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Evaluation of Human Papillomavirus Antibodies and Risk of Subsequent Head and Neck Cancer

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ABSTRACT

Purpose
Human papillomavirus type 16 (HPV16) infection is causing an increasing number of oropharyngeal cancers in the United States and Europe. The aim of our study was to investigate whether HPV antibodies are associated with head and neck cancer risk when measured in prediagnostic sera.

Methods
We identified 638 participants with incident head and neck cancers (patients; 180 oral cancers, 135 oropharynx cancers, and 247 hypopharynx/larynx cancers) and 300 patients with esophageal cancers as well as 1,599 comparable controls from within the European Prospective Investigation Into Cancer and Nutrition cohort. Prediagnostic plasma samples from patients (collected, on average, 6 years before diagnosis) and control participants were analyzed for antibodies against multiple proteins of HPV16 as well as HPV6, HPV11, HPV18, HPV31, HPV33, HPV45, and HPV52. Odds ratios (ORs) of cancer and 95% CIs were calculated, adjusting for potential confounders. All-cause mortality was evaluated among patients using Cox proportional hazards regression.

Results
HPV16 E6 seropositivity was present in prediagnostic samples for 34.8% of patients with oropharyngeal cancer and 0.6% of controls (OR, 274; 95% CI, 110 to 681) but was not associated with other cancer sites. The increased risk of oropharyngeal cancer among HPV16 E6 seropositive participants was independent of time between blood collection and diagnosis and was observed more than 10 years before diagnosis. The all-cause mortality ratio among patients with oropharyngeal cancer was 0.30 (95% CI, 0.13 to 0.67), for patients who were HPV16 E6 seropositive compared with seronegative.

Conclusion
HPV16 E6 seropositivity was present more than 10 years before diagnosis of oropharyngeal cancers.

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INTRODUCTION

Human papillomavirus type 16 (HPV16) is recognized as a cause of virtually all cervical cancers and of a substantial proportion of other anogenital cancers and oropharyngeal cancers.[1] The association between HPV16 and cancers of the oral cavity and larynx is less clear but, if associated, the attributable proportion is small.[1] HPV16 has been associated with a rapid increase in the incidence of oropharynx cancer in some parts of the world, notably in the United States, Sweden, and Australia, where it is now responsible for more than 50% of cases.[2-4] If current trends continue, the annual number of oropharyngeal cancers in the United States may soon surpass the number of cervical cancers.[2]

The only evidence for the temporal relationship between HPV exposure and development of head and neck cancers (HNC) comes from a study within the Nordic serum banks linked to tumor registries: a significant 14-fold increased risk for cancer of the oropharynx was reported for seropositivity to the L1 capsid protein of HPV16.[5] Antibodies against HPV L1 represent cumulative past HPV infection from multiple possible anatomic sites (ie, genital, anal, or oral), are common in controls, and

do not imply the presence of a HPV-related tumor.[6] Conversely, antibody markers against HPV E6 and E7 oncoproteins should occur in response to an underlying HPV-driven neoplastic process and would be expected at low levels among cancer-free individuals. Multiple case-control studies have validated this hypothesis for HPV16 E6 seropositivity, which was present in less than 1% of controls, but not for HPV16 E7 seropositivity, which was present in 2% to 4% of controls.[7-11] The presence of HPV16 E7 antibody reactivity among controls is currently not understood.

We investigated antibodies against the HPV oncogenes E6 and E7, other viral regulatory proteins (E1, E2, and E4), and the L1 antigen for multiple HPV types in prediagnostic plasma from patients with HNC and matched control participants from the European Prospective Investigation Into Cancer and Nutrition (EPIC) study, a cohort of more than 500,000 adults in 10 European countries.[12]

METHODS

Study Cohort

EPIC procedures have been previously described in detail.[12] In brief, 521,330 individuals were recruited to the cohort between 1992 and 2000 from 10 European countries, of whom 385,747 participants contributed a blood sample. Blood fractions were aliquoted into 0.5 mL straws, which were heat-sealed and stored in liquid nitrogen tanks at −196°C, except in Umeå, Sweden, where samples were stored in 1.8 mL plastic tubes in freezers at −80°C. Participants completed self-administered questionnaires on lifestyle factors and diet. All participants gave written, informed consent and the research was approved by the local ethics committees and the International Agency for Research on Cancer Ethics Committee.

Follow-Up for Cancer Incidence and Mortality Data

Incident patients with cancer were identified at regular intervals through population-based cancer registries (in Denmark, Italy except Naples, the Netherlands, Norway, Spain, Sweden, and the United Kingdom) or by active follow-up (France, Germany, Greece, and Naples), which involved a combination of methods, including a review of health insurance records, cancer and pathology registries, and direct contact with participants and their next-of-kin.

Mortality data, including vital status, cause of death, and date of death, were obtained from mortality registries at the regional or national level. Participants underwent follow-up from study entry until cancer diagnosis (except for diagnoses of nonmelanoma skin cancer), death, emigration, or the end of the follow-up period for the relevant study center. End of follow-up was defined as the latest date of complete follow-up for both cancer incidence and vital status and varied between study centers from December 2004 to June 2010. Over 98% of vital status follow-up is complete.

Selection of Patients With Cancer and Control Participants

After blood collection, 1,292 incident patients with HNC and esophagus cancer were identified according to the International Classification of Diseases for Oncology, Second Edition (ICD-O-2). The diagnoses included cancers of the oral cavity (ICD C02.0-C02.9, C04.0-C04.9, C03.0-C03.9, C05.0-C06.9, C14.0-C14.9), oropharynx (C01.9, C02.4, C09.0-C10.9), nasopharynx (C11.0-C11.9), hypopharynx (C13.0-C13.9), larynx (C32.0-C32.9), and esophagus (C15.0-C15.9). We excluded patients with a history of another cancer (n = 158, except for nonmelanoma skin cancer), who did not donate a blood sample (n = 152), and who were not histologically confirmed, were prevalent at the time of blood donation, or did not have questionnaire information available (n = 22), leaving a total of 960 eligible patients. All histologic subtypes of HNC (84.2% of which were squamous cell carcinoma) and esophagus cancer (45.7% of which were squamous cell carcinoma) were included.

Two control participants (one in Denmark) were randomly assigned for each patient with cancer from appropriate risk sets consisting of all cohort participants alive and free of cancer (except nonmelanoma skin cancer) at the time of diagnosis (and hence, age) of the index case. Matching criteria were: country, sex, date of blood collection (± 1 month, relaxed to ± 5 months for sets without available controls), and date of birth (± 1 year, relaxed to ± 5 years for sets without available participants). Two control participants were available for 677 participants, and one control participant for 282 participants.

After excluding participants who did not have a sufficient volume of plasma available for antibody analysis, the final study population included 938 patients with cancer and 1,599 control participants.

Serologic Analyses

Plasma samples were sent on dry ice to the German Cancer Research Center (DKFZ, Heidelberg, Germany) and testing was performed using multiplex assays by laboratory staff who were blinded to the patient-control status of the participants.[7-9,11] Antigens were affinity-purified, bacterially expressed fusion proteins with *N*-terminal Glutathione *S*-transferase. Samples were analyzed for antibodies to the major capsid protein (L1), the early oncoproteins (E6, E7), and other early proteins (E1, E2, E4) of the following carcinogenic mucosal types: HPV16 and HPV18 (L1, E1, E2, E4, E6, and E7); HPV31, HPV33, HPV45, and HPV52 (L1, E6, and E7); and of the noncarcinogenic mucosal types HPV6 and HPV11 (L1, E6, and E7). Mean fluorescence intensity (MFI) values were dichotomized as antibody positive or negative,[8-11] using predefined cutoff values (Appendix Table A1 [online only]) based on the mean ± 5 standard deviations (SD; for HPV early proteins) or the mean ± 3 SDs excluding positive outliers (for HPV late proteins) of the MFI values derived from serum samples of 117 female, HPV DNA-negative, self-reported virgins from a cross-sectional study among Korean students.[13] Values below 200 MFI were set to this minimum cutoff. For HPV6 E6, HPV11 L1, and HPV33 E7, an arbitrarily defined cutoff of 500 MFI was used.

We evaluated the reproducibility of HPV seropositivity among 114 samples that were randomly chosen at 5% within the current study population, as well as a parallel study on a different cancer site. This involved a total of 69 men and 45 women ages 34 to 77 years at recruitment. The intra-individual correlation coefficient of seropositivity for each antigen was acceptable, including for all HPV16-related proteins (L1, 0.78; E1, 0.86; E2, 0.72; E4, 0.87; E6, 1.0; E7, 0.83). A reference sample with known reactivity to three antigens (HPV16 L1, HPV16 E6, and HPV16 E7) was included on each plate as a measurement standard. Intra-individual correlation coefficients for all evaluated antigens are listed in Appendix Table A1.

Statistical Analyses

Characteristics of the patients with cancer (overall and by anatomic site of the cancer) and control participants were evaluated. Odds ratios (ORs) and 95% CIs were calculated by anatomic site using logistic regression for seropositivity by HPV type and protein. Because few control participants were seropositive for some markers, the final risk analysis was conducted using unconditional logistic regression including all 1,599 control participants to allow calculation of OR. Covariates included in the models comprised matching factors (country, sex, and age), smoking status (never/former/current), and alcohol intake (never/ever plus alcohol g/d intake at recruitment).

All-cause mortality between HPV16 E6 seropositive and seronegative patients with oropharyngeal cancer was evaluated by Cox proportional hazards regression analysis using years since diagnosis as the time variable. The hazard ratio (HR) for HPV16 E6 seropositivity was calculated after adjustment for age at oropharyngeal cancer diagnosis, sex, and country.

To provide estimates of the 10-year cumulative incidence of oropharyngeal cancer, age-specific incidence rates by sex and smoking status (never/former/current) were calculated using data from the entire EPIC cohort[14] and were standardized to EPIC participants ages 50 to 70 years by smoking status and sex (age-standardized rates [ASR]), as the rates were relatively stable in these age categories. These ASRs were multiplied by the smoking-specific ORs for HPV16 E6 and converted to 10-year cumulative incidence estimates.

RESULTS

Baseline Characteristics

Baseline characteristics of the study population are listed in Table 1. The study included 638 patients with HNCs, 300 with esophageal

Table 1. Characteristics of Patients With HNC (overall and by anatomic site) or Esophageal Cancer and Control Participants

Characteristic	Controls (n =1,599)		HNC (n =638)*		Oral Cavity Cancer (n = 180)		Oropharynx Cancer (n = 135)		Larynx Cancer (n =247)		Esophageal Cancer (n = 300)	
	No. of Participants	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
Sex												
Male	1,105	69.1	456	71.5	108	60.0	89	65.9	211	85.4	204	68.0
Female	494	30.9	182	28.5	72	40.0	46	34.1	36	14.6	96	32.0
Age at enrollment, years												
< 41	42	2.6	19	3.0	7	3.9	4	3.0	2	0.8	4	1.3
41-50	331	20.7	123	19.3	32	17.8	25	18.5	46	18.6	49	16.3
51-60	765	47.8	331	51.9	85	47.2	84	62.2	133	53.9	144	48.0
61-70	391	24.5	152	23.8	52	28.9	21	15.6	59	23.9	80	26.7
> 70	70	4.4	13	2.0	4	2.2	1	0.7	7	2.8	23	7.7
Country												
Denmark	285	17.8	189	29.6	39	21.7	54	40.0	81	32.8	92	30.7
France	14	0.9	4	0.6	1	0.6	3	2.2	0	0.0	3	1.0
Germany	208	13.0	84	13.2	20	11.1	22	16.3	32	13.0	20	6.7
Great Britain	265	16.6	65	10.2	23	12.8	9	6.7	26	10.5	67	22.3
Greece	44	2.8	19	3.0	3	1.7	1	0.7	11	4.5	3	1.0
Italy	140	8.8	59	9.3	20	11.1	8	5.9	22	8.9	11	3.7
The Netherlands	156	9.8	51	8.0	13	7.2	15	11.1	14	5.7	27	9.0
Norway	4	0.3	1	0.2	0	0.0	0	0.0	0	0.0	1	0.3
Spain	206	12.9	79	12.4	27	15.0	7	5.2	32	13.0	25	8.3
Sweden	277	17.3	87	13.6	34	18.9	16	11.9	29	11.7	51	17.0
Smoking†												
Never	631	39.5	121	19.0	45	25.0	34	25.2	16	6.5	61	20.3
Former	553	34.6	162	25.4	42	23.3	40	29.6	62	25.1	90	30.0
Current	385	24.1	350	54.9	92	51.1	60	44.4	166	67.2	143	47.7
Alcohol drinking†‡												
Never	171	10.7	79	12.4	23	12.8	18	13.3	31	12.6	42	14.0
Light	1,321	82.6	431	67.6	120	66.7	90	66.7	164	66.4	208	69.3
Heavy	107	6.7	127	20.0	37	20.6	27	19.9	51	20.7	49	16.3
Education†												
Primary	643	40.2	268	42.0	71	39.4	51	37.8	114	46.2	133	44.3
Higher than primary school	906	56.7	355	55.6	104	57.8	82	60.7	127	51.4	155	51.7
BMI†												
< 25	634	39.7	265	41.5	74	41.1	57	42.2	102	41.3	120	40.0
25-29	713	44.6	264	41.4	67	37.2	59	43.7	101	40.9	129	43.0
≥ 30	243	15.2	105	16.5	38	21.1	19	14.1	43	17.4	49	16.3

Abbreviations: BMI, body mass index; HNC, head and neck cancer.

*HNC included 180 cancers of the mouth (including oral cavity, n = 96; tongue, n = 52; floor of mouth, n = 32), 135 oropharynx cancers (tonsil, n = 82; base of tongue, n = 16; other oropharynx, n = 37), 247 larynx cancers (including hypopharynx, n = 31), 17 sinus cancers, 26 nasopharynx cancers, and 33 other HNCs (mainly overlapping sites).

†Self-reported smoking and drinking status at baseline. Columns do not add to 100% because of missing data.

‡Light drinkers were defined among men as those who consumed up to 60 gm/day and among women as those who consumed up to 30 gm/day; heavy drinkers were defined at those individuals who exceeded those thresholds.

cancers, and 1,599 cancer-free control participants. Median age at enrollment was 56.6 years, median age at cancer diagnosis was 62.8 years among patients with HNC, and their median time between blood draw and diagnosis was 6.3 years.

HPV Seropositivity and Cancer Risk

Seropositivity against HPV16 E6, one of the two HPV oncogenes that are preferentially retained and expressed in cancers, was present in prediagnostic plasma of 34.8% of patients with oropharyngeal cancer (n = 47) and 0.6% of control participants (n = 9; adjusted OR, 274; 95% CI, 110 to 681; Table 2). An increased risk was also observed for HPV16 E7 seropositivity and oropharyngeal cancer (OR, 2.4; 95% CI, 1.5 to 3.9), but the antibody was present in a substantial proportion of control participants (11%; n = 178), thus reducing the estimated OR. Risk of oropharynx cancer was elevated for HPV16 L1 (OR, 3.1; 95% CI, 2.1 to 4.5), E1 (OR, 5.7; 95% CI, 3.2 to 10.0), and E2 (OR, 9.5; 95% CI, 5.7 to 15.8), but not E4.

Among patients with HPV16 E6 seropositive oropharyngeal cancer (47 of 135 participants), 42.6% (n = 20) were also seropositive for HPV16 E7. None of the control participants or patients with oral cavity or esophageal cancer were seropositive for both HPV16 E6 and E7, but one patient with laryngeal cancer was dual positive for HPV16 E6 and E7.

No elevation in risk of oral cavity, larynx, or esophagus cancer was observed in relation to HPV16 antibodies except for HPV16 E1, with a significant two-fold increase in the risk of oral cavity and larynx

Table 2. ORs by HPV16 Serology Status for Cancer of the Oral Cavity, Oropharynx, Larynx, and Esophagus

Serology Status	Controls (n = 1,599)		Oral Cavity Cancer (n = 180)				Oropharynx Cancer (n = 135)				Larynx Cancer (n = 247)*				Esophageal Cancer (n = 300)			
	No. of Participants	%	No. of Patients	%	OR	95% CI	No. of Patients	%	OR	95% CI	No. of Patients	%	OR	95% CI	No. of Patients	%	OR	95% CI
HPV16 oncoproteins																		
E6																		
Seronegative	1,590	99.4	178	98.9	1		88	65.2	1		244	98.8	1		299	99.7	1	
Seropositive	9	0.6	2	1.1	1.3	0.3 to 6.9	47	34.8	274	110 to 681	3	1.2	3.8	0.8 to 17.6	1	0.3	0.6	.1 to 5.2
E7																		
Seronegative	1,421	88.9	155	86.1	1		108	80.0	1		217	87.9	1		272	90.7	1	
Seropositive	178	11.1	25	13.9	1.2	0.7 to 1.9	27	20.0	2.4	1.5 to 3.9	30	12.1	0.9	0.5 to 1.4	28	9.3	0.7	0.5 to 1.2
HPV16 other early proteins																		
E1																		
Seronegative	1,536	96.1	165	91.7	1		113	83.7	1		226	91.5	1		283	94.3	1	
Seropositive	63	3.9	15	8.3	2.1	1.1 to 3.9	22	16.3	5.7	3.2 to 10.0	21	8.5	2.2	1.2 to 3.9	17	5.7	1.7	0.9 to 3.0
E2																		
Seronegative	1,527	95.5	170	94.4	1		102	75.6	1		234	94.7	1		286	95.3	1	
Seropositive	72	4.5	10	5.6	1.0	0.5 to 2.1	33	24.4	9.5	5.7 to 15.8	13	5.3	1.0	0.5 to 1.9	14	4.7	0.9	0.5 to 1.7
E4																		
Seronegative	1,437	89.9	165	91.7	1		120	88.9	1		218	88.3	1		276	92.0	1	
Seropositive	162	10.1	15	8.3	0.8	0.5 to 1.5	15	11.1	1.3	0.7 to 2.4	29	11.7	1.2	0.7 to 1.9	24	8.0	0.8	0.5 to 1.2
HPV16 late protein																		
L1																		
Seronegative	1,270	79.4	138	76.7	1		79	58.5	1		187	75.7	1		231	77.0	1	
Seropositive	329	20.6	42	23.3	1.2	0.8 to 1.7	56	41.5	3.1	2.1 to 4.5	60	24.3	1.3	0.9 to 1.8	69	23.0	1.1	0.8 to 1.6

NOTE. All ORs were adjusted for sex, age at enrollment (in 5-year age categories), country, tobacco (never, former, current), and alcohol use (never/ever and continuous values in gm/day at recruitment).
Abbreviations: HPV, human papillomavirus; OR, odds ratio.
*The larynx cancer category includes 31 participants with hypopharyngeal cancer.

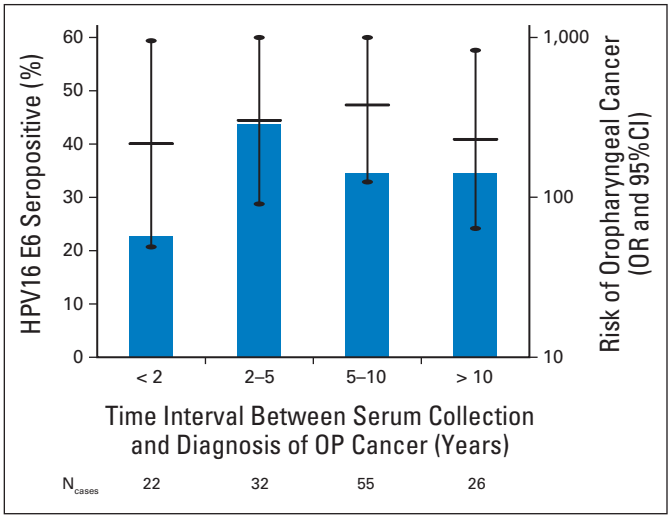

Fig 1. Proportion of human papillomavirus type 16 (HPV16) E6 seropositive patients with oropharyngeal (OP) cancer and corresponding odds ratios by lead time from blood draw to cancer diagnosis. Blue bars indicate the proportion of patients with OP cancer who were HPV16 E6 seropositive. Black lines indicate risk of OP cancer by HPV16 E6 serostatus using polytomous logistic regression after adjustment for age at enrollment, sex, country, and tobacco and alcohol use. Numbers at the bottom of the figure indicate how many patients with OP cancer in each time interval.

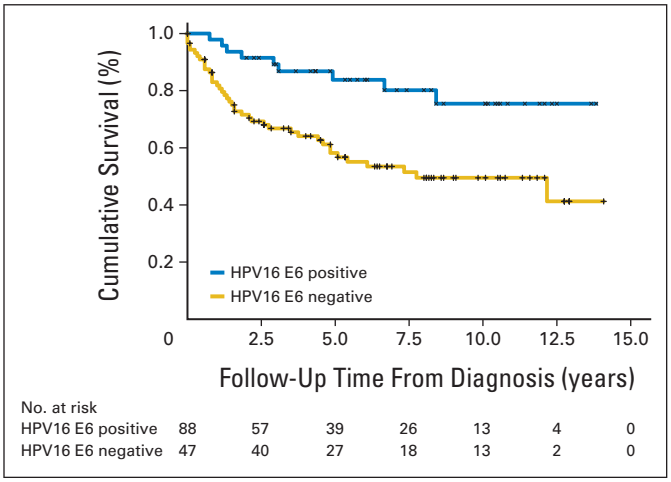

Fig 2. Cumulative survival of all-cause mortality among patients diagnosed with oropharyngeal cancer by prediagnostic human papillomavirus type 16 (HPV16) E6 serostatus. Patients who were seropositive (blue line; n = 47) and seronegative (gold line; n = 88) for HPV16 were compared for all-cause mortality. Numbers at the bottom of the figure indicate number of patients at the start of each time interval by HPV16 E6 serostatus.

cancer (Table 2). Risk of nasopharyngeal cancer was significantly elevated for HPV16 E6 seropositivity (two [7.7%] of 26 were positive; OR, 20.9; 95% CI, 3.4 to 128.4). Both HPV16 E6 seropositive patients with nasopharynx cancer were also positive for HPV16 E7.

OR for cancer associated with non-HPV16 carcinogenic genotypes was evaluated among patients and control participants who were HPV16 seronegative because of the concern over cross-reactivity. Only HPV-33 E6 significantly elevated the risk of larynx cancer (Appendix Table A2); no other mucosal (Appendix Table A2) or cutaneous (data not shown) HPV types were associated with risk.

Stratified Analysis of HPV16 E6 Seropositivity and Oropharyngeal Cancer

HPV16 E6 seropositivity and oropharyngeal cancer were further evaluated in four strata defined by lead-time between blood collection and cancer diagnosis ($<$ 2 years, 2 to 5 years, 5 to 10 years, and $\geq$ 10 years; Fig 1). HPV16 E6 seropositivity was common among patients in all lead-time categories, ranging from a minimum of 22.7% (n = 5; patients with a lead time less than 2 years) to a maximum of 43.8% (n = 14; patients with a lead time between 2 and 5 years; P for difference between categories was .81). Corresponding ORs were statistically significant in all lead-time categories, ranging from 218 (95% CI, 50 to 956), for cancer with a lead time of less than 2 years, and 231 (95% CI, 64 to 832), for cancer with a lead time of more than 10 years (P for trend across time categories was .89). The maximum lag time for an HPV16 E6 seropositive patient was 13.7 years.

HPV16 E6 seropositive patients with oropharyngeal cancer were more likely to be never-smokers (42.6%; n = 20; Appendix Table A3, online only) compared with HPV16 E6 seronegative patients with oropharyngeal cancer (15.9%; n = 14; $P \leq .001$) and thus were more similar to control participants in this instance (39.5%; n = 631). HPV16 E6 seropositive patients with oropharyngeal cancer also had greater body mass index (P = .005) and were older at the time of diagnosis (P = .03). HPV16 E6 seropositive and seronegative patients with oropharyngeal cancer were similar by sex (P = 1.00), calendar year of diagnosis (P = .80), region of Europe (P = .75), and alcohol drinking (P = .33).

All-Cause Mortality Among Oropharyngeal Cancer Participants by HPV16 E6 Seropositivity

Among patients with oropharyngeal cancer, the 5-year survival rates were 58% for those who were HPV16 E6 seronegative and 84% for those who were seropositive (Fig 2). The HR for HPV16 E6 seropositive patients was 0.30 (95% CI, 0.13 to 0.67; P = .003); further adjustment by smoking status did not affect this result (HR, 0.32; 95% CI, 0.14 to 0.73; P = .007). Individual-level data on treatment or other clinical prognostic factors including stage were not available for all patients and we were unable to further account for these variables.

Cumulative Incidence of Oropharyngeal Cancer by HPV16 E6 Seropositivity

The ASR of oropharyngeal cancer within EPIC, standardized to the EPIC cohort ages 50 to 70 years, was, among men, 4.5 per 100,000 person-years among never-smokers, 8.8 among former-smokers, and 14.6 among current-smokers. The corresponding incidence rates for women were 1.3, 2.1, and 5.8 per 100,000 person-years. ORs for HPV16 E6 positivity were 596 among never-smokers (95% CI, 137 to $>$ 1,000), 247 among former-smokers (95% CI, 67.8 to 902), and 39.7 among current-smokers (95% CI, 6.5 to 244). Among HPV16 E6 seropositive participants, the highest 10-year cumulative incidence for oropharyngeal cancer was estimated for men who were never-smokers (23.3%; 95% CI, 5.9% to 35.9%) and was lowest among female current-smokers (2.3%; 95% CI, 0.38% to 13.2%; Table 3).

DISCUSSION

HPV16 E6 seropositivity was present in 35% of patients with oropharyngeal cancer, in plasma specimens collected on average 6

Table 3. Ten-Year Cumulative Incidence Estimates for Oropharyngeal Cancer, Stratified by Sex and Smoking Status

Incidence	Men			Women		
	Never-Smokers	Former Smokers	Current Smokers	Never-Smokers	Former Smokers	Current Smokers
Age-standardized incidence rates of oropharyngeal cancer*	4.45	8.76	14.6	1.27	2.13	5.83
10-year cumulative incidence of oropharyngeal cancer for HPV16 E6 seronegative participants, %*	0.045	0.09	0.15	0.013	0.02	0.06
10-year cumulative incidence of oropharyngeal cancer for HPV16 E6 seropositive participants, %*†	23	20	5.60	7.30	5.10	2.30
95% CI	5.9 to 36	5.8 to 55	0.94 to 30	1.7 to 12	1.4 to 18	0.38 to 13

Abbreviations: EPIC, European Prospective Investigation Into Cancer and Nutrition study; OR, odds ratio.
*Per 100,000 person-years standardized to the EPIC cohort ages 50 to 70 years.
†The cumulative incidence rate and 95% CI were calculated based on the ORs and 95% CIs of the smoking-stratified ORs for HPV16 E6 seropositivity (never-smokers: OR, 596.2; 95% CI, 136.5 to > 1,000; former smokers: OR, 247.3; 95% CI, 67.8 to > 902.4; current smokers: OR, 39.7; 95% CI, 6.5 to 243.6).

years before cancer diagnosis, whereas fewer than 1% of control participants were positive for this biomarker, resulting in a high adjusted OR of 274 for diagnosis of subsequent oropharyngeal cancer. HPV16 was not associated with risk of oral cavity, larynx, or esophagus cancer.

Case-control studies that obtain blood samples at the time of diagnosis indicate that HPV16 E6 and E7 seropositivity are strongly associated with cancers of the oropharynx,[9,10,15-17] the penis,[18] and the uterine cervix.[19,20] In cervical cancer development, HPV E6 and E7 antibodies are late tumor markers that increase with clinical tumor stage.[19-21] In a prospective study of cervical cancer (follow-up time range, 1 to 20 years), fewer than 10% of patients showed antibodies to E6 and E7 proteins of HPV16 or HPV18, compared with approximately 1% of control participants, and an association with risk was only observed for cervical cancer diagnosed within 3.5 years of blood draw.[22] Other antibodies in the HPV16 proteome, specifically HPV E1 and E2, were also elevated in patients with oropharyngeal cancer in our study, a result previously noted in a case series of HPV16 DNA-positive patients with oropharyngeal cancer.[23]

HPV16 E6 seropositivity in the current prospective EPIC study was present more than 10 years before diagnosis of oropharynx cancer. Given that this was the longest interval analyzed for this cohort, the true lead time may be longer. It is unclear at what point the HPV16 E6 antibodies are generated and are detectable, be it a clinically important persistent oral HPV infection, an HPV-driven intraepithelial neoplasia (ie, a precursor lesion or preinvasive disease), or a slowly developing carcinoma. The fact that tonsils are lymphoid organs and rich in antigen-presenting cells may contribute to the relatively long time between seroconversion and cancer diagnosis, making this finding specific to the oropharynx and theoretically unlike other HPV-associated cancer sites. Specifically, immune presentation of infections at the tonsil/oropharynx may induce HPV16 E6 seroconversion in the absence of invasive disease.

The estimated 10-year risks of oropharyngeal cancer within EPIC were 7% and 23% for HPV16 E6-seropositive female and male never-smokers, respectively, though they were associated with wide CIs, and more accurate evaluations in larger studies are warranted. These estimates are comparable with the risk stratification achieved for HPV DNA testing in cervical cancer, for which the 10-year likelihood of developing cervical precancer among HPV16 DNA-positive women age older than 30 years was 17%.[24] The finding that the 10-year risk for HPV16 E6 seropositivity was higher among never-smokers than among current smokers is consistent with previous case-control studies, showing a strong negative interaction with HPV serology and tobacco smoking.[17,25] Development of oropharyngeal cancer may be driven by the carcinogenic effects of either tobacco- or HPV-induced genomic instability. As such, smokers may not need the HPV16-infection–induced pathway to cancer whereas nonsmokers do.

Our study raises several questions. First, the proportion of HPV16 E6 seropositive participants who were HPV16 DNA-positive in the tumor tissue is unknown, although efforts are currently underway to identify tumor blocks from a sample of the patients. Yet, the HPV16 E6 seropositive patients with oropharyngeal cancer were more likely to be never-smokers and have a better prognosis, as found in previous studies of HPV16 DNA-positive patients with oropharyngeal cancer,[26] thereby making it likely that most of the serologically detected oropharyngeal cancers were HPV16 DNA-positive as well. While the sensitivity of the HPV16 E6 antibody assay for detection of HPV16 DNA-driven oropharyngeal cancer is currently unknown, the capacity to detect oropharyngeal cancer overall could be higher in regions such as the United States where 70% of contemporaneous oropharyngeal tumors are thought to be caused by HPV infection. Second, further quantification of the lead time between HPV16 E6 seroconversion and cancer detection is warranted, to better understand how far in advance testing could occur. Analysis of repeat samples will also help determine the robustness of the serologic response. Third, of the nine HPV16 E6 seropositive control participants (of 1,599), we noted with interest that one developed anal cancer during the study period. This raises the question of whether the HPV16 E6 seropositivity rate among control participants in this study (0.6%) indicates the assay's false-positive rate, or if these controls may be at increased risk of eventually developing HPV-associated cancer (or a combination of the two). And finally, it will be important to more precisely evaluate the interaction between HPV16 E6 and smoking status, although a larger collaborative effort involving multiple prospective cohorts would seem necessary to obtain an adequate sample size.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Appendix

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Table A1. Intra-Individual Correlation Estimates of Seropositivity for HPV Antibody Assays

HPV Serology Marker	Intra-Individual Correlation Estimates	Cutoff (MFI)
HPV6 E6	1.00	500*
HPV6 E7	0.86	364
HPV6 L1	0.71	571
HPV11 E6	0.70	260
HPV11 E7	N/A	200
HPV11 L1	0.81	500*
HPV16 E1	0.81	200
HPV16 E2	0.72	679
HPV16 E4	0.87	876
HPV16 E6	1.00	484
HPV16 E7	0.83	548
HPV16 L1	0.78	422
HPV18 E6	1.00	243
HPV18 E7	1.00	789
HPV18 L1	0.71	394
HPV31 E6	0.89	890
HPV31 E7	N/A	200
HPV31 L1	0.66	712
HPV33 E6	0.70	253
HPV33 E7	0.81	500*
HPV33 L1	0.66	515
HPV45 E6	0.56	249
HPV45 E7	1.00	200
HPV45 L1	0.82	368
HPV52 E6	1.00	271
HPV52 E7	0.79	200
HPV52 L1	0.58	547

Abbreviations: HPV, human papillomavirus; MFI, mean fluorescence intensity; N/A, not available.
*Cutoff was arbitrarily defined.

Table A2. ORs by HPV Serology Status (E6 and E7 oncoproteins and L1) Among Study Participants Who Were Seronegative for HPV16 for the Specific Protein of Interest

	Controls		Oral Cavity Cancer				Oropharynx Cancer				Larynx Cancer				Esophageal Cancer			
Serology Status	No. of Participants	%	No. of Patients	%	OR	95% CI	No. of Patients	%	OR	95% CI	No. of Patients	%	OR	95% CI	No. of Patients	%	OR	95% CI
E6	1,590		178				88				244				299			
HPV6																		
Seronegative	1,572	98.9	174	97.8	1.0		86	97.7	1.0		241	98.8	1.0		289	96.7	1.0	
Seropositive	18	1.1	4	2.2	1.7	0.6 to 5.4	2	2.3	1.8	0.4 to 9.1	3	1.2	1.1	0.3 to 3.9	10	3.3	2.8	1.2 to 6.7
HPV11																		
Seronegative	1,547	97.3	173	97.2	1.0		85	96.6	1.0		238	97.5	1.0		294	98.3	1.0	
Seropositive	43	2.7	5	2.8	1.1	0.4 to 3.0	3	3.4	1.2	0.3 to 4.7	6	2.5	0.9	0.4 to 2.4	5	1.7	0.6	0.2 to 1.6
HPV18																		
Seronegative	1,560	98.1	175	98.3	1.0		85	96.6	1.0		238	97.5	1.0		295	98.7	1.0	
Seropositive	30	1.9	3	1.7	0.7	0.2 to 2.5	3	3.4	1.3	0.3 to 5.1	6	2.5	1.1	0.4 to 2.9	4	1.3	0.6	0.2 to 1.9
HPV31																		
Seronegative	1,550	97.5	175	98.3	1.0		87	98.9	1.0		240	98.4	1.0		295	98.7	1.0	
Seropositive	40	2.5	3	1.7	0.6	0.2 to 2.2	1	1.1	0.6	0.1 to 4.8	4	1.6	0.6	0.2 to 1.9	4	1.3	0.5	0.2 to 1.5
HPV33																		
Seronegative	1,568	98.6	176	98.9	1.0		84	95.5	1.0		238	97.5	1.0		294	98.3	1.0	
Seropositive	22	1.4	2	1.1	0.9	0.2 to 4.2	4	4.5	2.7	0.7 to 10.4	6	2.5	3.6	1.3 to 10.4	5	1.7	1.6	0.6 to 4.5
HPV45																		
Seronegative	1,565	98.4	176	98.9	1.0		87	98.9	1.0		240	98.4	1.0		292	97.7	1.0	
Seropositive	25	1.6	2	1.1	0.6	0.1 to 2.7	1	1.1	0.6	0.1 to 5.6	4	1.6	0.9	0.3 to 2.8	7	2.3	1.5	0.6 to 3.6
HPV52																		
Seronegative	1,563	98.3	175	98.3	1.0		85	96.6	1.0		238	97.5	1.0		293	98.0	1.0	
Seropositive	27	1.7	3	1.7	0.9	0.3 to 3.2	3	3.4	2.6	0.6 to 10.1	6	2.5	1.5	0.6 to 3.9	6	2.0	1.0	0.4 to 2.6
E7	1,421		155				108				217				272			
HPV6																		
Seronegative	1,363	95.9	147	94.8	1.0		107	99.1	1.0		205	94.5	1.0		265	97.4	1.0	
Seropositive	58	4.1	8	5.2	1.5	0.7 to 3.3	1	0.9	0.2	0.0 to 1.8	12	5.5	2.1	1.0 to 4.3	7	2.6	0.6	0.3 to 1.5
HPV11																		
Seronegative	1,403	98.7	154	99.4	1.0		105	97.2	1.0		214	98.6	1.0		266	97.8	1.0	
Seropositive	18	1.3	1	0.6	0.6	0.1 to 4.6	3	2.8	2.8	0.7 to 10.8	3	1.4	1.5	0.4 to 5.6	6	2.2	1.8	0.7 to 4.8
HPV18																		
Seronegative	1,403	98.7	153	98.7	1.0		108	100	—		213	98.2	1.0		270	99.3	1.0	
Seropositive	18	1.3	2	1.3	1.1	0.2 to 5.2	0	0.0			4	1.8	1.7	0.5 to 5.8	2	0.7	0.6	0.1 to 2.8
HPV31																		
Seronegative	1,395	98.2	152	98.1	1.0		105	97.2	1.0		212	97.7	1.0		261	96.0	1.0	
Seropositive	26	1.8	3	1.9	0.9	0.3 to 3.2	3	2.8	2.9	0.7 to 11.3	5	2.3	1.5	0.5 to 4.6	11	4.0	2.1	1.0 to 4.5
HPV33																		
Seronegative	1,349	94.9	145	93.5	1.0		101	93.5	1.0		203	93.5	1.0		260	95.6	1.0	
Seropositive	72	5.1	10	6.5	1.4	0.7 to 2.8	7	6.5	1.2	0.5 to 2.9	14	6.5	1.2	0.6 to 2.3	12	4.4	0.8	0.4 to 1.6
HPV45																		
Seronegative	1,403	98.7	148	95.5	1.0		107	99.1	1.0		214	98.6	1.0		271	99.6	1.0	
Seropositive	18	1.3	7	4.5	5.2	1.9 to 14.1	1	0.9	1.5	0.2 to 12.3	3	1.4	1.4	0.4 to 5.3	1	0.4	0.3	0.04 to 2.7

(continued on following page)

Table A2. ORs by HPV Serology Status (E6 and E7 oncoproteins and L1) Among Study Participants Who Were Seronegative for HPV16 for the Specific Protein of Interest (continued)

	Controls		Oral Cavity Cancer				Oropharynx Cancer				Larynx Cancer				Esophageal Cancer			
Serology Status	No. of Participants	%	No. of Patients	%	OR	95% CI	No. of Patients	%	OR	95% CI	No. of Patients	%	OR	95% CI	No. of Patients	%	OR	95% CI
HPV52																		
Seronegative	1,346	94.7	148	95.5	1.0		104	96.3	1.0		209	96.3	1.0		259	95.2	1.0	
Seropositive	75	5.3	7	4.5	0.9	0.4 to 2.2	4	3.7	0.8	0.3 to 2.5	8	3.7	0.8	0.4 to 1.9	13	4.8	1.1	0.6 to 2.0
L1	1,270		138				79				187				231			
HPV6																		
Seronegative	1,172	92.3	123	89.1	1.0		74	93.7	1.0		165	88.2	1.0		208	90.0	1.0	
Seropositive	98	7.7	15	10.9	1.3	0.7 to 2.4	5	6.3	0.6	0.2 to 1.7	22	11.8	1.5	0.8 to 2.6	23	10.0	1.3	0.8 to 2.1
HPV11																		
Seronegative	1,199	94.4	126	91.3	1.0		75	94.9	1.0		172	92.0	1.0		211	91.3	1.0	
Seropositive	71	5.6	12	8.7	1.4	0.7 to 2.8	4	5.1	0.6	0.2 to 1.9	15	8.0	1.1	0.6 to 2.2	20	8.7	1.6	0.9 to 2.8
HPV18																		
Seronegative	1,127	88.7	121	87.7	1.0		67	84.8	1.0		167	89.3	1.0		212	91.8	1.0	
Seropositive	143	11.3	17	12.3	1.0	0.6 to 1.8	12	15.2	1.2	0.6 to 2.5	20	10.7	0.7	0.4 to 1.3	19	8.2	0.6	0.4 to 1.0
HPV31																		
Seronegative	1,233	97.1	131	94.9	1.0		78	98.7	1.0		183	97.9	1.0		225	97.4	1.0	
Seropositive	37	2.9	7	5.1	1.7	0.7 to 4.1	1	1.3	0.4	0.1 to 3.3	4	2.1	0.7	0.2 to 2.2	6	2.6	0.9	0.4 to 2.3
HPV33																		
Seronegative	1,218	95.9	133	96.4	1.0		75	94.9	1.0		180	96.3	1.0		224	97.0	1.0	
Seropositive	52	4.1	5	3.6	0.8	0.3 to 2.2	4	5.1	1.4	0.5 to 4.3	7	3.7	0.8	0.3 to 1.8	7	3.0	0.6	0.3 to 1.4
HPV45																		
Seronegative	1,204	94.8	131	94.9	1.0		74	93.7	1.0		184	98.4	1.0		221	95.7	1.0	
Seropositive	66	5.2	7	5.1	1.0	0.4 to 2.2	5	6.3	1.2	0.5 to 3.3	3	1.6	0.3	0.1 to 0.9	10	4.3	0.8	0.4 to 1.6
HPV52																		
Seronegative	1,222	96.2	137	99.3	1.0		75	94.9	1.0		185	98.9	1.0		224	97.0	1.0	
Seropositive	48	3.8	1	0.7	0.2	0.02 to 1.3	4	5.1	1.3	0.4 to 4.0	2	1.1	0.3	0.1 to 1.3	7	3.0	0.8	0.3 to 1.8

NOTE. All ORs were adjusted for sex, age at enrollment (in 5-year age categories), country, tobacco (never, former, current), and alcohol use (never/ever and continuous values in gm/day at recruitment). The larynx cancer category includes 31 patients with hypopharyngeal cancer.

Abbreviations: HPV, human papillomavirus; OR, odds ratio.

Table A3. Comparison of Descriptive Characteristics of Patients With HPV16 E6 Seronegative and Seropositive Oropharyngeal Cancer

Characteristic	HPV16 E6 Seronegative Oropharyngeal Cancer (n = 88)		HPV16 E6 Seropositive Oropharyngeal Cancer (n = 47)	
	No. of Patients	%	No. of Patients	%
Sex				
Male	58	65.9	31	66.0
Female	30	34.1	16	34.0
Age at diagnosis, years*				
< 50	6	6.8	3	6.4
51-60	44	50.0	13	27.6
≥ 60	38	43.2	31	66.0
Calendar year at diagnosis				
Before 2000	29	33.0	14	29.8
2000-2004	35	39.8	20	42.6
2005 or later	24	27.3	13	27.7
Region†				
Northern	75	85.2	41	87.2
Southern	13	14.8	6	12.8
Smoking‡				
Never	14	15.9	20	42.6
Former	20	22.7	20	42.6
Current	53	60.2	7	14.9
Alcohol drinking‡				
Never	16	18.2	2	4.3
Light	47	53.4	43	91.5
Heavy	25	28.4	2	4.3
BMI§				
< 25	45	51.1	12	25.5
25-29	34	38.6	25	53.2
≥ 30	9	10.2	10	21.3

Abbreviations: BMI, body mass index; HNC, head and neck cancer; HPV, human papillomavirus.
*$P < .05$.
†Northern Europe includes Denmark, Germany, Great Britain, the Netherlands, and Sweden; Southern Europe includes France, Greece, Italy, and Spain.
‡$P < .001$.
§$P < .01$.

