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Pre-diagnostic Dynamic HPV16 IgG Seropositivity and Risk of Oropharyngeal Cancer

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Introduction

Human papillomavirus (HPV) is associated with a subset of head and neck squamous cell carcinomas (HNSCC) (1), with a strong association with cancers of the oropharynx (OPC), including cancers of the tonsils and base of tongue (2). It is now estimated that HPV type 16 is associated with 65–80% of OPC (3; 4) and incidence rates of HPV-related OPC are rapidly rising (5).

There now is strong evidence that IgG immune responses to the HPV16-derived oncoproteins E6 and E7 can be selectively detected in the blood of 60–85% of patients with HPV-related OPC (3; 6–10), as well as Abs to other HPV16-derived early antigens (11–14). In the ARCAGE study, HPV16 E6 seropositivity was detected in 30.2% of OPC cases at the time of diagnosis (OR=132) (15). In a study of 135 oropharynx cancers, HPV16 Abs to E6 were detected in 34.8% of patients up to 10 years prior to diagnosis with a low frequency (0.6%) in 1,599 controls, and no association with the time prior to diagnosis (16). Tumor HPV status was not available for that study, so the estimated frequencies are expected to be

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Conflict of Interest Statement

Dr. Anderson is a consultant, hold stock options, and serves on the scientific advisory board of Provista Diagnostics. Both Drs. Anderson and Posner have a patent submission on HPV serologic biomarkers. All other authors report no conflicts of interest.

higher within the HPV+ subset. As a result, HPV16 serology warrants further evaluation in the pre-diagnostic setting prior to use as an early detction biomarker for OPC (17).

Here, we sought to determine the association of a panel of HPV16-specific IgG Abs and the subsequent risk of OPC. The Janus Serum Bank is a population-based biorepository integrated into the Cancer Registry of Norway since 2004, and contains serum samples collected from 318,628 Norwegians in the time period 1972–2004 (18). By linking the Janus Cohort to the Cancer Registry we identified 92 participants who were diagnosed with OPC up to 15 years after serum collection. We determined the association of individual HPV16 Abs, as well as a pre-defined signature of a panel of Abs, with the risk of subsequent OPC.

Material and Methods

Patient and Controls Sera Selection

The Janus Serum Bank Cohort was queried for cases of histologically or cytologically confirmed squamous cell carcinoma of the oropharynx for which blood samples were collected within 15 years prior to OPC diagnosis. Oropharyngeal site was defined according to the following codes of the *International Classification of Diseases, Seventh Revision* (International classification of diseases, 7th rev. Geneva: World Health Organization, 1955): 145 (oropharynx including tonsils) and 141.0 (base of tongue). Ninety-two patients were identified. Patients with HPV related cancers diagnosed prior to the OPC diagnosis were not eligible (cervical, anal, vulvar, penile, or a previous OPC). The minimum time from blood draw to diagnosis was 1 month. Five matched controls were selected per case (n=460) using the incident density sampling method. All controls were required to be alive and residing in Norway at the date of diagnosis of the case (to ensure similar follow-up time as the cases) and were free of HPV-related cancers at the time of case diagnosis (see above). For cases with multiple time points (n=10), controls were matched on the oldest sample collection time point of the case. Controls were matched 5:1 (n=460) to cases based on age at blood draw (+/− 2 years), date of blood draw (+/− 2 years), gender, geography (same county), and the source of blood collection. Ten cases had samples available from multiple pre-diagnosis time points (2, n=5; 3, n=3; 4, n=1; 6, n=1) spanning an average of 3.9 years (SD = 2.3 years); the most proximate sample was used for risk analysis. All serum samples were provided as coded, blinded samples, and remain blinded to the laboratory investigators. The donors have given a broad consent for the use of the samples in cancer research. The study was approved by the Norwegian regional committee for medical and health research ethics.

Programmable Protein (RAPID) ELISA

RAPID ELISAs were performed essentially as described (12; 14). Recombinant GST-tagged HPV16 proteins were expressed from template cDNA using human cell lysate (#88882; Thermo Scientific, Waltham, MA), and captured onto 96-well plates coated with anti-GST Ab ((#27-4577-01; GE Healthcare, Piscataway, NJ). Sera were diluted 1:100 and analyzed as blinded specimens in duplicate. Bound IgG was measured as a ratio to GST control using HRP anti-human IgG Abs and chemiluminescence (relative light units (RLU)) at 425 nm. All recombinant DNA research was performed in accord with NIH guidelines under institutional biologic safety review and approval.

Tumor HPV DNA Detection by qPCR

Archived tumor specimens from the 92 OPC were requested from 15 pathology departments in Norway. DNA was extracted from formalin-fixed paraffin-embedded tissue using the QIAamp DNA FFPE Tissue Kit (#56404; Qiagen, Hilden, Germany) according to manufacturer's guidelines with the following modifications: after DNA elution using 50 µl AE buffer followed by centrifugation, the eluate was added the filter once more, incubated for 3 minutes, centrifugated and collected.

Multiplex quantitative polymerase chain reaction (qPCR) assays were performed on the Roche Lightcycler 480 II using LightCycler 480 Probes Master reagent (#04 887 301 001; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. qPCR assays targeting the conserved region E6/E7 in the HPV genome were used. Hydrolysis probes labeled with FAM, HEX or Cy-5 distinguished HPV type 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68a and 68b in six multiplex reactions. Human genomic DNA was co-amplified and used as internal control in each sample. Our method was validated against 46 control samples in the WHO HPV LabNet Proficiency Study, and found to be proficient for detection of the above-mentioned HPV types.

Smoking status

Information on smoking habits from baseline questionnaire data were requested from the Norwegian Institute of Public Health. Smoking information were categorized in never, former or current smokers and pack-years of smoking were calculated (19)

Statistical Analysis

Characteristics of cases and healthy controls were compared using Firth's penalized logistic regression for categorical and t-tests for means for continuous variables. Cut-off values for positive serology were pre-determined, and defined as the mean + 3 standard deviations of the RLU ratio observed among healthy volunteers from the United States (n=247) (13). These levels were E1: 2.16; NE2: 2.39; CE2: 2.32; E4: 2.34; E5: 1.66; E6: 2.38; E7: 2.21; L1: 1.95; L2: 1.93. A multiparametric model for seropositivity based on E1, E2, E4, E5, E6, and E7 Abs as continuous variables was predetermined from a set of 111 cases and 247 age- and gender-matched controls (13). ORs were calculated using Firth's penalized-likelihood logistic regression (20). Calculations were performed using the logistf package in R (http://www.R-project.org/).

RESULTS

Subject Characteristics

The demographics of cases and controls and clinical characteristics of cases are presented in Table 1. No significant differences between cases and controls were observed. The mean age of cases was 45.0 and controls was 44.6, and 81.6% of cases and controls were male. Overall, cases had a higher frequency of current smokers ($p<0.001$) and greater than 10 pack-year history of smoking ($p=0.007$) than controls. Out of 47 cases where biopsies had valid qPCR results, 39 (83%) had HPV16+ tumors. There was no significant difference in

age, gender, or smoking between the HPV16+ OPC cases and the subset of their matched controls (n=195).

Serum sample and Tumor Characteristics

Sixty-three patients (68%) had cancer of the oropharynx incl. tonsils (ICD-7: 145) and 29 patients (32%) had cancer of the base of tongue (ICD-7: 141.0).. Thirteen patients (14%) had localized disease, while metastasis to regional lymph nodes was found in 72 (78%) patients. Distant spread was seen in four patients (4%), and for three patients (3%) metastasis status was unknown. We identified tissue blocks from 62 of the 92 cases (67%) that were available for HPV typing by qPCR. Of these, 15/62 (24%) resulted in invalid qPCR results, likely due to DNA degradation from these older samples. Of the remaining 47 cases, 39 were HPV16+ by qPCR (83.0%), and three were positive for other HPV types (HPV33, 35, and 59; 6.4%).

Association of HPV16 Seropositivity with Case/Control Status and Tumor HPV Status

Serum IgG Abs to the HPV16-derived early proteins E1, E4, E5, E6 and E7, as well as the capsid proteins L1 and L2 were measured as a ratio of specific signal to GST control protein. Antibodies to the HPV16 E2 protein were measured to N-terminal and C-terminal E2 fragments (NE2 and CE2) and are reported separately. Cut-off values for seropositivity for individual Abs and a logistic regression classifier of seropositivity, based on the combined signal of IgG from all early antigens, were predetermined. A classifier score >0.5 is considered positive, and all results are reported in Table 2. The individual HPV16 Abs E1, E2, E6, and E7 were associated with an increased risk of OPC compared with controls ($p<0.05$). The binary classifier was strongly associated with case status, with an overall sensitivity of 12% at 99.1% specificity ($p<0.001$).

We observed a strong association of tumor HPV16 status with HPV seropositivity for each of the early antigens E1, CE2, E4, E6, and E7 ($p<0.05$, Table 2, right), and with the binary classifier (sensitivity, 20.5%, $p<0.001$) when compared against the matched controls. There was no association of NE2 or E5 seropositivity with tissue HPV16 status. There was no L1 or L2 seropositivity detected in cases. Of the 5 cases that were known to be negative by HPV PCR tissue typing, all had very low scores by the classifier (not shown).

Detection of HPV16 Abs and Risk of OPC

The association of serologic status and risk of OPC was determined separately for the individual HPV Abs, the binary classifier, and within the subset of known HPV16+ OPC cases and their matched controls (Table 3). Abs to E1, E2, E6, E7, and the binary classifier were associated with OPC cases. Abs to the C-terminal half of E2 (CE2) were most strongly associated with OPC cases (OR 20.9, 95% CI, 5.8–110.6), and when adjusted for age, sex, and smoking status (OR 20.7, 95% CI, 5.5–113.5). A positive score on the binary classifier was most strongly associated with OPC status, with an OR of 35.0 (95% CI, 7.5–336.8) within the HPV16+ subset of OPC cases.

Association of HPV16 Abs and Time to Clinical Diagnosis

The crude odds ratio of seropositivity as measured by the classifier for all cases was 15.8 (95% CI=5.6–53.4, p<0.001, Table 4). When adjusted for age, sex, and smoking status, the odds ratio of seropositivity for all cases was 15.3 (95% CI=5.2–53.1). Although only 14 cases had samples collected within 5 years of clinical diagnosis, the odds ratio increased for samples collected closer to diagnosis of OPC. For samples collected within 2 years before diagnosis, OR=83.0 (CI 17.5–419.2, p<0.0001), which decreased to OR=72.5 at 2–5 years prior to diagnosis (p=0.0001), OR=16.0 at 5–10 years prior to diagnosis (p=0.0005), and down to 5.7 from >10 years prior to diagnosis (p=0.0913). This inverse association with the time from serum collection and diagnosis was statistically significant in a logistic regression model (OR, 0.8 per additional year; 95% CI: 0.7–0.9, p=0.0034). The percent positivity at each time interval is shown in Figure 1. Six of fourteen cases (42.9%) were positive within 5 years of diagnosis.

Increase in HPV16 Abs Over Time

Multiple timepoints were available for 10 cases. We determined the probability of HPV positivity for these cases and determined the association with years prior to clinical diagnosis and tumor HPV status (Figure 2). Two cases were seropositive (probability of HPV ≥ 0.5) at the initial blood draw and remained seropositive in all subsequent draws. One case was seronegative at the initial draw 14 years prior to diagnosis but seropositive at the final pre-diagnostic draw 9 years prior to diagnosis. Seven cases were seronegative in all pre-diagnostic draws; however, the probability of seropositivity of one of the two cases known to be HPV16+ increased 4-fold but did not reach the threshold for seropositivity. There was no evidence of decline from seropositivity to seronegativity in the cases.

Discussion

We have measured multiple HPV16-specific Abs in sera up to 15 years prior to the diagnosis of OPC in serum samples from the Janus Serum Bank Cohort in Norway. We observed a strong association of Abs to the early HPV16 antigens E1, E2, E6, and E7 with OPC status, and with HPV16 tumor status. Using a pre-determined multiparametric classifier, the OR association of cases with seropositivity increased for samples closer to the time of clinical diagnosis. These data support further investigation of these serum biomarkers for the early detection of OPC.

One strength about the cohort we have investigated is the availability of serial pre diagnostic samples from the same individuals. A key challenge in the field is to determine the true frequency of seropositivity prior to clinical diagnosis of OPC. The relative prevalence of HPV in OPC in Scandinavia in the past is estimated to be lower than it is today, as smoking rates have declined and HPV prevalence has increased, now approaching 80% in 2008–2012 (21). In addition, there has been no published data yet on the rate of change of seropositivity over time prior to clinical diagnosis. We had tumor tissue available from 62/92 of our cases, but 15 samples were not of sufficient integrity for HPV DNA analysis. Thirteen of those tissue samples were from 1996 or earlier, and likely represent a lower HPV frequency. Therefore, the true prevalence of HPV16 in the overall samples likely lies between 63–83%.

Our data are consistent with a recent series from the Cleveland Clinic which found that 86.6% (252/291) of OPC from 1996–2013 (22) were HPV+ as defined by ISH or p16 positivity.

One unique finding in our data is that seropositivity increases closer to the time of clinical diagnosis both overall and in selected cases for which serial samples were available. Data published by Kreimer et al did not find an association with HPV16 E6 Abs and the time prior to diagnosis of OPC (16), but Abs were associated with the time prior to diagnosis of anal cancer (23). We did not observe a strong association of E6 serology with the time prior to diagnosis; the majority of the impact of the increase in seropositivity is derived from an increase in the rates of E1 and E2 seropositivity (data not shown).

In the Kreimer study, antibodies to other HPV16-derived antigens, including E1, E2, and E7, were detected at lower frequencies in prediagnostic OPC sera (16–24%), but were also detected in normal individuals (3.9–11.1%), limiting clinical utility as early detection biomarkers. In contrast, our data suggests that Abs to the HPV16 antigens E1 and E2 are highly specific for cases. The multiparametric algorithm that incorporates the serology for all early antigens improves the specificity of detection. The differences between Kreimer et al´s findings (16) and the present data are likely related to technical differences in the protein production and display for the serologic measurements.

Our finding that seroconversion appears up to 15 years prior to diagnosis supports a model of long lag time between infection and clinical cancer, and is consistent with the main findings of Kreimer et al (16). HPV16 E6 seropositivity has similarly been identified years prior to diagnosis of invasive cervical cancer with an odds ratio of 10.2 (24). High HPV16 E6 seroreactivity in healthy controls is associated with Abs to multiple HPV16 proteins, suggesting these infections may also be biologically relevant but not clinically detectable (25). These results should encourage the search for clinical precursor lesions to HPV+ OPC, analogous to the disease progression found in cervical cancer (26). Our serologic data suggest that multiple early HPV-derived proteins, especially the E1 and E2 antigens, would be expressed in early OPC carcinogenesis.

There are several challenges facing the use of Abs as a predictive biomarker for OPC carcinogenesis (27). While the biomarker panel has the high specificity (>99%) that is needed for early detection in a high-risk population, there is a need for integrated clinical, pathologic, and imaging strategies for the assessment of positive results. HPV+ OPC remains a relatively rare disease with a favorable prognosis, and stage at diagnosis seems to be less associated with survival than what is the case for other head and neck cancers (28–30). Morbidity however may be altered by early detection if treatment options, such as vaccines and targeted immunotherapy are more effective in the setting of minimal disease burden or before additional malignant evolution has taken place. Our data suggest that serial measurements of rising Ab levels to multiple HPV antigens may aid in risk assessment and early detection strategies for OPC.

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Abbreviations

Abs	antibodies
HPV	Human Papilloma Virus
qPCR	qualitative polymerase chain reaction
OPC	oropharyngeal cancer
OR	odds ratio
SD	standard deviation

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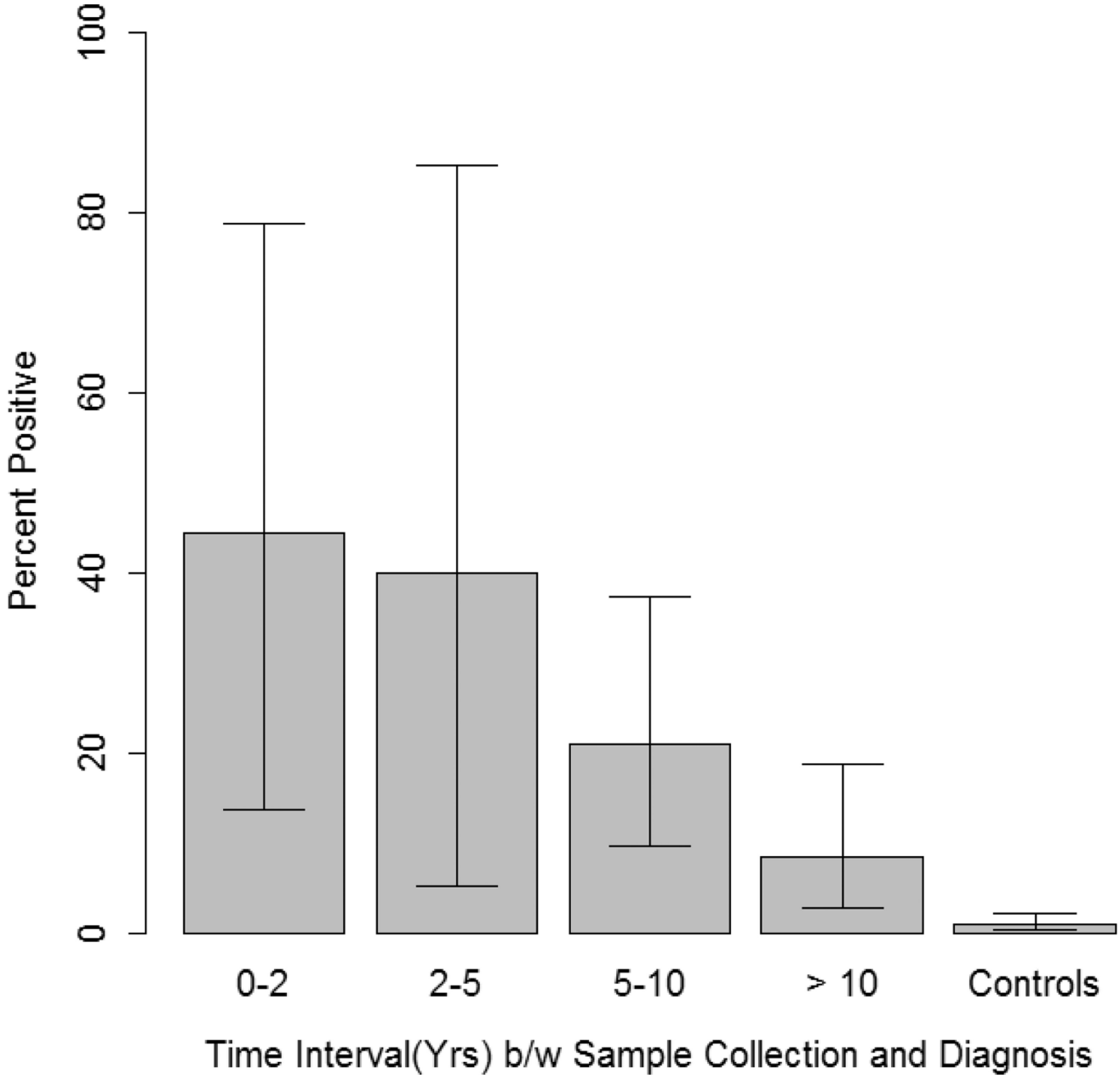

Figure 1. Percent Positive at Fixed Cutoff (Prob HPV Pos = 0.5)

Cases Prior to Diagnosis: 0–2 yrs (N=9), 2–5 yrs (N=5), 5–10 yrs (N=38), >10 yrs (N=59), Controls (N=460). Error bars indicate 95% confidence intervals.

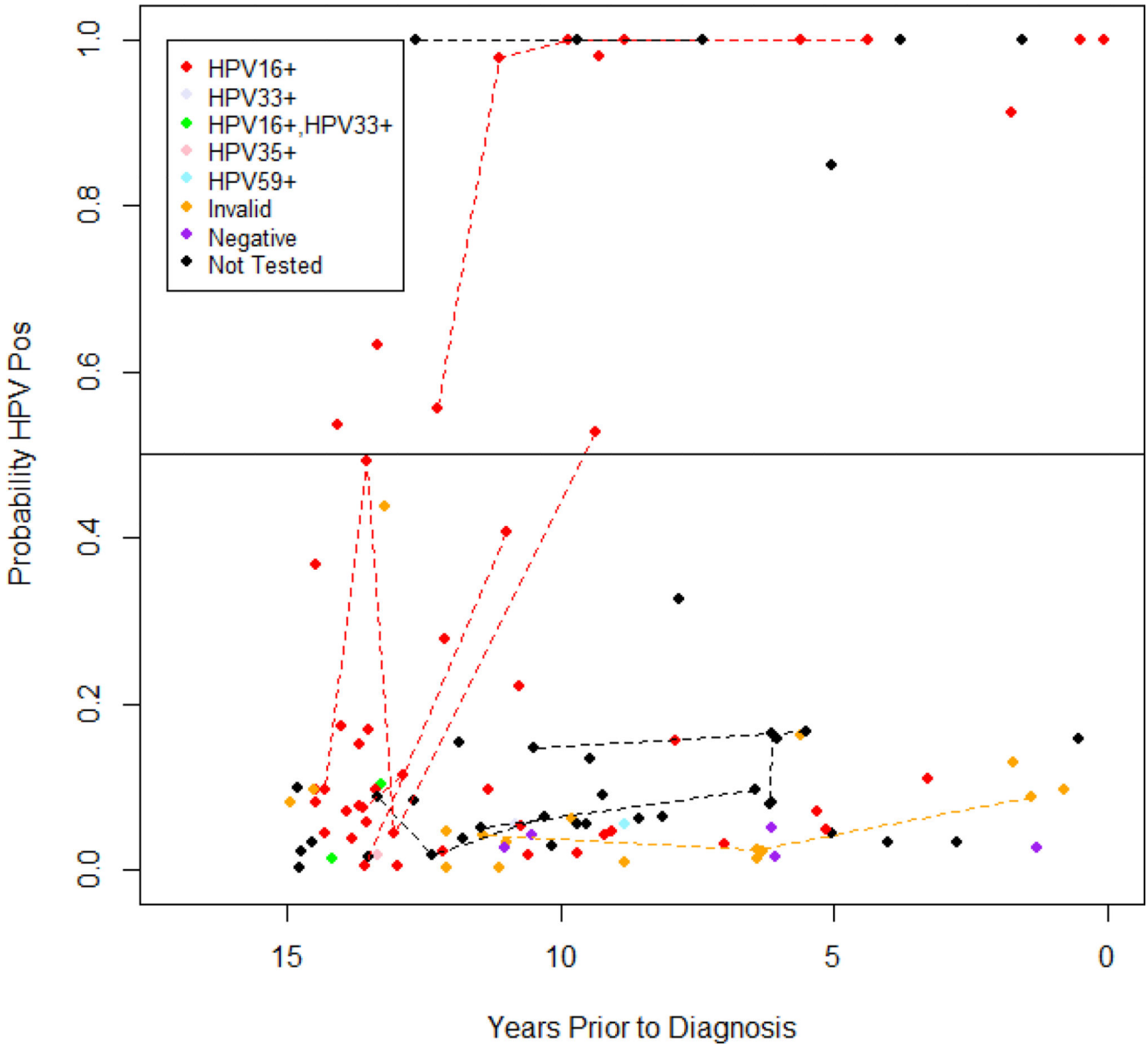

Figure 2. Probability of HPV Abs increases prior to clinical diagnosis

The classifier score is represented as the probability of HPV positivity vs Number of Years Prior to Diagnosis. The horizontal line at 0.5 indicates the positivity cutoff for the classifier.

Table 1

Demographics of serum sample cases and controls and tumor characteristics

	Controls (N=460)	OPC (N=92)	P	Controls[a] (N=195)	HPV16 DNA+ OPC Patient (N=39)	P
Age at blood draw, mean (SD), years	44.6 (6.7)	45.0 (7.0)	.600	42.4 (6.3)	42.6 (6.2)	.798
	N (%)	N (%)		N (%)	N (%)	
Sex			.951			.0.900
Male	375 (81.5)	75 (81.5)		170 (87.2)	34 (87.2)	
Female	85 (18.5)	17 (18.5)		25 (12.8)	5 (12.8)	
Smoking			<.001			.068
Never	120 (30.9)	13 (16.3)		59 (35.5)	8 (22.9)	
Former	94 (24.2)	9 (11.3)		44 (26.5)	6 (17.1)	
Current	174 (44.8)	58 (72.5)		63 (38.0)	21 (60.0)	
Missing	72 (−)	12 (−)		29 (−)	4 (−)	
Pack-years of smoking			.007			.134
≤10	173 (45.1)	23 (28.8)		84 (51.2)	13 (37.1)	
>10	211 (54.9)	57 (71.3)		80 (48.8)	22 (62.9)	
Missing	76 (−)	12 (−)		31 (−)	4 (−)	
Tumor Status	HPV16 DNA+ OPC Patients (N=39)	HPVDNA+ HPV16− OPC Patients (N=3)	HPV DNA-OPC Patients (N=5)	HPV DNA Invalid OPC Patients (N=15)	HPV DNA Not Tested OPC Patients (N=30)	
	N (%)	N (%)	N (%)	N (%)	N (%)	
Site of OPC						
Oropharynx incl. tonsils([b])	32 (82.1)	3 (100.0)	4 (80.0)	7 (46.7)	17 (56.7)	
Base of tongue([c])	7 (17.9)	0 (0.0)	1 (20.0)	8 (53.3)	13 (43.3)	
Metastasis						
None	1 (2.6)	0 (0.0)	3 (60.0)	3 (20.0)	6 (20.0)	
Regional	36 (92.3)	2 (66.7)	2 (40.0)	12 (80.0)	20 (66.7)	
Distant	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	3 (10.0)	
Unknown	1 (2.6)	1 (33.3)	0 (0.0)	0 (0.0)	1 (3.3)	

[a]Subset of controls matched to the known HPV16+ OPC cases

OPC: oropharyngeal cancer; HPV: human papillomavirus

[b]ICD-7-code: 145

[c]ICD-7-code: 141.0

Matching criteria for controls were age at blood draw (+/− 2 years), date of blood draw (+/− 2 years), gender, geography (same county), and the source of blood collection

Table 2

Sensitivity and specificity of seropositivity up to 15 years prior to diagnosis

	Controls		OPC			HPV16 DNA+ OPC		
	N+ (%)	Specificity (%)	N+ (%)	P	Sensitivity (%)	N+ (%)	Sensitivity (%)	P
E1+	19 (4.1)	95.9 (93.6 – 97.5)	9 (9.8)	.030	9.8 (4.6 – 17.8)	6 (15.4)	15.4 (5.9 – 30.5)	.040
NE2+	4 (0.9)	99.1 (97.8 – 99.8)	4 (4.3)	.021	4.3 (1.2 – 10.8)	2 (5.1)	5.1 (0.6 – 17.3)	.086
CE2+	2 (0.4)	99.6 (98.4 – 99.9)	9 (9.8)	<.001	9.8 (4.6 – 17.8)	6 (15.4)	15.4 (5.9 – 30.5)	<.001
NE2+ and/or CE2+				<.001				.001
E4+	9 (2.0)	98.0 (96.3 – 99.1)	5 (5.4)	.060	5.4 (1.8 – 12.2)	5 (12.8)	12.8 (4.3 – 27.4)	.002
E5+	8 (1.7)	98.3 (96.6 – 99.2)	0 (0.0)	.303	0.0 (0.0 – 3.9)	0 (0.0)	0.0 (0.0 – 9.0)	.535
E6+	3 (0.7)	99.3 (98.1 – 99.9)	7 (7.6)	<.001	7.6 (3.1 – 15.1)	4 (10.3)	10.3 (2.9 – 24.2)	.001
E7+	4 (0.9)	99.1 (97.8 – 99.8)	9 (9.8)	<.001	9.8 (4.6 – 17.8)	3 (7.7)	7.7 (1.6 – 20.9)	.020
L1+	7 (1.5)	98.5 (96.9 – 99.4)	0 (0.0)	<.001	0.0 (0.0 – 3.9)	0 (0.0)	0.0 (0.0 – 9.0)	<.001
L2+	3 (0.7)	99.3 (98.1 – 99.9)	0 (0.0)	<.001	0.0 (0.0 – 3.9)	0 (0.0)	0.0 (0.0 – 9.0))	.001
E6+ and/or E7+	5 (1.1)	98.9 (97.5 – 99.6)	14 (15.2)	<.001	15.2 (8.6 – 24.2)	6 (15.4)	15.4 (5.9 – 30.5)	<.001
E2+, E6+ and/or E7+	9 (2.0)	98.0 (96.3 – 99.1)	16 (17.4)	.368	17.4 (10.3 – 26.7)	7 (17.9)	17.9 (7.5 – 33.5)	.655
Any Early	35 (7.6)	92.4 (90.0 – 94.6)	23 (25.0)	.811	25.0 (16.6 – 35.1)	13 (33.3)	33.3 (19.1 – 50.2)	.990
Any Late	8 (1.7)	98.3 (96.6 – 99.2)	0 (0.0)	.303	0.0 (0.0 – 3.9)	0 (0.0)	0.0 (0.0 – 9.0)	.535
Binary Classifier[a]	4 (0.9)	99.1 (97.8 – 99.8)	12 (13.0)	<.001	13.0 (6.9 – 21.7)	8 (20.5)	20.5 (9.3 – 36.5)	<.001

[a]Binary classifier is a combination of antibodies based on ROC analysis; ≥0.5 is considered positive

OPC: oropharyngeal cancer; HPV: human papillomavirus; E: early protein; L: late protein; SD: standard deviation

Table 3

Association of pre-diagnostic antibody status with case-control status

Antibody	OPC cases versus controls Crude Odds Ratio (95% CI)	HPV16 DNA+ OPC cases vs matched controls Crude Odds Ratio (95% CI)	OPC cases versus controls Adjusted Odds Ratio (95% CI)	HPV16 DNA+ OPC cases vs matched controls Adjusted Odds Ratio (95% CI)
E1+	2.6 (1.1 – 5.7)	3.1 (1.1 – 8.5)	3.0 (1.2 – 6.8)	3.4 (1.1 – 9.5)
NE2+	5.2 (1.3 – 20.3)	5.2 (0.8 – 34.4)	4.0 (1.0 – 16.2)	4.8 (0.7 – 36.2)
CE2+	20.9 (5.8 – 110.6)	15.0 (3.7 – 84.5)	20.7 (5.5 – 113.5)	12.6 (3.0 – 72.7)
NE2+ and/or CE2+	8.9 (3.3 – 25.7)	8.3 (2.4 – 31.3)	8.0 (2.9 – 23.9)	7.0 (2.0 – 27.1)
E4+	3.0 (0.9 – 8.5)	8.8 (2.2 – 39.4)	3.3 (1.0 – 9.8)	8.5 (2.1 – 39.9)
E5+	0.3 (0.0 – 2.3)	0.4 (0.0 – 4.0)	0.3 (0.0 – 2.6)	0.4 (0.0 – 3.7)
E6+	11.5 (3.3 – 48.0)	16.4 (2.9 – 167.5)	13.2 (3.6 – 58.5)	18.3 (3.0 – 199.6)
E7+	11.5 (3.8 – 40.3)	7.4 (1.4 – 45.9)	10.2 (3.3 – 36.8)	5.3 (1.0 – 34.3)
L1+	0.3 (0.0 – 2.7)	0.5 (0.0 – 5.2)	0.3 (0.0 – 2.7)	0.6 (0.0 – 5.7)
L2+	0.7 (0.0 – 7.4)	1.0 (0.1 – 12.3)	0.7 (0.0 – 8.4)	0.8 (0.0 – 10.7)
E6+ and/or E7+	15.3 (5.9 – 46.0)	15.0 (3.7 – 84.5)	15.3 (5.7 – 47.6)	12.5 (3.0 – 71.9)
E2+, E6+ and/or E7+	10.3 (4.5 – 24.5)	8.0 (2.5 – 27.0)	10.0 (4.3 – 24.6)	6.8 (2.1 – 24.0)
Any Early	4.1 (2.3 – 7.2)	4.9 (2.1 – 11.0)	4.6 (2.5 – 8.5)	4.8 (2.1 – 10.9)
Any Late	0.3 (0.0 – 2.3)	0.4 (0.0 – 4.0)	0.3 (0.0 – 2.2)	0.4 (0.0 – 3.8)
Binary Classifier	15.8 (5.6 – 53.4)	35.0 (7.5 – 336.8)	15.3 (5.2 – 53.1)	28.7 (6.0 – 278.4)

Odds ratios and confidence intervals computed using Firth's penalized likelihood logistic regression
Adjusted odds ratios adjusted for age, sex and smoking status
OPC: oropharyngeal cancer; E: early protein; L: late protein

Table 4

Association of Seropositivity with Years prior to Cancer Diagnosis

Cases (N)	Crude Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)	P-value[a]
All Cases (92)	15.8 (5.6 – 53.4)	15.3 (5.2 – 53.1)	<0.0001
0–2 Years Pre-diagnosis (9)	83.0 (17.5 – 419.2)	85.2 (13.7 – 68.5)	<0.0001
2–5 Years Pre-diagnosis (5)	72.5 (10.1 – 490.4)	55.5 (6.9 – 466.1)	0.0005
5–10 Years Pre-diagnosis (33)	16.0 (3.9 – 65.2)	14.9 (3.6 – 62.8)	0.0005
>10 Years Pre-diagnosis (45)	5.7 (1.0 – 26.5)	4.6 (0.8 – 22.2)	0.0913

[a] p-value for odds ratio adjusted for sex, age and smoking status