



## Human papillomavirus infection and oral cancer: A case-control study in Montreal, Canada

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**Summary** The objective of the present study was to examine the association between human papillomavirus (HPV) infection and risk of developing oral cancer. The investigation followed a hospital-based case-control design. Cases consisted of newly diagnosed patients with squamous cell carcinoma of the oral cavity and oropharynx. Controls were frequency matched to cases on gender, age, and hospital. Subjects were interviewed to elicit information on putative risk factors. Oral exfoliated cells were tested for detection of HPV DNA by the PGM09/11 polymerase chain reaction protocol. Serum antibodies against HPV 16, 18, and 31 viral capsids were detected using an immunoassay technique. Logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CI) of oral cancer according to HPV exposure variables. HPV DNA was detected in 19% of cases (14 out of 72), and 5% of controls (six out of 129). Among tonsil-related cancers (palatine tonsil and base of tongue) viral DNA was detected in 43% of cases

**Abbreviations:** CI, confidence interval; ELISA, enzyme linked immunosorbent assay; HPV, human papillomavirus; ICD-O, International Classification for Diseases in Oncology; OD, optical density; OR, odds ratio; PCR, polymerase chain reaction; VLP, virus-like-particles.

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(nine out of 21). The OR for tonsil-related cancers for high-risk HPV types was 19.32 (95%CI: 2.3–159.5), after adjustment for socio-demographic characteristics, tobacco, and alcohol consumption. The equivalent OR for HPV 16 seropositivity was 31.51 (95%CI: 4.5–219.7). The ORs of non-tonsillar oral cancers for high risk HPV DNA in oral cells and for seropositivity were 2.14 (95%CI: 0.4–13.0) and 3.16 (95%CI: 0.8–13.0), respectively.

These results provide evidence supporting a strong causal association between HPV infection and tonsil-related cancers. The evidence for an etiologic link is less clear for non-tonsillar oral cancers.

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## Introduction

Cancers of the oral cavity and of the oropharynx are a major health problem worldwide, accounting for over 300,000 incident cases annually.<sup>1</sup> Incidence rates of oral cancer have been rising in most regions of the world.<sup>2</sup> These cancers are more frequent in less developed countries and more common among males than among females.<sup>1</sup> Several studies have identified tobacco and alcohol consumption, and their joint effect, as the most important determinants for the development of oral cancers.<sup>3–5</sup> Other factors have been linked to these neoplasms, such as diet low in carotenoids and vitamin A,<sup>6,7</sup> poor oral hygiene,<sup>8</sup> and indoor air pollution.<sup>9</sup>

The role of human papillomavirus (HPV) in the etiology of cervical and other anogenital cancers is now firmly established.<sup>10,11</sup> HPV infection has also been postulated as a potential risk factor for at least some forms of oral cancer. Several studies have detected HPV DNA in a substantial proportion of malignant tumors of the oral cavity, with detection rates ranging from 0% to 100% (reviewed by Franceschi et al., Miller and White, and McKaig et al.).<sup>12–14</sup>

Recently, some studies have consistently identified a distinct subset of cancers of the oral cavity that appears to be etiologically linked to HPV infections. These HPV-associated cancers are predominantly located in the oropharynx, especially palatine tonsils and base of tongue,<sup>15</sup> and they present more frequent basaloid morphology and less frequent p53 mutations.<sup>16</sup> Epidemiological studies have assessed the association between HPV infection and oral cancer using only viral DNA detection techniques,<sup>17–19</sup> only serological assays,<sup>20–22</sup> or using both techniques but without examining subsite specific associations, e.g., buccal cavity vs. oropharynx.<sup>23</sup>

The primary objective of this study was to examine the association between HPV infection – assessed by detection of HPV DNA in the oral cavity as well as serological response – and risk of developing oral cancer, and whether the risk differs between tonsil-related carcinomas (palatine tonsils and base of tongue) and other oral cancers.

## Patients and methods

This investigation, as part of a multi-center study coordinated by the International Agency for Research on Cancer (IARC), followed a hospital-based case-control design. The design and methods of the investigation have been recently published.<sup>24</sup> The Montreal component of the investigation used an independent protocol for testing and typing for viral

DNA and obtaining serological evidence of HPV infection, the results of which are reported here.

## Study subjects

All patients with newly diagnosed squamous cell carcinoma of the mouth (International Classification for Diseases in Oncology [ICD-O]) (rubrics C00–C06), palatine tonsils (C09), and oropharynx (C10) referred to three head-and-neck surgery services in Montreal (Jewish General Hospital, Royal Victoria Hospital, and Notre-Dame Hospital) between October 1997 and May 2001, were considered eligible for the study. Patients with tumors of the salivary glands (C07–C08) were excluded from the investigation. All diagnoses were confirmed histologically.

Control subjects were selected from the same hospitals where cases had been recruited, and frequency matched to cases on the basis of gender, age (five-year group), and hospital. Controls were selected among individuals without a personal history of cancer, and with admitting conditions not related to tobacco and/or alcohol consumption. Efforts were made to ensure a good balance in the distribution of diseases among controls, with no single diagnostic group contributing more than 20% of all controls. The most common diagnostic groups were diseases of the nervous system and sensory organs (19%), diseases of the digestive system (19%), and symptoms, signs, and ill-defined conditions (18%).

## Interview of study subjects

Research nurses specially trained for this study interviewed all subjects using a standardized questionnaire to elicit information on socio-demographic factors, lifetime history of smoking and alcohol drinking and cancer in the family, recent intake of selected dietary items, oral hygiene, and detailed information on lifetime sexual practices. All interviews were carried out before treatment was initiated.

## Detection of HPV DNA in the oral cavity

Presence of HPV DNA was examined in oral exfoliated cells. The buccal cavity of both case and control subjects was brushed using a soft toothbrush with five gentle strokes in the following areas: right and left buccal mucosa; left, dorsal, and right side of the tongue; upper and lower gum; and upper and lower inner lip. The toothbrush was then introduced in a tube containing 20 ml of phosphate buffered saline (PBS), and was shaken to release exfoliated cells.

Patients were asked to perform vigorous washing of the oral cavity, including the throat by performing gargarisms, with 10 ml of saline solution, which was then poured in the same tube.

The tube containing the exfoliated cells was centrifuged, the supernatant discarded, and the cell pellet resuspended in 2 ml of PBS and frozen until viral testing by a polymerase chain reaction (PCR) technique. Cell suspensions were thawed, lysed, and digested with proteinase K.<sup>25</sup> Five  $\mu$ l of each lysate were tested with PC04 and GH20 primers for the presence of  $\beta$ -globin DNA.<sup>26</sup> DNA was extracted with phenol/chloroform and precipitated in ethanol for the 11 samples that tested negative for  $\beta$ -globin.  $\beta$ -globin was then detected in all 11 processed samples. Lysates or extracted DNA testing positive for  $\beta$ -globin were tested for HPV with consensus primers PGMY09 and PGMY11.<sup>27,28</sup> HPV DNA amplification consisted of the following steps: AmpliTaq Gold activation at 95 °C for 9 min, denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, DNA synthesis at 72 °C for 1 min for 40 cycles, followed by a 5-min terminal extension step at 72 °C.

HPV amplicons were detected and typed with the line blot assay as described previously.<sup>27,29</sup> HPV types were classified into low-risk (6, 11, 26, 40, 42, 53, 54, 55, 57, 66, 83, and 84) and high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) according to Muñoz et al.<sup>30</sup>

### HPV serology

Serological response to HPV infection was examined in patients' plasma samples using an enzyme linked immunosorbent assay (ELISA) using HPV virus-like-particles (VLP) as antigens, as described by Viscidi et al.<sup>31</sup> Briefly, production of VLPs was performed infecting *Trichoplusia ni* cells with recombinant baculovirus expressing HPV L1 or L1 and L2 proteins. For the ELISA assays the wells of PolySorp microtiter plates (Nunc, Naperville, IL) were coated overnight at 4 °C with 50 ng, 40 ng, or 40 ng of HPV 16, HPV 18, or HPV 31 VLP protein, respectively. Diluted plasma samples (1:100) were tested in duplicate on separate antigen coated plates for 1 h at 37 °C. Bound immunoglobulin was detected with peroxidase-conjugated goat antibodies to human IgG (Zymed, San Francisco, CA). After 30 min at 37 °C, color development was initiated by addition of 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) hydrogen peroxide solution (Kirkegaard and Perry, Gaithersburg, MD). After 20 min incubation, plates were monitored until the weak positive control reached a predetermined optical density (OD) value. The positive controls were human serum samples previously shown to be reactive in the assay. Three controls were included on each plate, a weak positive, a moderate to strong positive and a negative control. After stopping the enzyme reaction absorbance was measured at 405 nm. The cutpoint for positive results was determined from the reactivity of plasma samples from previously established negative control samples. The mean and standard deviation (SD) of OD values for the control samples was calculated. After excluding outliers, the cut point was set as an OD value greater than the mean OD of the negative controls plus either 3 SD or 0.030 OD units, whichever value was higher. The cut points for the HPV 16,

18 and 31 assays were 0.050, 0.040, and 0.045 OD units, respectively.

### Statistical analysis

In addition to considering oral cancer as the main outcome, two subsite analyses were performed: (i) tonsil-related cancers (palatine tonsil and base of tongue), and (ii) other oral cancers (non-tonsillar tumors). The odds ratio (OR) was the measure of association to estimate the rate ratio of disease for each study factor. Multivariate logistic regression was used to assess the effect of candidate risk factors with mutual adjustment for confounders. Adjustment for tobacco and alcohol consumption was based on the lifetime cumulative exposure using the pack-years equivalent of cigarette smoking and the sum over all alcoholic beverage types in kilograms of ethanol consumption. A pack-year was defined as the cumulative exposure equivalent to smoking one pack of cigarettes daily during one year. Doses were calculated as follows: 20 commercial-brand cigarettes = 4 hand-rolled, black tobacco cigarettes = 4 cigars = 5 pipefuls with pipe tobacco = 1 pack; ethanol concentration in beer = 5%, wine = 10%, hard liquor = 40%.

Both HPV DNA and serological assays were performed blindly with respect to disease status. The Institutional Review Boards of McGill University, Université de Montréal, and of the three participating hospitals approved this project. All participating subjects provided an informed consent.

### Results

A total of 72 cases and 129 controls were recruited in the study. Eighteen eligible cases were not enrolled: we failed to contact four patients before beginning of treatment, four had extreme weakness or were unable to give consent due to mental conditions, and 10 cases refused to participate. Among cases, the most common cancer site was tongue (excepting base of tongue), with 21 cases (29.2%), followed by floor of the mouth and palatine tonsil, with 12 cases each (16.7%), base of tongue, and other and unspecified parts of mouth with 9 cases each (12.5%), palate with four cases, gum, and oropharynx with two cases each, and inner lip with only one case.

Table 1 summarizes the distribution of subjects according to selected characteristics. By design, the distributions of gender and age were comparable between cases and controls. Most patients were aged 55–74, and men accounted for over 70% of participating subjects. On average, cases had fewer years of schooling than controls, and the proportion of white subjects was slightly higher among cases. As expected, the consumption of tobacco smoking and alcohol drinking was higher among cases. Heavy smokers (more than 49 pack-years) represented 39% of cases and 16% of controls, whereas heavy drinkers (lifetime alcohol consumption of more than 400 kg of ethanol) represented 53% and 17%, respectively.

### Detection of HPV DNA in the oral cavity

The distribution of cases and controls according to oral HPV DNA positivity is shown in Table 2. HPV DNA was detected in

**Table 1** Distribution of study subjects according to selected characteristics

Variable	Categories	Controls (N = 129)		Cases (N = 72)	
		N	%	N	%
Age	25–54	38	29.5	20	27.8
	55–64	37	28.7	19	26.4
	65–74	33	25.6	20	27.8
	75–84	21	16.3	13	18.1
Sex	Male	92	71.3	51	70.8
	Female	37	28.7	21	29.2
Schooling	<10 years	24	18.6	28	38.9
	10–14 years	47	36.4	29	40.3
	15+ years	58	45.0	15	20.8
Race	White	118	92.2	68	94.4
	Non-white	10	7.8	4	5.6
Smoking status	Never	41	31.8	11	15.3
	Former	70	54.3	26	36.1
	Current	18	14.0	35	48.6
Total tobacco (in pack-years)	Never	41	31.8	11	15.3
	≤19	40	31.0	12	16.7
	20–48	27	20.9	21	29.2
	49+	21	16.3	28	38.9
Lifetime alcohol consumption (in kgs)	Non drinker	15	11.6	7	9.7
	1–80	47	36.4	10	13.9
	81–400	45	34.9	17	23.6
	≥401	22	17.1	38	52.8

oral exfoliated cells of six out of 129 controls (4.7%) and 14 out of 72 cases (19.4%). The distribution of subjects according to HPV oncogenicity showed that most viral infections among cases harbored high-risk HPV types (13 out of 14 samples) compared with four of the six HPV positive controls. HPV 16 was not detected among controls, whereas 13 of the 14 positive samples from cases harbored HPV 16.

Table 3 shows the ORs of oral cancer from crude and two adjusted analyses using detection of oral HPV DNA defined in two ways: (i) dichotomous (positive versus negative), and (ii) by oncogenicity (high risk types, low risk types, and negative). For all oral cancers, the crude OR for overall HPV DNA positivity was 4.95 (95%CI: 1.8–13.5). Adjustment for socio-demographic variables reduced the OR to 3.04 (95%CI: 1.0–9.3). Further adjustment for tobacco smoking and alcohol drinking did not modify the point estimate but imposed a loss of precision (OR = 3.14; 95%CI: 0.9–10.9). The analysis of detection of HPV DNA according to oncogenic types showed that the ORs of oral cancer for high-risk types were substantially higher than for low-risk types. The estimates for low-risk types were very close to unity, whereas the fully adjusted OR for high risk types was 4.81 (95%CI: 1.2–19.4).

In subsite analysis, HPV DNA was detected in nine out of 21 tonsil-related cancers. The crude OR of these cancers for high-risk HPV types was 23.1 (95%CI: 6.2–86.2). Adjustment for socio-demographic variables and tobacco and alcohol slightly decreased the estimate (OR = 19.3; 95%CI: 2.3–

159.5). The ORs of other oral cancers (not tonsil-related) for high risk HPV types were considerably lower: 2.67 (95%CI: 0.6–11.1) for the crude model, and 2.14 (95%CI: 0.4–13.0) for the more adjusted model.

### HPV serology

Plasma samples were obtained from 66 of the 72 cases, and 128 of the 129 controls. Table 4 presents the mean OD values for the ELISA assays carried out for three anti-VLP antibodies: HPV 16, HPV 18, and HPV 31. The mean OD values were higher among cases than controls for the three antibodies tested. The highest difference was seen for HPV 16: 0.067 (95%CI: 0.021–0.113).

The association between serological response to HPV 16 as a dichotomous variable, using the predefined cutoff value, and risk of oral cancer is summarized in Table 5. For all oral cancers, the OR of disease for HPV 16 seropositivity after adjustment for socio-demographic variables and tobacco smoking and alcohol drinking (model 3), was 7.5 (95%CI: 2.1–27.2). Despite the correlation between seropositivity and oral HPV DNA status, further adjustment for detection of HPV DNA slightly decreased the estimates for HPV 16 seropositivity (model 4): OR = 6.5 (95%CI: 1.6–25.3).

The subsite analysis for tonsil-related cancers showed that the magnitude of association between HPV 16 seropositivity and these tumors was extremely high. The fully adjusted model, including adjustment for detection of HPV

**Table 2** Distribution of cases and controls according to HPV DNA detection in oral exfoliated cells

HPV exposure variable	Categories	Controls		Cases	
		N	%	N	%
Overall positivity	Negative	123	95.3	58	80.6
	Positive, any types	6	4.7	14	19.4
Oncogenic risk grouping	Positive, low risk types	2	1.6	1	1.4
	Positive, high risk types	4	3.1	13	18.1
Positivity by type <sup>a</sup>	11	1	0.8		
	16			8	11.1
	55	1	0.8		
	58	2	1.6		
	66	1	0.8		
	84			1	1.4
	16, 31			1	1.4
	16, 35			1	1.4
	56, 58	1	0.8		
	16, 39, 53			1	1.4
	16, 51, 55			1	1.4
6, 16, 39, 53			1	1.4	

<sup>a</sup> Empty entries denote instances of no detection for a particular type or type combination.

**Table 3** Odds ratios (and 95% confidence intervals) of oral cancer according to HPV DNA positivity in oral exfoliated cells

Outcome	HPV DNA	Number of cases/controls	Crude	Adjusted <sup>a</sup>	Adjusted <sup>b</sup>
All oral cancers	Negative	58/123			
	Positive	14/6	4.95 (1.8–13.5)	3.04 (1.0–9.3)	3.14 (0.9–10.9)
	Negative	58/123			
	Low risk	1/2	1.06 (0.1–11.9)	0.41 (0.0–5.3)	0.27 (0.0–4.4)
	High risk	13/4	6.89 (2.2–22.1)	4.41 (1.3–15.5)	4.81 (1.2–19.4)
Tonsil-related cancers <sup>c</sup>	Negative	12/123			
	Positive	9/6	15.38 (4.7–50.6)	12.70 (2.4–66.8)	18.43 (2.2–154.5)
	Negative	12/123			
	Low risk	0/2	0.00 (0.0–ND)	0.00 (0.0–ND)	0.00 (0.0–ND)
	High risk	9/4	23.06 (6.2–86.2)	18.45 (3.3–104.5)	19.32 (2.3–159.5)
Other oral cancers <sup>d</sup>	Negative	46/123			
	Positive	5/6	2.23 (0.6–7.7)	1.42 (0.4–5.7)	1.29 (0.3–6.3)
	Negative	46/123			
	Low risk	1/2	1.34 (0.1–15.1)	0.55 (0.0–7.3)	0.33 (0.0–5.5)
	High risk	4/4	2.67 (0.6–11.1)	2.01 (0.4–9.7)	2.14 (0.4–13.0)

<sup>a</sup> Adjusted for age, sex, schooling, race, religion, and language.

<sup>b</sup> Adjusted for age, sex, schooling, race, religion, language, tobacco smoking, and alcohol drinking.

<sup>c</sup> Palatine tonsils and base of tongue.

<sup>d</sup> Not tonsil-related.

DNA in oral cells, yielded an OR of 99.3 (95%CI: 3.2–3089.9). OR estimates were rather imprecise due to the low number of cases in the subsite analysis and the low number of seropositive controls. The risk estimates for other oral cancers were much smaller: the OR for the fully adjusted model was 3.9 (95%CI: 0.9–18.0).

The adjusted (covariates as in model 3) ORs for seropositivity to HPV 18 and HPV 31 were 2.3 (95%CI: 0.4–13.4) and 2.2 (95%CI: 0.8–6.3), respectively. Likewise, HPV 18 and HPV 31 seropositivity was not significantly associated with

tonsil-related cancers or with lesions in other oral sites (data not shown).

### Combination of serological assay and HPV DNA detection

Among controls, most subjects were negative for both detection techniques (117 out of 128: 91%) whereas none was positive for both assays. It was not possible to estimate ORs of oral cancer due to positivity for both measurements

**Table 4** HPV capsid serum antibodies (optical density values) according to disease status

Anti-VLP antibody	Disease status	N	Mean OD	Difference in means	95%CI	p-Value <sup>a</sup>
HPV16	Controls	128	0.014	0.067	0.021–0.113	0.005
	Cases	66	0.082			
HPV18	Controls	128	0.011	0.004	0.000–0.007	0.048
	Cases	66	0.015			
HPV31	Controls	128	0.026	0.036	–0.009–0.081	0.115
	Cases	66	0.062			

<sup>a</sup> p-Value for independent t-test.

**Table 5** Odds ratios (and 95% confidence intervals) of oral carcinoma according to HPV 16 seropositivity in different covariate adjustment models<sup>a</sup>

Outcome	Serostatus	Number of cases/controls	Model 1	Model 2	Model 3	Model 4
All oral cancers	Negative	58/123	1 (referent)	5.79 (1.7–19.3)	7.48 (2.1–27.2)	6.45 (1.6–25.3)
	Positive	14/5	6.62 (2.3–19.3)			
Tonsil-related cancers <sup>b</sup>	Negative	8/123	1 (referent)	31.51 (4.5–219.7)	182.27 (7.0–4753.0)	99.34 (3.2–3089.9)
	Positive	9/5	27.68 (7.5–102.2)			
Other oral cancers <sup>c</sup>	Negative	44/123	1 (referent)	3.16 (0.8–13.0)	3.87 (0.9–17.5)	3.93 (0.9–18.0)
	Positive	5/5	2.8 (0.8–10.1)			

<sup>a</sup> Model 1: crude analysis; model 2: adjusted for socio-demographic variables; model 3: adjusted for socio-demographic variables plus tobacco and alcohol; model 4: adjusted for socio-demographic variables, tobacco, alcohol, and additionally for detection of HPV DNA  
<sup>b</sup> Palatine tonsils and base of tongue.  
<sup>c</sup> Not tonsil-related.

since none of the control subjects was in this category. To circumvent this problem, a case-only analysis was performed to assess the magnitude of association of markers of HPV infection with tonsil-related cancers, compared to other oral cancers; these results are shown in Table 6. The magnitude of the association for both markers of HPV infection (HPV DNA and serology) with tonsil-related cancers was substantially higher than for either marker alone. These results suggest that not only the association of HPV infection with tonsil-related cancers is much stronger than

for other oral cancers, but also that assessment of HPV infection with serological assays supplements the information on HPV exposure that is obtained with detection of viral DNA in the oral cavity.

## Discussion

After controlling for the confounding effect of socio-demographic factors, tobacco smoking, and alcohol drinking we

**Table 6** Case-only analysis: odds ratios (ORs) of tonsil-related<sup>a</sup> cancers (compared with non tonsil-related) for markers of HPV infection

HPV marker	Categories	Number of non-tonsillar cases/tonsillar cases	OR (95%CI)
HPV DNA	Negative	46/12	1 (referent)
	Positive	5/9	6.90 (1.9–24.4)
HPV 16 serology	Negative	44/8	1 (referent)
	Positive	5/9	9.90 (2.6–37.3)
Combined tests	Both negative	42/7	1 (referent)
	Only HPV DNA positive	2/1	3.00 (0.2–37.7)
	Only serology positive	2/1	3.00 (0.2–37.7)
	Either test positive	7/10	8.57 (2.4–30.0)
	Both tests positive	3/8	16.00 (3.4–75.3)

<sup>a</sup> Tonsillar cases: include palatine and lingual tonsils; non tonsillar cases: other than palatine and lingual tonsils.

found a strong association between high-risk HPV types and risk of tonsil-related cancers, using both detection of DNA and serological response to HPV 16. Moreover, this association did not decrease after adjustment for markers of sexual activity (data not shown), suggesting that genital infections are not likely to explain the serological response to HPV 16. Our finding that the relatively high magnitude of the ORs for HPV 16 seropositivity was not materially affected by adjustment for oral HPV DNA indicates that seropositivity contributes substantial independent risk prediction. HPV 16 seropositivity may thus serve as a surrogate marker for the totality of HPV exposure that is relevant in oral carcinogenesis.

The tonsils appear to be infected preferentially in the oral cavity. The epithelium of the deep tonsillar crypts, in close contact with lymphoid tissue, could be more susceptible to HPV infection or transformation. One of the first observations suggesting that tonsillar cancers are linked to HPV was provided by Snijders et al.<sup>32</sup> The authors detected HPV DNA in all of the 10 biopsies of tonsillar carcinomas and in none of the seven biopsies of tonsillitis used as controls. Since then, several reports found that tonsillar cancers, or cancers of the oropharynx, presented a higher prevalence rate of HPV infection than other oral cancers.<sup>33</sup> Gillison et al.<sup>34</sup> found that oropharyngeal tumors were six times more likely to harbor HPV DNA than other oral cancers (OR = 6.2; 95%CI: 3.1–12.1). Mork et al.<sup>22</sup> found an OR of 14.4 (95%CI: 3.6–58.1), which is comparable in magnitude with that of our study. In addition to the epidemiological evidence, there is consistent biological evidence that HPV-positive cancers arising from the palatine and lingual tonsils are a distinct entity etiologically linked to infection by high-risk HPV types, especially HPV 16. Gillison et al.<sup>34</sup> showed that HPV-positive oropharyngeal cancers had predominantly basaloid morphology and fewer p53 mutations. The majority harbored HPV 16, which was localized by *in situ* hybridization within the nuclei of cancer cells, and Southern blot hybridization patterns were consistent with viral integration.<sup>34</sup> Van Houten et al.<sup>35</sup> found that HPV-positive oral tumors, which were also positive for expression of the viral E6 gene (E6 mRNA), lacked a p53 mutation. On the other hand, p53 mutations were very common among HPV negative tumors and HPV positive tumors that did not express the E6 gene. In agreement with this study, a report by Wiest et al.<sup>36</sup> showed that among HPV positive tumors of the head and neck regions, most tumors of the oropharynx expressed the viral oncogenes E6 and E7 and presented the wild type p53, whereas most tumors arising from other oral regions presented a mutated p53 and did not express the viral oncogenes.

The analysis for oral cancers other than palatine tonsil and base of tongue showed a non significant, positive association of low magnitude with detection of high-risk HPV DNA, which is in agreement with other studies.<sup>18,19,37</sup> Herrera et al.,<sup>24</sup> who included patients with cancers of the mouth and of the oropharynx, failed to find an association with detection of viral DNA in oral cells. Two case-control studies that included a considerable proportion of patients already treated for oral cancer found a relatively low detection rate (less than 10%) of high-risk HPV DNA in both cases and controls.<sup>17,23</sup> A meta-analysis by Miller and Johnstone<sup>38</sup> reported a pooled OR of 5.37 (95%CI: 2.5–11.6) for studies

comparing prevalence of HPV in normal mucosa and squamous cell carcinomas of the mouth. An association between detection of HPV DNA and non-tonsil-related oral cancers does not necessarily mean that the virus plays an etiologic role. HPV appears to be a common comensal in the oral mucosa,<sup>39</sup> and there is the possibility that the virus infects preferentially already premalignant or malignant tissues.

Studies of serological response to HPV may better represent the temporal relationship between the virus and the tumor, since serological response is more a marker of past cumulative HPV exposure rather than current HPV infection.<sup>20,40</sup> In our study we found a positive association between serological response to the HPV 16 capsid and cancers of the oral cavity other than tonsil and base of tongue (adjusted OR = 3.87, 95%CI:0.9–17.5). These findings are in agreement with other investigations.<sup>22–24</sup>

The biological evidence for a firm etiologic link between HPV infection and cancers of the oral cavity not related to the lingual and palatine tonsils is not well established. The extreme sensitivity of PCR could detect latent infections not related to the tumor. Gillison et al.<sup>34</sup> found that non tonsillar oral tumors positive for HPV by PCR were rarely positive by Southern blot or *in situ* hybridization, suggesting a lack of clonal association between the virus and cancer cells. Nevertheless, some reports have shown that the HPV 16 genome was integrated into the genome of oral cancer cells and that the viral genome was transcriptionally active.<sup>41</sup> This latter evidence, plus the association between HPV seropositivity and cancers of the mouth found in this and other studies, gives support to the idea that at least some squamous cell carcinomas not related to the tonsils may be etiologically linked to HPV infection. However, given that the biological evidence is scarce, the proportion of non-tonsillar cancers of the mouth attributable to HPV infection is likely to be small.

Given the strong association between markers of sexual activity and cervical cancer, some studies have examined the relationship between sexual activity and oral carcinomas. Whereas some researchers<sup>23,42</sup> found a positive correlation between markers of sexual activity and oral cancers, Smith et al.<sup>18</sup> failed to find any association. In our study we did not find any association with such markers (data not shown).

One of the limitations of the present study is the relatively small number of patients with oral cancer, which limited the precision of risk estimates and prevented us from assessing effect modification between HPV and other factors, such as tobacco smoking and age. Another potential limitation, intrinsic to case-control studies in which exposure and outcome are assessed at the same point in time, is the uncertainty to confirm whether the exposure precedes the outcome. It is extremely unlikely that reverse causality bias could explain the magnitude of the association between HPV infection and cancers of the palatine and lingual tonsils. Since serological response is more a marker of past than recent infection, the positive association between seropositivity and these cancers – in addition to the biological evidence – further supports a causal link between HPV and these malignancies.

Among the strengths of this study is that HPV infection was assessed using two markers: detection of HPV DNA in the oral cavity and serological response to the virus. Individually, both markers showed similar association with the dis-

ease, whereas combined they supplemented each other. Also we showed that the association between the virus and oral cancer is subsite specific.

In summary, the association found in this investigation between HPV and cancers of the palatine tonsils and base of tongue seems to be genuine. The association is independent from the influence of smoking and alcohol, the two established causal factors for oral cancers. In addition, the association was consistently detected using both PCR and serological techniques, which further supports its validity. The prospect of therapeutic and/or preventive HPV vaccines, currently being developed for cervical cancer, eventually may be used for prevention of at least certain types of oral tumors.

### Conflict of Interest Statement

None declared.

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