resultados muestran una prevalencia del 38% para el VPH y confirma el aumento de supervivencia entre estos pacientes ( $p=0,035$ ). El subtipo 16 fue el más común (50%), seguido por 59, 58 y 53. El 38% de los pacientes están infectados por múltiples subtipos lo que contrasta con otras cohortes (5%). Además, no vimos diferencias significativas en la supervivencia cuando miramos a los pacientes positivos para VPH y Ciclina D1 ( $p=0,933$ ). Sin embargo, debido al bajo número de muestras y diferencias en el corte en la expresión de Ciclina D1 no podemos afirmar esto por lo que nuevos análisis deben de hacerse.

**Palabras Clave:** Cáncer de orofaringe, Ciclina D1, virus del papiloma humano

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## **Abbreviations**

<b>CDK4/6</b>	Cyclin Dependent Kinase 4 and 6
<b>ENE</b>	Extranodal Extension
<b>HPV</b>	Human Papillomavirus
<b>OPC</b>	Oropharyngeal Carcinomas
<b>Rb</b>	Retinoblastoma Protein



## 1. INTRODUCTION

Oropharyngeal cancers are a common malignancy in the head and neck region and more than 90% are squamous cell carcinomas (1). In 2018, there were estimated 92,887 newly diagnosed oral cancer patients and 51,005 deaths from them worldwide which shows their significant incidence and mortality (2). The anatomic location of these cancers largely influences their associated risk factors, treatment options, and related epidemiologic characteristics (3). The term “oropharynx” refers to the posterior 1/3 of the tongue, palatine and lingual tonsils, soft palate, and the posterior pharyngeal wall (4) (5).

Both oral cavity and oropharyngeal cancer have been related to cigarette smoking and alcohol consumption (6-8). However, although documented decreases in both these factors only oral cavity carcinomas incidence rates are decreasing while oropharyngeal carcinomas (OPC) are reaching epidemic levels (7, 9). This suggests differences in their biology and the presence of other major risk factors. Indeed, oropharyngeal cancer is tightly related to chronic latent infections of the human papillomavirus (HPV) (10-12). For instance, HPV detection in OPC has increased from 16% to 73% in the United States indicating a profound epidemiologic shift from carcinogen-induced to HPV-associated OPC (11, 13). HPV infection has been related to cervix cancer and the main HPV-types accounting for 70% of the cases are Type 16 and Type 18 (14-16). However, HPV-positive OPCs are HPV-16 in 95% of the cases (11, 17, 18). HPV infection is sexually transmitted and the increase of incidence can be explained by the changes in sexual behaviors that have occurred in the last years (16, 19-21). HPV+ OPC patients have remarkably higher overall survival than those without HPV-disease (13, 22-24). In a multicenter cohort study researchers saw that 5-year overall survival was similar for 7th edition TNM stage I, II, III, and IVA but was lower for stage IVB and that it did not differ among N0, N1–N2a and N2b subsets, but was significantly lower for those with N3 disease (13). Therefore, in the 8th edition TNM staging it was included a separate classification for p16-positive and p16-negative oropharyngeal cancer, a surrogate marker for oncogenic HPV. The T classification remains the same, except for carcinoma in situ (Tis) and T4b category that have been removed from HPV-positive disease. A key change is in the N category. In HPV-related carcinoma it is based on lymph node size (>6 cm in greatest dimension; cN3) and presence in the contralateral neck (cN2). Whereas, in HPV-negative cancer, extranodal extension defines cN3 category, independently of single ipsilateral lymph node ≤3 cm (cN1) or single/multiple metastasis in ipsilateral/bilateral/contralateral lymph nodes <6 cm (cN2). These modifications result in relative downstaging for HPV-positive patients – due to N criteria – and upstaging for HPV-negative patients – due to extranodal extension (25). However, the biological phenotypes of tumors are often divergent despite identical staging, resulting in different clinical outcomes and response to the selected treatment so new prognostic biomarkers are key to better stratify the risk in HPV-positive patients for determining appropriate therapy (26-28).

One molecular marker that has been related to HPV-related cancer aggressiveness has

been Cyclin D1 by either gene amplification (26), rearrangement (29) or overexpression (28, 30). Cyclin D1 functions as a mitogen sensor and allosteric activator of cyclin dependent kinase 4 and 6 (CDK4/CDK6) regulating cell cycle transition from G1 to S phase, which in turn phosphorylate the retinoblastoma protein (Rb) which results in its functional inactivation releasing E2F family transcription factors from Rb-dependent repression. This allows the activation of a variety of genes promoting the entry and progression through S phase (31-33). In addition, cyclin D1 is capable to sequester CDK inhibitors, such as the INK4 family where p16- INK4a plays a crucial role CIP and KIP family that includes p21CDKN1A and p27-CDKN1B, increasing signalling efficiency (32, 34, 35). E6 and E7 HPV viral oncoproteins inactivate p53 and the retinoblastoma protein pRb causing p16 protein overexpression that leads to an inhibition and reduce levels of Cyclin-D1-CDK4/6 due to loss of the pRb negative feedback loop (36-38). Therefore, cyclin D1 downregulation has been linked to HPV-positive cancers and its overexpression has been seen to confer poor outcome (28, 39, 40). However, there is not a large amount of evidence suggesting its prognostic significance in oropharyngeal cancer due to high heterogeneity among studies.

Given that HPV associated oropharyngeal cancers are becoming an epidemic worldwide growing problem, characterization of HPV-associated molecular markers is key for determining new prognostic markers that would allow to develop new therapeutically approaches. Due to that the main objective of our study was to determine the association of cyclin D1 and HPV status in OPC patients and its relevance on their outcome. Our second objective was to find another clinical prognostic markers such as patients age, sex, alcohol and tobacco exposure, previous tumors, secondary tumors, tumor recurrence and type and treatment.

## **2. MATERIAL AND METHODS**

### **2.1. Patients and tumour tissue**

This is a retrospective case series. A total of 54 patients (n = 54) with primary OPC diagnosed at Hospital Universitario Marqués de Valdecilla between 2014-2018 were retrospectively selected. The years were chosen so that all patients have had at least 2 years of follow-up after diagnosis. Patients information was collected through electronic medical records review from the hospital Database including age, gender, smoking status, alcohol intake, previous tumors, tumour location, treatment received, secondary tumours, tumor recurrence and type, follow-up time and survival status. Tumour staging using the TNM classification (41) was determined by review of clinical notes, radiology reports, surgical notes and pathology reports.

### **2.2. P16 and Cyclin D1 immunohistochemistry**

Immunopathological study was carried out on formalin-fixed 4- $\mu$ m-thick paraffin-embedded tissue sections using the EnVision FLEX Visualization System (Dako, Agilent

Technologies, SL, Las Rozas, Madrid, Spain). Antibodies used in the immunohistochemical study are detailed in Table 2.1. The immunohistochemical reactions were performed using appropriate tissue controls. Automatic staining was accomplished on a Dako Omnis autostainer (Agilent Technologies, SL) Appropriate controls were used including normal tonsil (negative) and a known positive OPC case. P16 staining was considered positive only if there was strong nuclear and cytoplasmic staining that accounted more than 70% of tumor cells. Negative staining was considered when the staining was only nuclear, had a patch mosaic expression, had a marginal expression pattern or was absent (42). P16 Immunohistochemistry was used as a HPV surrogate marker and only implies positivity for HPV not for p16 subtype specifically. Cyclin D1 staining was considered positive when >50% of the specimen stained positive for it.

ANTIBODY	SOURCE	CLONE	DILUTION	RETRIEVAL SOLUTION
P16	DB Biotech	R-19-D	1:100	High
CYCLIN D1	Dako	EP-12	FLEX-RTU	High

**Table 2.1.** Immunohistochemical antibodies used.

### 2.3. HPV molecular study

HPV molecular study was carried out on DNA extracted from 5 µm thick paraffin embedded sections. CLART HPV 4 kit (Genomica, Madrid) was used. This is based in viral DNA specific fragments amplification and hybridation with specific probes for each HPV type; it has 35 HPV subtypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68a y b, 70, 71, 72, 73, 81, 82, 83, 84, 85 y 89). Visualization was done with CLART- Strip.

### 2.4. Statistical analysis

Data was tabulated using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and analyzed using SPSS/PASW Statistics (version 19, SPSS Inc., Chicago, IL). In order to compare dichotomous and categorical variables we used the Fisher's exact test. Oropharyngeal cancer-specific survival was taken as time from cancer diagnosis until date of death or last medical consult. Kaplan-Meier method with log-rank test for determining statistical significance was used to estimate overall survival. Statistical significance was defined as  $p < 0.05$ .

### 3. RESULTS

#### 3.1 Clinicopathological data

54 patients were identified to have primary oropharyngeal squamous cell carcinoma from the year 2014 to 2018 from the Hospital database of Hospital Universitario Marqués de Valdecilla. Several clinicopathological characteristics were analyzed and are summarized in **table 3.1** and **3.2**. Patients were categorized into two outcome groups based on disease status after treatment: Group 0 represented disease-free survival (54,5%) and deaths for other causes (10,9%); Group 1 represented disease-specific deaths (32,7%). Median age at onset was 64 years old (range 39 to 88 years). From all 54 patients 83,6% were males and 21,8% had previous tumours. Most of the tumors were tumor stage T2 or T4, 17 cases (31%) and 16 cases (29%) respectively, followed closely by tumors larger than 4 cm or

<i>Clinical Characteristics</i>	<b>Total (n=54)</b>	<b>Group 0 (n=37)</b>	<b>Group 1 (n=18)</b>	<b>P value</b>
<b>Sex</b>				
Female	9	6	3	1
Male	46	31	15	
<b>Smoking</b>				
Yes	50	34	16	0,594
no	4	2	2	
<b>Alcohol intake</b>				
Yes	46	30	16	0,704
no	8	6	2	
<b>Tumoral site</b>				
Tongue base	21	14	7	
Palatine tonsil	12	8	4	
Lateral wall	5	3	2	0,292
Posterior wall	2	2	0	
Palate	6	2	4	
Others	9	8	1	

**Table 3.1. Clinicopathological characteristics.** Group 0: disease-free survival and patients who died for other causes; Group 1 represented disease-specific deaths.

affecting the lingual surface of the epiglottis (T3) that were 14 (25%). Tumors of 2 cm or smaller (T1) were only 15% of the cases. Around one third of the patients did not present metastatic lymph nodes (36%), 15% had a metastatic ipsilateral lymph node 3 cm or smaller without extranodal extension (ENE) and most of patients were N2 (45%), which means that they have metastatic ipsilateral or bilateral positive lymph nodes between 3-6 cm without ENE. Only 4% of the patients had N3

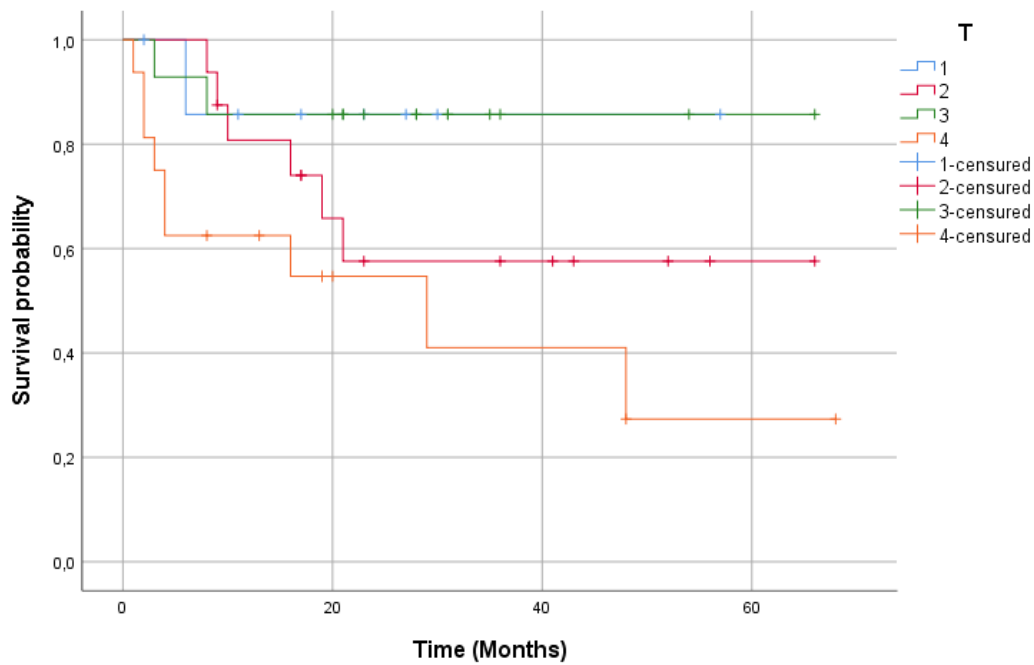
<i>Tumor Characteristics</i>	<b>Total (n=54)</b>	<b>Group 0 (n=37)</b>	<b>Group 1 (n=18)</b>	<i>P value</i>
<b>T</b>				
1	1	7	1	
2	17	11	6	0,04
3	14	12	2	
4	16	7	9	
<b>N</b>				
0	20	13	7	
1	8	6	2	0,882
2	25	17	8	
3	2	1	1	
<b>M</b>				
0	53	37	16	0,000
1	2	2	0	
<b>Tumoral Stage</b>				
1	5	5	0	
2	7	4	3	0,362
3	6	5	1	
4	37	23	14	

**Table 3.2. Tumoral characteristics.** Group 0: disease-free survival and patients who died for other causes; Group 1 represented disease-specific deaths.

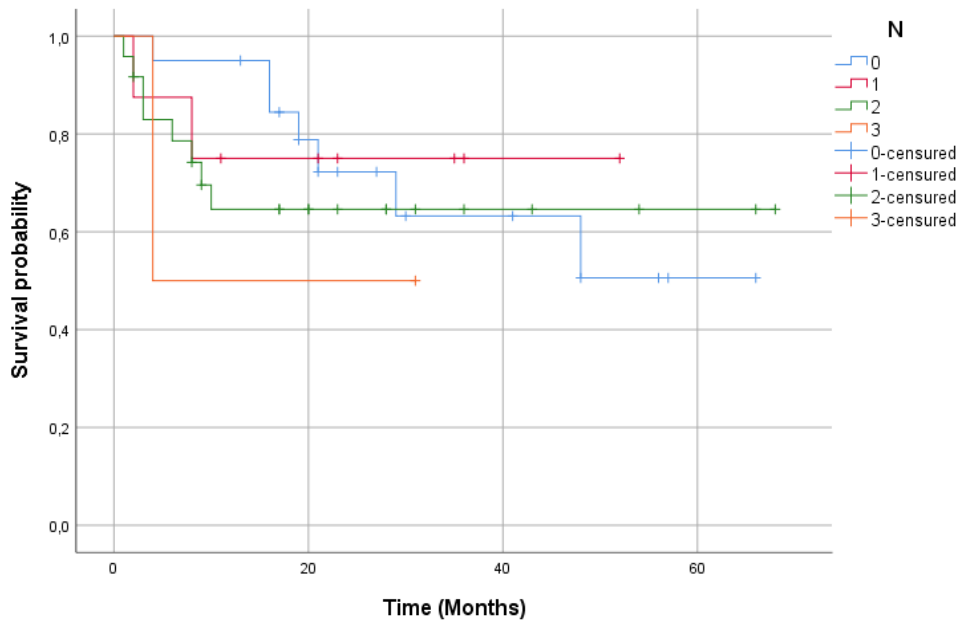
<i>Clinical Characteristics</i>	<b>Total (n=54)</b>	<b>Group 0 (n=37)</b>	<b>Group 1 (n=18)</b>	<b>P value</b>
<b>Treatment</b>				
Surgery	10	9	1	
Surgery+CM	1	0	1	<b>SG vs CM+RT</b>
Surgery+RT	1	0	1	p=0,268
CM+RT	28	20	8	<b>CM+RT vs</b>
Paliative CM	3	0	3	<b>SG+CM+RT</b>
RT	5	2	3	p=0,526
Surgery+CM+RT	7	5	2	
<b>Relapse</b>				
yes	40	32	8	0,003
no	15	5	10	
<b>Type of relapse</b>				
Locoregional	3	0	3	
Metastasis	3	2	1	0,17
LR+MT	2	0	2	
Local	1	1	2	
Regional	2	2	4	

**Table 3.3. Patients treatment and relapse.** Group 0: disease-free survival and patients who died for other causes; Group 1 represented disease-specific deaths. CM: chemotherapy, RT: radiotherapy, LR: locoregional.

stage disease. Only 4% presented metastasis at diagnosis. When looking at patients overall survival (OS) it was, surprisingly, only related to its T stage (Fig. 3.1) but not its N stage (Fig. 3.2). Indeed, when we compared N1 (75% OS), N2 (56,7 OS) and N3 (50% OS) patients to N0 (65% OS) ones we did not find a significant decrease in survival (p=0,856), (p=0,585) and (p=0,505) respectively. Moreover, T1 and T3 stage tumors exhibited the same survival (87,5% and 85.6% respectively) whereas T2 tumors showed a poorer survival 64,7% after at least two years follow-up although not statistically significant. T4 tumors survival was 43,8%. The vast majority of patients were classified as stage IV disease at diagnosis (67,3%), while only 9,1%, 12,7% and 10,9% were stage I, II and III respectively. In regards to survival, all patients diagnosed with stage I disease survived whilst stage II patients had the worse survival rate (57,1%) even in comparison with stage IV patients (62,2%) although not significant. Stage III patients survival was (83,3%).



**Fig. 3.1. Kaplan-Meier log-rank test survival analysis for comparison between T stage groups. 1 (Blue): T1, 2 (Red): T2, 3 (Green) T3, 4 (Orange): T4 ( $p=0,04$ ).**



**Fig. 3.2. Kaplan-Meier log-rank test survival analysis for comparison between N stage groups. 0 (Blue): N0, 1 (Red): N1, 2 (Green) N2, 3 (Orange) N3. When comparing N1, N2 and N3 to N0 we did not find a significant decrease when looking at survival ( $p=0,856$ ), ( $p=0,585$ ) and ( $p=0,505$ ) respectively.**

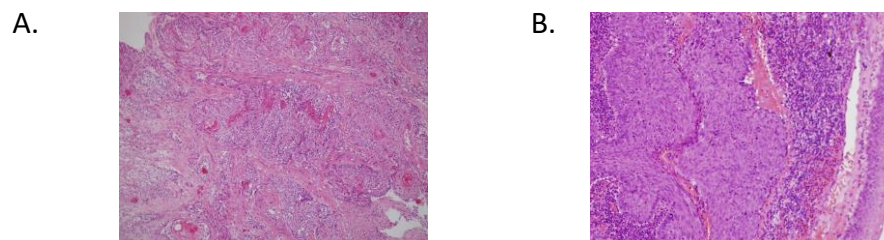
Patient treatment and relapse data is summarized in **table 3.3**. Most patients (50%) were

treated with chemotherapy and radiotherapy and exhibited a survival rate of 71.4% that was even slightly higher although not significant when surgery was added to the regime. 18% of patients were treated solely with surgery with a 90.1% survival rate but it was not significantly higher than when treated with chemotherapy and radiotherapy alone ( $p=0,268$ ). During the follow-up recurrence was observed in 15 cases, while 10 patients died of the disease ( $p=0,003$ ). Survival did not differ among different types of relapse mostly due to a small sample so further patients should be studied to confirm this. No statistical significance was found among patients including alcohol intake, smoking, age, sex, tumor location and tumoral stage between the two groups.

Histopathological characteristics are summarized in **table 3.4**. Only 20% of tumors were keratinizing tumors (Fig 3.3 A) and there were not significant differences in overall survival compared with nonkeratinizing tumors (Figure 3.3 B) ( $p=1$ ).

<i>Histological Characteristics</i>	<b>Total (n=54)</b>	<b>Group 0 (n=37)</b>	<b>Group 1 (n=18)</b>	<b>P value</b>
<b>Keratinizing</b>				
yes	11	8	3	1
no	44	29	15	
<b>HPV</b>				
Positive	18	15	3	0,035
negative	36	16	20	
<b>Cyclin D1</b>				
Positive	18	10	8	0,422
negative	36	26	10	

**Table 3.4. Histological characteristics.** Group 0: disease-free survival and patients who died for other causes; Group 1 represented disease-specific deaths. HPV: human papillomavirus.

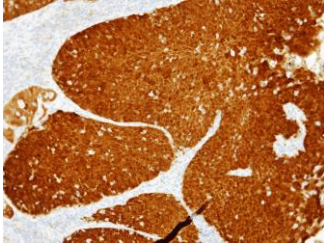


**Figure 3.3. Tumor keratinizing state. A)** Keratinizing tumor with tumor cells nets presenting round to oval nuclei, inconspicuous nucleoli, and high mitotic activity (Hematoxylin and eosin, magnification 10X) **B)** Nonkeratinizing tumor with well circumscribed tumor cells nets that have round to oval nuclei, inconspicuous nucleoli, and high mitotic activity (Hematoxylin and eosin, magnification 20x).



### 3.2 HPV status and p16 expression

HPV status was analyzed by both immunohistochemistry (Fig. 3.4) and by molecular study allowing HPV subtypes determination. P16 analysis is summarized in table 3.2.

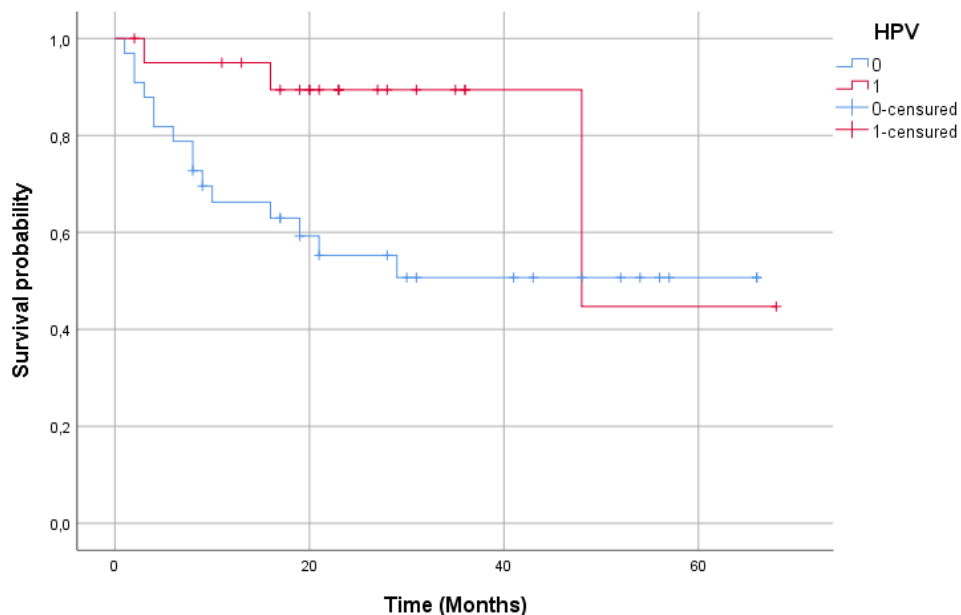


**Figure 3.4. p16 expression.** p16 positive specimen showing strong and diffuse expression in cytoplasm and nucleus in > 70% of tumor cells (x20).

IHC HPV	POSITIVE		NEGATIVE	
	14		40	
DNA		PCR		PCR
YES	11	Positive 11 Negative 0	14	Positive 7 Inhibited 7
NO	3		26	

**Table 3.2. HPV status and P16 analysis resume.** DNA: yes: there was sufficient sample to perform PCR based *in situ* hybridization. No: there was no DNA in the sample. PCR: positive: DNA sample amplified and hybridized with any of the HPV probes. PCR: inhibited: It was not possible to obtained a PCR product to hybridized. IHC: immunohistochemistry.

We were not able to isolate DNA from 50% of samples most of them collected from older periods. Seven patients that were negative in the immunohistochemistry were positive for p16 when analyzed by molecular study so they were considered false negatives. A 33,3% of patients (n=18) tested positive for HPV with a survival rate of 85,7% compared to the 58,3% that exhibited HPV negative patients (p=0,035) (Figure 3.5). Moreover, when we look for high risk HPV subtypes we found that 50% of the patients did not tested positive for p16 but for other HR-HPV subtypes. 22,2% patients were also positive for 58 and 53, 16,6% 59 and 11% 84, 66, 60, 31 and 18. In addition 38,8% of patients were coinfecting by 2 or more HR-HPV subtypes and 2 patients were coinfecting by 6 different HPV subtypes.

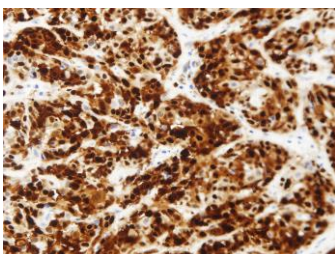


**Figure 3.5. Kaplan-Meier log-rank test survival analysis for comparison between HPV positive and negative subgroups. 0 (Blue): HPV negative, 1 (Red): HPV positive ( $p=0,035$ ).**

Absence of alcohol intake or smoking was not significantly related to HPV positive tumors ( $p=0,24$ ) and ( $p=0,28$ ) respectively. 50% of patients that have a significant alcohol intake and 56% of smokers were also positive for HPV. In addition, HPV expression was not seen to have tropism to any anatomical site.

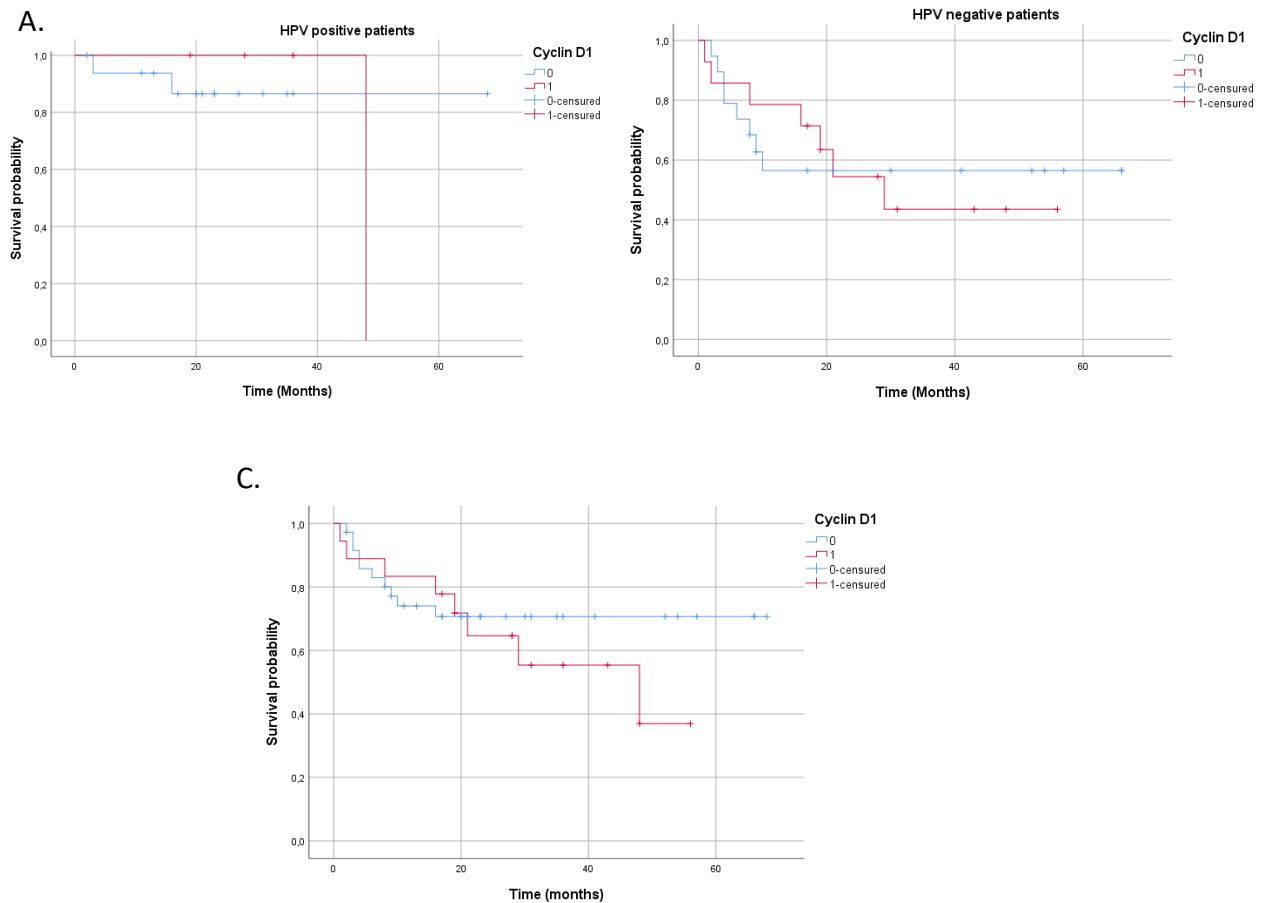
### 3.3 Cyclin D1 expression

Cyclin D1 expression was analyzed by immunohistochemistry. Positivity was considered when more than 50% of tumoral cells were positive for this protein (Fig. 3.6). 33,3% of the patients samples stained positive for cyclin D1. Media was  $38,18 \pm 26,46$  ED. Cyclin D1 positive patients overall survival was 55,6% compare to 72,2% for Cyclin D1 negative patients although not significant ( $p=0,422$ ) (Fig. 3.7).



**Figure 3.6. Cyclin D1 immunohistochemistry.** Positive cyclin D1 immunohistochemistry showing an intense nuclear brown staining in tumor cells (> 90%) (40x).

From 21 patients that were high risk HPV positive only 4 were cyclin D1 positive too. Overall survival for HPV+/cyclin D1 positive patients was 75% in contrast to 88,2% for HPV+/cyclin D1 negative patients ( $p=0,933$ ).



**Figure 3.7. Kaplan-Meier log-rank test survival analysis for comparison between A) HPV+ patients cyclin D1 positive or negative B) HPV- cyclin D1 positive or negative C) Cyclin D1 positive and negative subgroups. 0 (Blue): Cyclin D1 negative, 1 (Red): Cyclin D1 positive ( $p=0,933$ ).**

42,4% of HPV negative patients were cyclin D1 positive and its overall survival was 50% compare to 57,1% for cyclin D1 negative patients ( $p=0,896$ ). Cyclin D1 expression was not related with either T or N status or to any anatomical site location.

## 5. Discussion

In the last decades considerable effort has been made in order to determine new oropharynx cancer prognostic markers. Thanks to it, HPV infection has been associated in a not negligible group of patients with OPC carcinogenesis and has been widely related to better survival rates. Indeed, that has led to a TNM understratification for HPV positive patients as embodied in the last TNM guidelines (8<sup>o</sup> edition). However, differences in the techniques used, IHC or PCR based *in situ* hybridization arrays suggest variability among groups. Our results support the impact of HPV in OPC patients overall survival. 38,8% of our cohort was positive for HPV and their OS was greater than patients without HPV infection although they share other main risk factors as tobacco smoking and alcohol which weren't different among groups. Strikingly, our cohort had a higher number of HPV patients (38,8%) compared to others studies contradicting previous findings that indicated

Southern Europe to have the lower rates of OPC HPV positive tumors. One of them prevalence was 7.6%-9.4% and globally around 20% (12) and another metanalysis found a 24.2% in Southern Europe, around 18% in Spain and globally 45.8% (43). This suggest that HPV OPC infection is rising rapidly in the last years even passing Western Europe countries prevalence that is around 32-38% (12, 43). It is necessary to point out that we were not able to obtained DNA for analysis in 50% of our samples suggesting that our HPV positive percentage might be underestimated and therefore further analysis should be done in order to obtain a reliable data. The discrepancies may be explained by differences in the geographic origin of the samples, as well as the techniques used among studies to obtain HPV data. For instance, some studies only assess as said in the guidelines p16<sup>ink4a</sup> by immunohistochemistry as a read out for HPV positivity. However, several studies have determined the necessity to another marker in order to determine an active HPV infection changing the paradigm of HPV positive to HPV-driven OPC. p16<sup>ink4a</sup> determination together with E6-E7 RNA (43) or HPV PCR based molecular analysis (44) are currently the gold standard as done in our study. Furthermore when using PCR based molecular analysis with different HR-HPV probes we were able to determine that 50% of HPV-driven OPC are not only due to 16 subtype but due to other subtypes. This might explain the low prevalence of HPV in some studies as some of them only determine HPV-16 subtype. In addition several cohorts have pointed HPV-16 as the main subtype accounting for more than 95% (11, 17) or 85% (45) followed by 33 and 35 or 60% of cases (46) followed by HPV 33, HPV 26, HPV 35, and HPV 18 (12) which contrast with our results that found 59, 58 and 53 as the most prevalent ones after p16 (50%). Moreover, other studies have seen that multiple HPV infections in OPC patients are rare 5% that is in high contrast with our results that found 38% of tumors co-infected with 2 or more subtypes. This might be explained as HPV subtypes different distribution among populations, sexual behaviors among them (47), and the rising epidemic of HPV.

In this study, we observed that OPC patients disease specific survival rate was 67.3 % which was significantly associated besides to HR-HPV presence with T status. However we have not found a significant correlation between N status and survival which can be explained as we took patients initial TNM for the study that was obtained using the 7<sup>o</sup> edition TNM guidelines. This might be explained considering that the T classification remains the same, except for carcinoma in situ (Tis) and T4b category that have been removed from HPV-positive disease whereas in HPV-related carcinoma a downstaging in N criteria, as explained in the introduction, has been done (25). Indeed, only 30% of N0 patients were HPV-positive whereas 62% were N1, 41% were N2 and 0% were N3 indicating overstaging as a possibility to explain the lack of differences in OS among groups.

Even though HPV status has improve prognostic stratification there are still some patients that have a more aggressive and lethal disease within this group suggesting the need for new biomarkers to better predict HPV positive patients outcome. Cyclin D1, as a cell cycle regulator, is striking marker to consider since its overexpression has been related to worse prognosis in several cancers including some types of breast cancer (48, 49), esophageal

(50), gastric (51) and most importantly HPV positive cervix cancer (52-54). Indeed several authors have consistently related cyclin D1 overexpression or amplification with poor prognosis of different head and neck tumors (55) (56) including oropharyngeal ones (57, 58). Authors strongly converge in that cyclin D1 expression is downregulated in most HPV positive tumors (28, 50, 59, 60). Indeed, when we look at Cyclin D1 expression in HPV positive patients only 19% of them stained positive for cyclin D1 in more than 50% of tumoral cells, which matches other cohorts percentages (28, 57, 61). However, few studies have evaluated HPV and cyclin D1 relationship, and findings are not consistent and diverge, although several authors have pointed Cyclin D1 overexpression as a poor outcome predictor in 16 positive OCP patients (28, 59, 60, 62). When we looked at Cyclin D1 implication in HPV positive patients prognosis in our cohort we did not find any significant association. This can be mostly explained due to our limited patient number so further patients should be checked in order to affirm this firmly. However, there is another important factor to take into consideration since there is not a consensus about cyclin D1 cutoff for positivity since it ranges between 5-50% depending on the cohorts. Differences in immunohistochemistry protocols as well as in cyclin D1 antibodies can also influence. Moreover, cyclin D1 overexpression has been related with radioresistance (63) and patients treatment regimes varies among them. In addition patients economic status might influence affecting OS, whereas in Spain this last variable is not present. Lastly differences in HPV status evaluation, as explained before, might also influence in these results.

To sum up, HR-HPV infection is present in 38% of patients, which correlates with better outcome, being 16 the most prevalent subtype (50%) and multiple HPV infections are seen in 38% of patients. In addition low cyclin D1 expression is related to HPV positive patients but its overexpression is not related with an increased risk of death in HPV positive patients although further patients should be analyzed in order to corroborate it.

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