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Double Positivity for HPV DNA/P16^{INK4a} Does Not Influence Survival of Patients With Oral Squamous Cell Carcinoma

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Abstract. *Background/Aim: We investigated the prevalence of human papillomavirus (HPV) in a prospective cohort of patients with squamous cell carcinoma of the oral cavity (OSCC) using both p16^{INK4a} and HPV DNA, i.e., double positivity, as a definition criterion. Additionally, we examined the association of HPV with survival. Patients and Methods: Samples from 280 OSCC patients were analyzed for HPV-positivity using p16^{INK4a} immunohistochemistry (IHC) and in situ hybridization (ISH)/LCD arrays, for HPV low and high-risk types. Only patients positive for both p16^{INK4a} and HPV DNA were considered as HPV-positive. Survival probabilities and 95% confidence intervals were estimated using the Kaplan-Meier method. Cox proportional hazards models were used to assess HPV association with disease-free survival (DFS), cause-specific survival (CSS) and overall survival (OS) in a competing risks scenario. Results: Specimen from 30 (10.7%) patients were p16+ and HPV DNA+, while 31 (11.0%) were either p16+ or HPV DNA+ only. OS probabilities at five years for HPV-positive and -negative groups were 50.9% (35.4%-73.1%) and 52.9% (47.0%-59.5%), respectively. HPV double positivity influenced neither OS, CSS nor DFS: HR=0.84 (0.43-1.63), 1.64 (0.76-3.54) and 1.13 (0.55-2.35), respectively. Conclusion: In contrast to oropharyngeal cancer, the prevalence of HPV in OSCC is low and the presence of HPV does not influence survival outcomes. Hence, there is no evidence to support a parallel transfer of therapy regimen for HPV-positive OPC to OSCC, in terms of therapy de-escalation and/or vaccination.*

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Human papillomavirus (HPV) infection represents an independent risk factor for the development of several neoplasms, including cervical, anal and oropharyngeal cancer (OPC) (1-3). However, the role of HPV infection in oncogenesis and progression of squamous cell carcinoma of the oral cavity (OSCC) has drawn less attention and is still unclear.

The etiological involvement of HPV in OSCC, is challenged by several associated and mostly coexisting risk factors, such as tobacco smoking and alcohol consumption. Furthermore, the detection methods used to define HPV-positive tumors are heterogeneous (4, 5), ranging from single p16^{INK4a} assessment using immunohistochemistry (IHC) (6), to HPV DNA detection in p16-positive samples (7).

Currently, it is accepted that neither sole HPV DNA nor immunohistological detection of p16^{INK4a} in carcinomas can provide definitive evidence of HPV causality in oral cancer. This is because the presence of HPV DNA could reflect a transient infection rather than a genuine HPV-driven malignant transformation and the immune histologic expression of p16^{INK4a} is not specific for HPV activity in these neoplasms (3, 8-12).

However, the simultaneous demonstration of HPV DNA and p16^{INK4a} in carcinoma cells, so-called "double positivity" is considered reliable for a biologically active HPV infection (9, 13). We evaluated HPV-positive OSCC

prevalence in a large single-center cohort. Additionally, we examined the association of HPV with overall and disease-free survival after accounting for competing risks.

Patients and Methods

Study population. First, we identified all patients (n=418) presenting with primary malignant disease of the head and neck who referred for treatment to Department of Maxillofacial Surgery of the University Medical Center of Lübeck, Germany between January 2002 and December 2011.

From the 418 patients, we included only patients with OSCC who underwent a curative therapy. Patients with histologic entities other than squamous cell carcinoma, cancer site other than the oral cavity, or those who were not treated were excluded. The oral cavity was identified using the following ICD-10 codes: C00, C02 (excluding C02.4), C03, C04, C05 (excluding C05.2), and C06. We excluded patients previously irradiated and patients with second primaries to rule out artifact related staining errors. The final cohort included specimens from 280 patients in which HPV involvement in oncogenic transformation was assessed. All included patients were staged using the 7th edition of the UICC classification of cancer (14), and the histologic entities were classified using the World Health Organization Classification of Head and Neck Tumors (15).

P16^{INK4a} immunohistochemistry. Tumor samples (Ø=1.5 mm) were transferred from the donor onto the recipient block and processed using the tissue microarray method (TMArrayer, Pathology Devices Inc., Westminster, MD, USA). Monoclonal mouse antibodies were used (Klon JC8, 1:100 dilution, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) and immunohistochemical reactions were conducted using the automated Bond™ Enzyme Pretreatment Kit (Leica Biosystems, Newcastle Ltd, Newcastle, UK), Bond™ Epitope Retrieval Solution 1, Bond™ Wash Solution and Bond™ Polymer Refine Detection. Procedures were performed according to manufacturer's instructions. Tissues that tested negative for high-risk HPV DNA served as negative controls. Only tissues showing strong nuclear and cytoplasmic staining in more than 70% of tumor cells were considered p16-positive (Figure 1A). IHC (p16^{INK4a} staining) was evaluated conventionally and controlled by the "two-man rule" to ensure validity.

Chromogenic in situ hybridization (CISH). Tissue micro array (TMA) of tumor sections were DNA investigated for high-risk HPV types, 16, 18, 31, 33 and 51, as well as low-risk types, 6 and 11 by chromogenic *in situ* hybridization (CISH). This qualitative detection was performed using the automated Bond™ system (Leica Microsystems, Wetzlar, Germany) according to manufacturer's instructions. The following kit components were also used; Bond™ enzyme pretreatment kit, enzymes 1, Bond™ DNA-ISH HPV specimen (types 16, 18, 31, 33, 51, Bond™ DNA-ISH HPV specimen (types 6, 11), Bond™ anti-biotin antibody, Bond™ stringency wash solution, the Bond™ polymer refine detection kit and Bond™ wash solution. Tissues testing negative for high-risk HPV DNA served as negative controls. High-grade squamous intraepithelial lesions of the cervix uteri served as positive controls for high risk HPV types. A condyloma accuminatum specimen was used as a positive control for low-risk HPV types 6/11. The results of CISH were considered HPV-positive when intranuclear, solitary, and multiple signals were observed (Figure 1B).

LCD-array HPV-chip type 3.5C. For further HPV-subtyping, or in case of unspecific patchy patterns of p16^{INK4a} in IHC staining, the LCD-Array HPV-Chip Type 3.5C (Chipron GmbH, Berlin, Germany) was additionally performed for HPV-types 6, 11, 16, 18, 31, 33, 35, 39, 42, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68, 70, 72, 73, 81, 82, 83, 84, 90 and 91 to confirm the results of HPV-positivity.

Using two HPV-specific biotinylated primer mixtures (Pimer Mix A: My 11/09 and Primer Mix B: "125"), amplicons with a length of approx. 430 base pairs and approx. 125 base pairs were generated by PCR. The biotinylated PCR products subsequently hybridized to type -specific probes on the LCD chip surface. At the positions where the PCR amplicons had bound, the reaction resulted in dark blue precipitates on the LCD chip. The LCD chips were then scanned with the CHIP scanner PF3650u (Chipron GmbH) and analyzed using the manufacturer's analysis software.

Interpretation of histological findings. To define the HPV-associated oncogenic transformation, tumors were analyzed based on the following patients' criteria:
1) Patients with positive p16^{INK4a} staining and,
2) Patients with both positive p16^{INK4a} staining and HPV DNA detection.

Follow-up and statistical analysis. We assessed demographics, risk factors, clinical tumor characteristics and treatment decisions for all patients at baseline, and at each follow-up. The association between HPV status and several demographic and clinical factors was examined using *t*-test for continuous variables and Chi-square test for categorical variables. Smoking and alcohol consumption were assessed at baseline using questionnaire. Patients were considered current or former smokers if they have smoked at least one cigarette a day for at least one year. We considered patients who consumed more than 5 alcoholic beverages weekly as excessive drinkers. General health condition at baseline was quantified using the updated version of Charlson's comorbidity index (CCI) score (16), which was categorized into no significant comorbidities (score=0), and an least one score point (CCI score ≥1).

All survival outcomes were measured from the timepoint of initial diagnosis. Patients were examined regularly every three months in the first two years, and every six months for at least three more years, until they fulfilled five years of complete remission, suffered a recurrence, decided to drop out from regular follow-up, or died. For this study, follow-up ended on the 31st of March 2019, and statistical analyses were conducted afterwards. Date and causes of death were obtained from patients' general physician or next of kin and were validated through the cancer registry in the state of Schleswig-Holstein in Germany. All survival durations were measured starting date of diagnosis.

The endpoint for overall survival (OS) was death from any cause, while the endpoint for cause-specific survival (CSS) was death from oral cancer progression. The endpoint of disease-free survival (DFS) was diagnosis of local or regional recurrence, or distant metastasis. We estimated survival probabilities using Kaplan-Meier survival analyses and cumulative incidence functions.

We used univariable and multivariable Cox's proportional hazards regression models to estimate crude and adjusted hazards ratios (HR) and corresponding 95% confidence intervals (CI). Adjusted models included age at diagnosis, gender, smoking and alcohol consumption, CCI score, tumor size, nodal status, resection

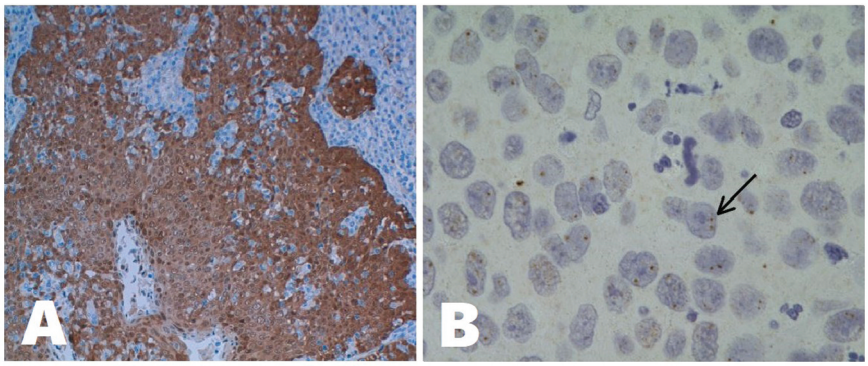

Figure 1. *An example of tissue showing strong nuclear and cytoplasmic stating (A), and HPV-positive tissues showing solitary intranuclear signals in CISH (B).*

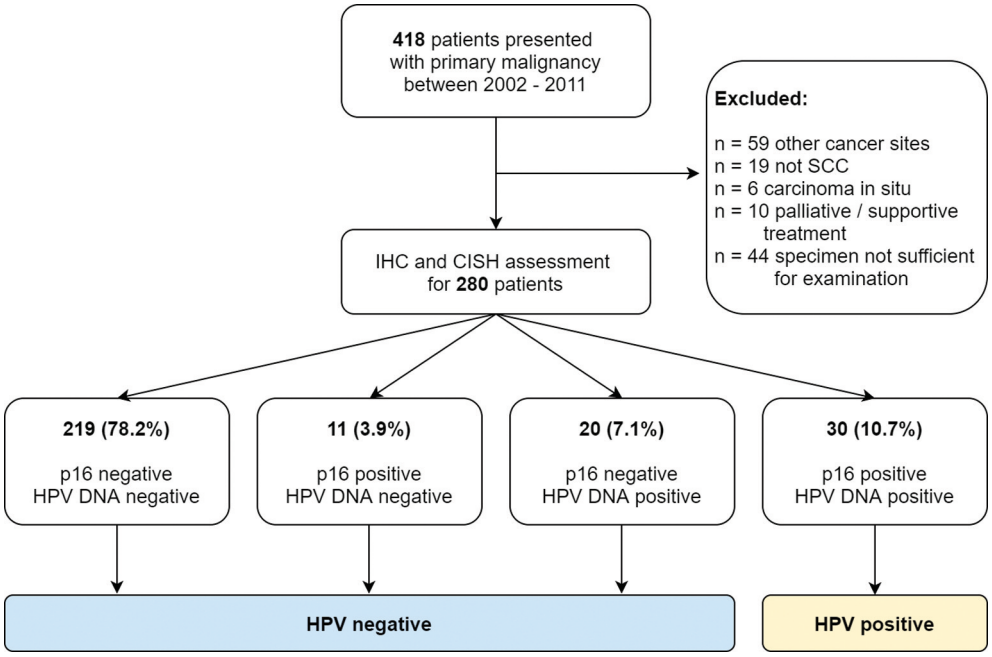

Figure 2. *Flow-chart of patient selection accompanied by HPV-positivity.*

margins. HPV DNA, $p16^{INK4a}$ status and double HPV/p16 positivity were each separately introduced to the regression models as binary variables. We examined the assumption of proportional hazards using Schoenfeld residual plots. The models for CSS and DFS included death from any cause other than oral cancer as a competing risk. We used Fine and Gray's method to estimate the hazards ratio for survival outcomes with competing risks (17). All analyses were performed using SAS (Version 9.04.01M6, SAS Institute, Cary, NC, USA).

Ethics. On admission, all participants signed informed consent forms allowing their data to be collected and used anonymously for academic research. The ethics review committee of the University of Lübeck approved the study prior to initiation (ID: 12-079A).

Results

We evaluated 280 patients, of which 41 (14.6%) were $p16^{INK4a}$-positive and 50 (17.9%) showed HPV DNA in CISH. Only 30 (10.7%) of patients were positive for both $p16^{INK4a}$ and HPV DNA and those were considered truly HPV-positive (*i.e.*, double positive). Among the 50 HPV DNA-positive specimens, the vast majority, 48 (96%), were positive for high-risk HPV type 16. None of the specimens were positive for the other high-risk types and five were positive for low-risk HPV types 6 and 11. However, only 2 (4%) of those were additionally $p16^{INK4a}$-positive. Figure 2 shows the criteria for

Table I. *Characteristics of study population by HPV double positivity.*

Variables	Total (N=280)	HPV– (N=250)	HPV+ (N=30)	*p*-Value
Age at diagnosis				0.8189
Mean±SD	62.8±12.0	62.8±11.9	63.3±13.2	
Minimum - Maximum	18.7-89.3	26.9-88.9	18.7-89.3	
Median (IQR)	63.1 (54.5-71.7)	62.6 (54.3-71.7)	64.5 (55.2-70.4)	
Gender				0.0457
Male	188 (67.1)	163 (65.2)	25 (83.3)	
Female	92 (32.9)	87 (34.8)	5 (16.7)	
Smoking				0.0406
Unknown	8 (2.9)	8 (3.2)		
Non-smoker	92 (33.8)	88 (36.4)	4 (13.3)	
Previous or current smoker	180 (66.2)	154 (63.6)	26 (86.7)	
Alcohol consumption				0.0743
Unknown	1 (0.4)	1 (0.4)		
None or moderate consumption	115 (41.2)	105 (42.2)	10 (33.3)	
Excessive consumption	164 (58.4)	144 (57.4)	20 (66.7)	
CCI score				0.0277
0	173 (61.8)	160 (64.0)	13 (43.3)	
≥1	107 (38.2)	90 (36.0)	17 (56.7)	
Cancer subsite				0.5413
Lip	19 (6.8)	17 (6.8)	2 (6.7)	
Anterior tongue	63 (22.5)	59 (23.6)	4 (13.3)	
Gum	50 (17.9)	45 (18.0)	5 (16.7)	
Floor of Mouth	93 (33.2)	80 (32.0)	13 (43.3)	
Palate	23 (8.2)	19 (7.6)	4 (13.3)	
Cheek, Vestibule, Retromolar	32 (11.4)	30 (12.0)	2 (6.7)	
Tumor size				0.4057
T1	105 (37.5)	98 (39.2)	7 (23.3)	
T2	94 (33.6)	82 (32.8)	12 (40.0)	
T3	30 (10.7)	26 (10.4)	4 (13.3)	
T4	51 (18.2)	44 (17.6)	7 (23.3)	
Nodal status				0.5544
N0	192 (68.8)	170 (68.0)	23 (76.7)	
N1	20 (7.2)	19 (7.6)	1 (3.4)	
N2a/b	43 (15.4)	38 (15.2)	5 (17.2)	
N2c/N3	24 (8.6)	23 (9.2)	1 (3.4)	
Pathological grade				0.3117
Well	26 (9.3)	24 (9.6)	2 (6.7)	
Moderate	183 (65.4)	166 (66.4)	17 (56.7)	
Poor	71 (25.4)	60 (24.0)	11 (36.7)	
Resection margins				0.0868
Missing	18 (6.4)	17 (6.8)	1 (3.3)	
R0	225 (85.9)	204 (87.6)	21 (72.4)	
R1/2	19 (7.3)	15 (6.4)	4 (13.8)	
Primary RT	18 (6.9)	14 (6.0)	4 (13.8)	
p16 status				<0.0001
p16 negative	239 (85.4)	239 (95.6)		
p16 positive	41 (14.6)	11 (4.4)	30 (100)	
HPV DNA positivity				<0.0001
HPV DNA-	230 (82.1)	230 (92.0)	0 (0)	
Only HPV16 DNA+	45 (16.1)	17 (6.8)	28 (93.3)	
Only HPV6/11 DNA+	2 (0.7)	0 (0)	2 (6.7)	
Both HPV16 and HPV6/11 DNA+	3 (1.1)	3 (1.2)	0 (0)	
Treatment				0.2640
Surgery only	182 (65.0)	164 (65.6)	18 (60.0)	
Adjuvant R(C)T	80 (28.6)	72 (28.8)	8 (26.7)	
Primary R(C)T	18 (6.4)	14 (5.6)	4 (13.3)	

Table I. *Continued*

Table I. *Continued*

Variables	Total (N=280)	HPV– (N=250)	HPV+ (N=30)	*p*-Value
Tumor resection				0.1027
No	18 (6.4)	14 (5.6)	4 (13.3)	
Yes	262 (93.6)	236 (94.4)	26 (86.7)	
Neck dissection				0.1154
No	39 (13.9)	32 (12.8)	7 (23.3)	
Yes	241 (86.1)	218 (87.2)	23 (76.7)	
Free flap reconstruction				0.4783
No	123 (43.9)	108 (43.2)	15 (50.0)	
Yes	157 (56.1)	142 (56.8)	15 (50.0)	
Chemotherapy				0.3798
No	239 (85.4)	215 (86.0)	24 (80.0)	
Yes	41 (14.6)	35 (14.0)	6 (20.0)	
Death from any cause at 5 years				
Alive or censored	181 (64.6)	164 (65.6)	17 (56.7)	
Dead	99 (35.4)	86 (34.4)	13 (43.3)	
Cause of death at 5 years				
Unknown cause of death	10 (3.6)	10 (4.0)		
Alive or censored	181 (67.0)	164 (68.3)	17 (56.7)	
Death from oral cancer	48 (17.8)	40 (16.7)	8 (26.7)	
Death from other causes	41 (15.2)	36 (15.0)	5 (16.7)	
Recurrence pattern at 5 years				
Local recurrence	82 (29.3)	76 (30.4)	6 (20.0)	
Regional recurrence	37 (13.2)	34 (13.6)	3 (10.0)	
Distant metastasis	33 (11.8)	30 (12.0)	3 (10.0)	

SD: Standard deviation; IQR: interquartile range; CCI: updated Charlson Comorbidity Index score; R(C)T: Radio(chemo)therapy.

patients' exclusion and the distribution of p16 and HPV positivity in the cohort.

HPV-positive tumors showed a higher prevalence in males, independent of HPV's definition criteria. The majority of patients were current or previous smokers n=180 (66.2%). Most tumors were localized in the floor of the mouth (n=93, 33.2%), followed by the anterior tongue (n=63, 21.5%), the gum (n=50, 17.8%), cheek (n=32, 11.4%), palate (n=23, 8.2%) and lip (n=19, 6.7%). T1 tumors were dominant in 105 patients (37.5%), followed by T2 in 94 patients (33.6%), T4 in 51 patients (18.2%) and T3 in 30 patients (10.7%) (Table I). Cervical lymph node metastases were clinically and/or histologically evident in 87 (31.2%) patients. According to the current guidelines for the treatment of oral cancers (18), only 182 patients (65%) were treated surgically, whereas 80 patients (28.6%) underwent adjuvant radiotherapy or radio-chemotherapy. Among these, R0 resections were achieved in 225 patients (85.9%). Eighteen patients received primary chemo-radiation. Table I provides the characteristics, treatment and outcomes of the study population stratified by HPV status.

Survival analysis. Follow-up ranged from two months to 19 years, with a median of 5.6 years amongst survivors, and a cumulative follow-up of 2,311 person-years. After five years of follow-up, 181 (64.6%) patients were alive, and 99 (35.4%) had died, of which 48 (17.8%) died from oral cancer, and 41 (15.2%) died from other causes. Eighty-two patients (29.3%) suffered a local recurrence and 37 (13.2%) suffered a regional recurrence. Thirty-three patients (11.8%) developed distant metastasis.

The median OS durations for HPV-positive and HPV-negative groups were 5.2 and 5.5 years, respectively. The OS probabilities at five years were 50.9% (CI=35.4-73.1%) and 52.9% (CI=47.0-59.5%) for HPV-positive and negative groups, respectively. The probability of suffering a disease recurrence and dying with oral cancer within five years were 28.1% (CI=15.3-51.5%) and 21.7% (CI=10.5-45.0%) in the HPV-positive group, and 36.6% (CI=31.0-43.3%) and 24.2% (CI=19.3-30.4%) in the HPV-negative group, respectively (Figure 3).

HPV status as shown by double-positivity did not impact on DFS, CSS or OS; HR=1.13 (CI=0.55-2.35; p=0.74), HR=1.64 (CI=0.76-3.54; p=0.21), and HR=0.84 (CI=0.43-1.63; p=0.60), respectively (Table II). Furthermore, neither $p16^{INK4a}$ nor HPV DNA positivity, each evaluated as a single risk factor, showed any significant impact on survival outcomes (Figure 4 and Figure 5, Table III).

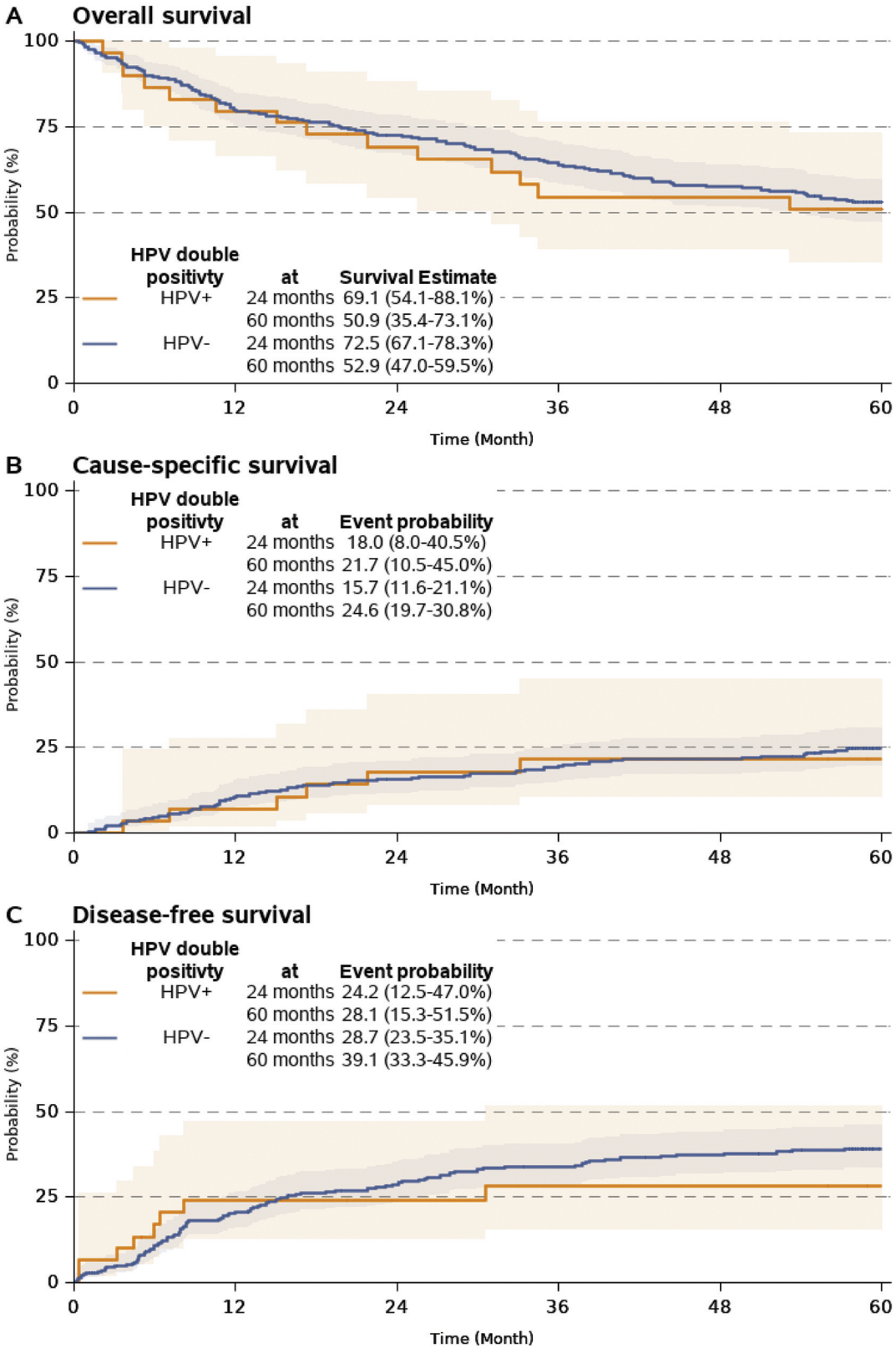

Figure 3. *Survival outcomes stratified by HPV double positivity.*

Table II. *Hazards ratios and 95% confidence intervals for different prognostic factors on overall survival, and disease-free survival.*

Variable	Level	HR for DFS	*p*-Value	HR for CSS	*p*-Value	HR for OS	*p*-Value
Age	1-year increments	1.01 (0.99-1.03)	0.49	1.02 (0.99-1.06)	0.18	1.06 (1.03-1.09)	**<0.01**
Gender	Male	Ref.		Ref.		Ref.	
	Female	1.02 (0.62-1.67)	0.95	1.67 (0.77-3.62)	0.20	1.17 (0.71-1.94)	0.54
Smoking	Non-smoker	Ref.		Ref.		Ref.	
	Previous or current smoker	2.06 (1.20-3.53)	**<0.01**	2.92 (1.24-6.89)	**0.01**	4.66 (2.38-9.12)	**<0.01**
Alcohol	None or moderate consumption	Ref.		Ref.		Ref.	
	Excessive consumption	1.43 (0.54-3.78)	0.46	1.3 (0.6-2.79)	0.50	1.14 (0.69-1.9)	0.61
CCI score	0					Ref.	
	≥1					1.51 (0.97-2.37)	0.07
Tumor size	T1	Ref.		Ref.		Ref.	
	T2	1.08 (0.62-1.9)	0.78	1.05 (0.4-2.76)	0.92	1.09 (0.62-1.94)	0.76
	T3	1.93 (0.91-4.08)	0.08	2.74 (0.83-9)	0.10	2.99 (1.38-6.47)	<0.01
	T4	1.74 (0.9-3.34)	0.10	2.26 (0.81-6.3)	0.12	1.54 (0.77-3.1)	0.22
Nodal status	N0	Ref.		Ref.		Ref.	
	N1	1.45 (0.7-3)	0.32	2.36 (0.9-6.19)	0.08	1.8 (0.89-3.61)	0.10
	N2a/b	1.84 (0.99-3.42)	**0.05**	2.95 (1.25-6.96)	**0.01**	1.96 (1.03-3.69)	**0.04**
	N2c/N3	2.55 (1.21-5.39)	**0.01**	2.79 (0.75-10.36)	0.13	5.58 (2.32-13.44)	**<0.01**
Resection Margins	R0	Ref.		Ref.		Ref.	
	R1/2	2.18 (1.01-4.69)	**0.05**	3.13 (1.04-9.43)	**0.04**	3.01 (1.41-6.41)	**<0.01**
	Primary RT	1.52 (0.59-3.93)	0.39	2.36 (0.64-8.69)	0.20	1.18 (0.45-3.11)	0.73
HPV double positivity	HPV–	Ref.		Ref.		Ref.	
	HPV+	1.13 (0.55-2.35)	0.74	1.64 (0.76-3.54)	0.21	0.84 (0.43-1.63)	0.60

HR: Hazard ratio; DFS: Disease-free survival; CSS: cancer-specific survival; OS: overall survival; Ref: reference level. Bold values indicate statistical significance.

Table III. *Hazards ratios and 95% confidence intervals for p16, HPV DNA and HPV double positivity for overall survival, and disease-free survival.*

Variable	Model	Level	HR for DFS	*p*-Value	HR for CSS	*p*-Value	HR for OS	*p*-Value
p16	Crude model	p16–	Ref.		Ref.		Ref.	
		p16+	0.99 (0.57-1.74)	0.99	1.52 (0.76-3.02)	0.23	1.14 (0.67-1.94)	0.63
	Adjusted model	p16–	Ref.		Ref.		Ref.	
		p16+	1.29 (0.72-2.33)	0.39	1.51 (0.77-2.96)	0.23	0.73 (0.4-1.34)	0.31
HPV DNA	Crude model	HPV DNA–	Ref.		Ref.		Ref.	
		HPV DNA+	0.91 (0.54-1.55)	0.73	1.25 (0.62-2.53)	0.53	1.07 (0.65-1.78)	0.78
	Adjusted model	HPV DNA–	Ref.		Ref.		Ref.	
		HPV DNA+	1.06 (0.63-1.79)	0.82	1.17 (0.58-2.34)	0.66	0.81 (0.46-1.43)	0.47
HPV double positivity	Crude model	HPV–	Ref.		Ref.		Ref.	
		HPV+	0.80 (0.39-1.61)	0.52	1.73 (0.80-3.70)	0.16	1.37 (0.77-2.46)	0.28
	Adjusted model	HPV–	Ref.		Ref.		Ref.	
		HPV+	1.13 (0.55-2.35)	0.74	1.64 (0.76-3.54)	0.21	0.84 (0.43-1.63)	0.60

Adjusted models were adjusted for age, gender, updated Charlson's Comorbidity Score, smoking, alcohol consumption, tumor size, nodal status and resection margins. HR: Hazard ratio; DFS: disease-free survival; CSS: cancer-specific survival; OS: overall survival; Ref: reference level.

On the other hand, smoking history, positive resection margins and advanced nodal involvement were all relevant prognostic factors of DFS, CSS and OS and were all associated with poor survival (Table II). Smokers showed a higher risk of poor prognosis compared to non-smokers for all survival outcomes. Using non-smokers as a reference, the HR for OS, CSS and DFS were 2.06 (CI=1.2-3.53; $p<0.01$), HR=2.92 (CI=1.24-6.89; p=0.01) and HR=4.66 (CI=2.38-9.12; $p<0.01$), respectively. And as expected, there was a significant decrease in DFS, CSS, and OS for patients with positive resection margins; R1/R2, HR=2.18 (CI=1.01-4.69; p=0.05), HR=3.13 (CI=1.04-9.43; $p<0.04$), and HR=3.01 (CI=1.41-6.41; $p<0.01$), respectively.

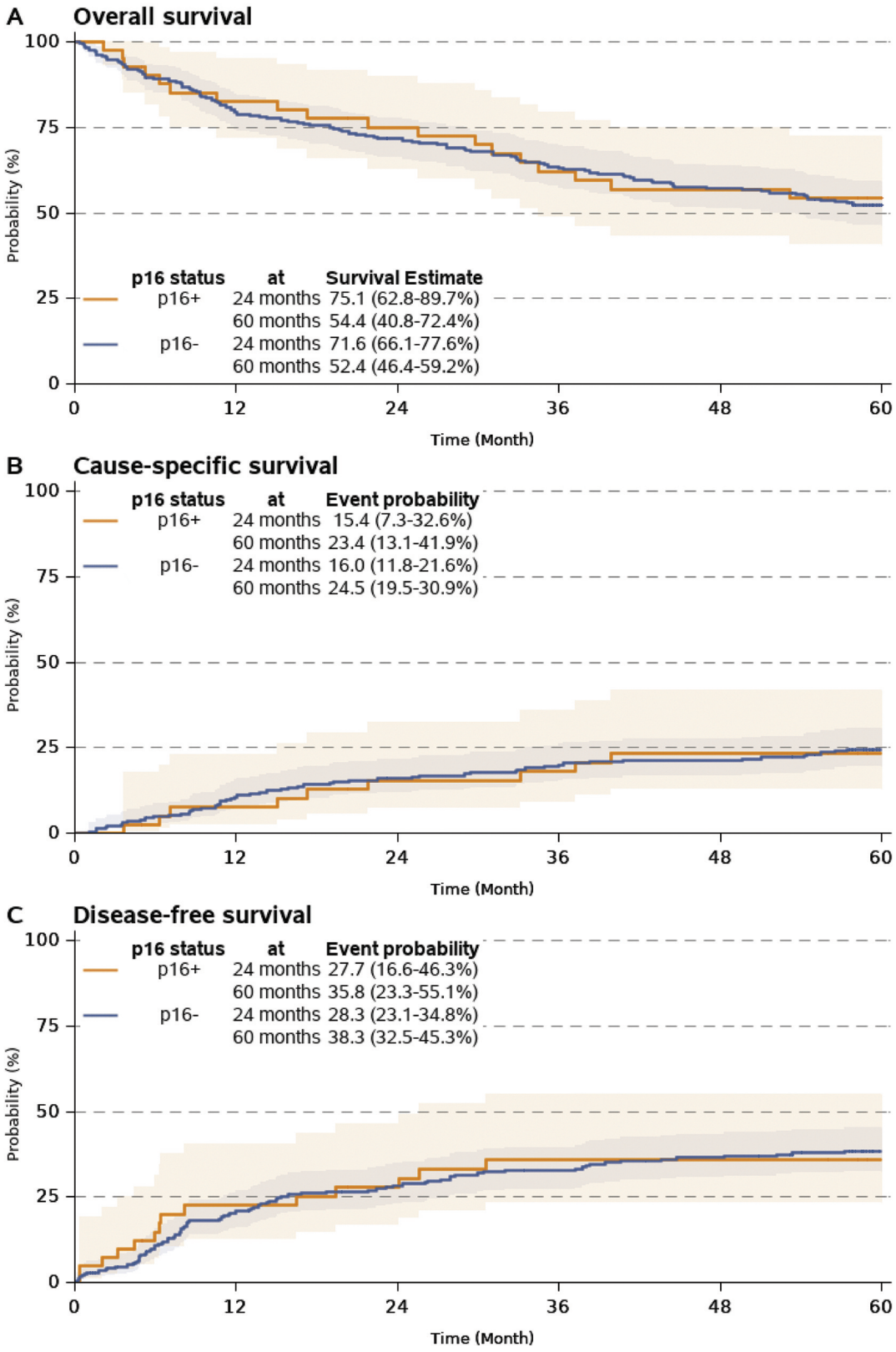

Figure 4. *Survival outcomes stratified by p16 status*.

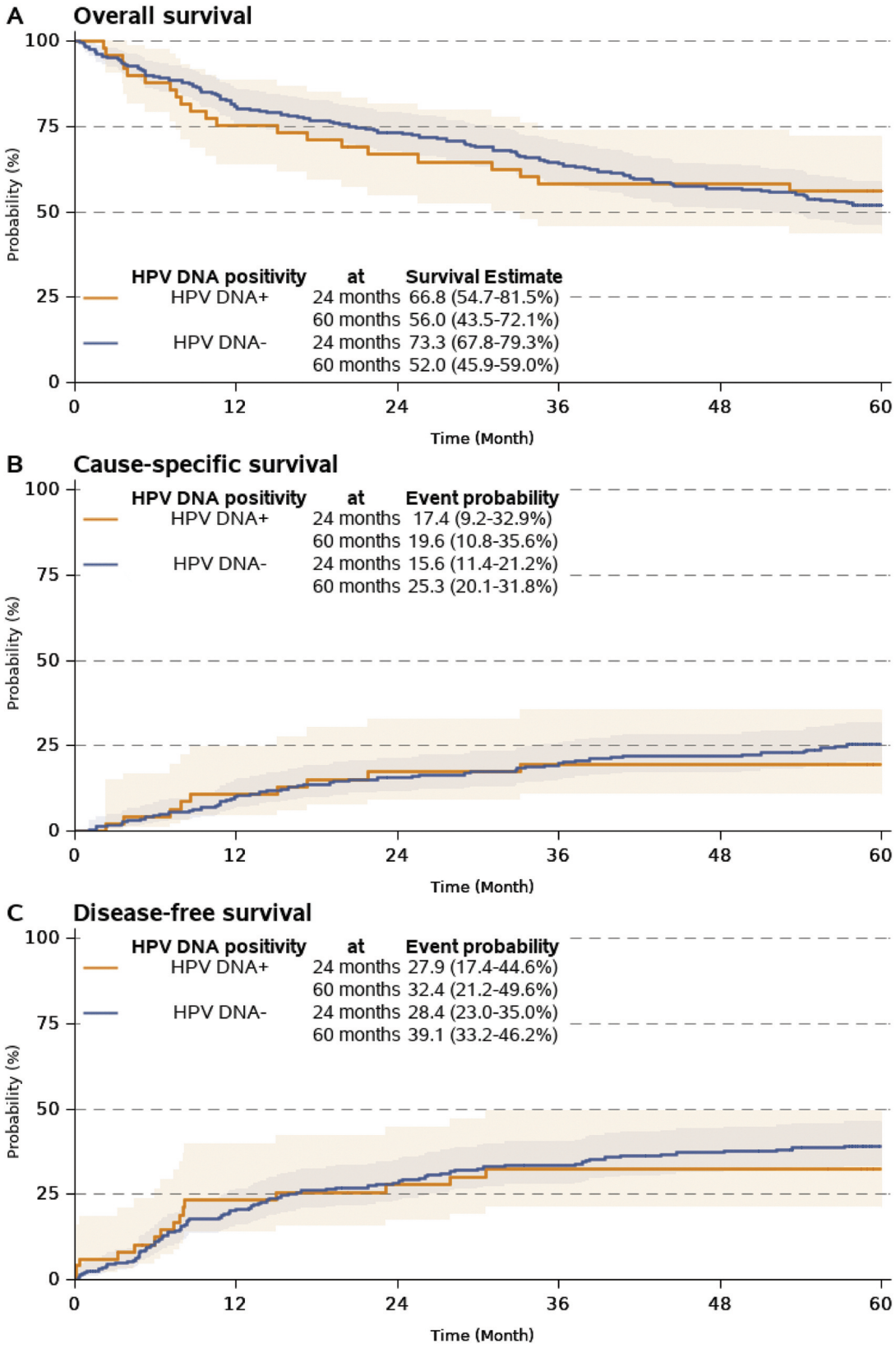

Figure 5. *Survival outcomes stratified by HPV DNA status.*

Discussion

There is sufficient evidence of the causal association between HPV infection and the initiation as well as the development of OPC. This has established HPV as predictive and prognostic marker of OPC in addition to tobacco smoking and alcohol consumption (19). At this anatomical location, it is widely accepted that HPV-positive squamous cell carcinomas constitute a new entity, with distinct clinical and histopathological features (3, 13, 20).

However, there is a dearth of information on the prevalence and impact of HPV infection on development of OSCC. For OSCC, the HPV-status is generally set by different surrogate parameters, such as immunostaining by $p16^{INK4a}$ or by detection of HPV DNA using PCR or ISH. It is noteworthy that each detection method alone lacks reliability for several reasons (13, 21-25). A recent report suggested that "double positivity", *i.e.*, considering both HPV DNA and $p16^{INK4a}$ was a stronger indicator for the prognosis of tonsillar cancer (26, 27). Few small studies assessed HPV using this definition and investigated the association with the survival outcomes (28-30), but none of the published studies compared survival outcomes using the different HPV positivity definitions for OSCC.

This study highlights HPV-attributed OSCC from two perspectives. Firstly, we assessed the prevalence of HPV attributed OSCC, by $p16^{INK4a}$ IHC or HPV DNA/RNA (CISH/LCD-array) analyses, or both (double positivity). Secondly, we report estimates for different survival endpoints while accounting for prognostic risk factors and competing survival risks. As far as we are aware, this study is the first to investigate these aspects in an adequate number of prospectively included patients in a single-center cohort, supported by relevant clinical survival data.

We showed that the prevalence of HPV-associated OSCC is low, accounting for 10.7%, if double positivity (HPV DNA and $p16^{INK4a}$) is considered. This prevalence is comparable with data estimated for HPV-positive OSCC in a 3,680 sample study (7.8%) (3), and a meta-analysis (6.8%) (31).

This prevalence remains relatively low when considering only HPV DNA or $p16^{INK4a}$ as a surrogate parameter for HPV induced oncogenic transformation (17.9% and 14.6%, respectively), in comparison to OPC, which is as high as 33%-45.8% (3, 31, 32).

For regular OPC treatment, primary radiotherapy or chemo-radiation is often recommended in advanced stages (33). Thus, the pathological assessment of tumor size and related "resection margins" are not encountered and cannot be evaluated per se. On the contrary, for OSCC, primary surgery and subsequent radiotherapy (also for advanced stages) still represent the first treatments of choice, since reconstruction of soft and hard tissue is mandatory to maintain function and allow postoperative adjuvant radiation.

From this point of view, the results of the present study illuminate the role of associated risk factors in the assessment of HPV-oncologic transformation, and this for HPV-DNA, $p16^{INK4a}$, and for both. Our data show that smoking, involved resection margin (R1), increased tumor size (T3/T4), as well as advanced nodal dissemination (N2a/b) play the main role in determining OS, CSS and DFS regardless of the assigned HPV-status.

Conclusion

In contrast to cancers of the oropharynx, the prevalence of HPV-positive squamous cell carcinoma of the oral cavity is low. In addition, HPV involvement does not influence survival outcomes in patients with OSCC. Accordingly, to date there is no evidence that supports or justifies a parallel transfer of therapy regimen for HPV-positive OPC to OSCC with regard to therapy de-escalation and/or vaccination.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

SH and PS conceptualized the study. MG, LT and UA developed the methods. MH and MG ran ISH procedures and evaluated immunohistochemistry. UA, SH and DR acquired clinical and outcome data, conducted statistical analyses and interpreted the data. UA and SH drafted the main manuscript and all Authors revised the final manuscript. PS and DR provided further administrative support. SH and PS supervised the study. MH and UA contributed equally to this study.

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