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## A LONGITUDINAL STUDY OF HPV16 L1, E6 AND E7 SEROPOSITIVITY AND ORAL HPV16 INFECTION

Daniel C. Beachler, PhD<sup>1</sup>, Raphael Viscidi, MD<sup>2</sup>, Elizabeth A. Sugar, PhD<sup>1,3</sup>, Howard Minkoff, MD<sup>4</sup>, Howard D. Strickler, MD<sup>5</sup>, Ross D. Cranston, MD<sup>6</sup>, Dorothy J. Wiley, PhD<sup>7</sup>, Lisa P. Jacobson, ScD<sup>1</sup>, Kathleen M. Weber, MS<sup>8</sup>, Joseph B. Margolick, MD<sup>2</sup>, Susheel Reddy, MS<sup>9</sup>, Maura L. Gillison, MD, PhD<sup>10</sup>, and Gypsyamber D'Souza, PhD<sup>1</sup>

<sup>1</sup>Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, US

<sup>2</sup>Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, US

<sup>3</sup>Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, US

<sup>4</sup>Department of Obstetrics and Gynecology, Maimonides Medical Center, Brooklyn NY, US

<sup>5</sup>Departments of Epidemiology and Population Health, Albert Einstein College of Medicine, Bronx, NY, US

<sup>6</sup>Department of Medicine, University of Pittsburgh, Pittsburgh, PA, US

<sup>7</sup>School of Nursing, University of California-Los Angeles, Los Angeles, CA, US

<sup>8</sup>Hektoen Institute of Medicine, The CORE Center at John H. Stroger Jr. Hospital of Cook County, Chicago, IL, US

<sup>9</sup>Department of Infectious Disease, Northwestern University, Chicago, IL, US

<sup>10</sup>Viral Oncology Program, Ohio State University Comprehensive Cancer Center, Columbus, OH, US

### Abstract

**Background**—Individuals with HPV infections can develop IgG antibodies to HPV proteins including the L1 capsid and E6 and E7 oncoproteins. Evidence on whether L1 antibodies reduce the risk of cervical HPV infection is mixed, but this has not been explored for oral HPV infections. Antibodies to HPV16's E6 oncoprotein have been detected in some oropharyngeal cancer cases years prior to cancer diagnosis, but it is unknown if these antibodies are associated with oral HPV16 DNA.

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**Corresponding Author:** Gypsyamber D'Souza, 615 N Wolfe St. E6132B, Baltimore, MD 21205, Phone: 410-502-2583, Fax: 410-614-2632, gdsouza2@jhu.edu.

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The data were presented in part during the 2014 International Papillomavirus (IPV) conference in Seattle, Washington.

**Methods**—Enzyme linked immunosorbent assays tested for serum antibodies to HPV16's L1 capsid in 463 HIV-infected and 293 HIV-uninfected adults, and for antibodies to recombinantly expressed E6 and E7 oncoproteins to HPV16 in 195 HIV-infected and 69 HIV-uninfected cancer-free participants at baseline. Oral rinse samples were collected semi-annually for up to three years and tested for HPV DNA using PGMY 09/11 primers. Adjusted Poisson, logistic, and Wei-Lin-Weissfeld regression models were utilized.

**Results**—HPV16 L1 seroreactivity did not reduce the subsequent risk of incident oral HPV16 infection in unadjusted (HR=1.4, 95%CI=0.59–3.3) or adjusted (aHR=1.1, 95%CI=0.41–3.0) analysis. Antibodies to HPV16 E6 and E7 oncoproteins were detected in 7.6% and 3.4% of participants respectively, but they were not associated with baseline oral HPV16 DNA prevalence or oral HPV16 persistence (each p-value>0.40).

**Conclusions**—Naturally acquired HPV16 L1 antibodies did not reduce the risk of subsequent oral HPV16 infection. HPV16 E6 and E7 seropositivity was not a marker for oral HPV16 infection in this population without HPV-related cancer.

### Keywords

HPV; Oropharyngeal Cancer; L1 Seropositivity; E6 Seropositivity

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### Introduction

HPV16 is the most commonly detected HPV type in the oral region,<sup>1,2</sup> and causes most HPV-positive Head and Neck Squamous Cell Carcinomas (HNSCC).<sup>3</sup> HPV16 infections, along with other HPV types, can lead to the production of type-specific antibodies to HPV proteins including to the L1 capsid. These naturally acquired IgG antibodies have been shown to reduce the risk of the subsequent acquisition of cervical HPV infection in some studies,<sup>4–8</sup> but not others.<sup>9,10</sup> Whether these antibodies impact the subsequent risk of *oral* HPV infection has not been explored.

Long-term persistent HPV16 infections are known to sometimes lead to the development of antibodies to HPV16's E6 and E7 oncoproteins, usually late in carcinogenesis.<sup>11</sup> E6 antibodies are strongly associated with HPV-positive oropharyngeal cancer<sup>12</sup> and have been detected in some cases more than ten years prior to their cancer diagnoses,<sup>13</sup> but it is unknown if these antibodies are common among cancer-free individuals currently infected with oral HPV16.

Therefore, we examined the relationship between HPV16 L1, E6 and E7 seropositivity and oral HPV16 infection utilizing a longitudinal cohort study of HIV-infected and at-risk HIV-uninfected individuals known to have a higher oral HPV16 prevalence.<sup>1</sup>

### Materials and Methods

#### Study Participants

These analyses included individuals from the Persistent Oral human Papillomavirus Study (POPS), a study nested within the Multicenter AIDS Cohort Study (MACS) of men who have sex with men (MSM) and the Women Interagency HIV Study (WIHS).<sup>1,14,15</sup> There

were 463 HIV-infected and 293 at risk HIV-uninfected participants who were tested for HPV16 L1 antibodies. These participants met the following criteria: enrolled in 2009–2010, not vaccinated with a prophylactic HPV vaccine by study baseline, and had four or more POPS follow-up visits. Banked serum was obtained from participant's baseline POPS visit and tested for HPV L1 antibodies to HPV16, and to the other two most common oncogenic oral HPV types in the POPS: HPV33 and HPV45. The detected HPV L1 antibodies may have developed after an HPV infection at any number of anatomic (genital, anal, oral) regions.

HPV16 E6 and E7 antibody testing was performed on a subgroup of 273 participants without a history of any HPV-related cancer (cervical, anal, penile, or oropharyngeal). Participants with a detectable oral HPV16 infection at any POPS visit were included (n=91) along with twice as many oral HPV16-negative controls (n=182). These controls were a random sample selected after stratification by cohort and HIV-status to match the distribution among oral HPV16-positive individuals. The MACS/WIHS executive committees and the Institutional Review Boards from each site approved the study protocol, and participants provided written informed consent.

### Laboratory testing

Antibody testing was performed on banked serum samples from participant's POPS baseline visit by using virus-like particle-enzyme linked immunosorbent assays (VLP-ELISAs) with HPV 16, 33, and 45 capsids produced in insect cells from recombinant baculoviruses, following previously published methods.<sup>16,17</sup> Seropositivity was defined as an optical density (OD) over three standard deviations above the mean OD of sera from two year-old children.<sup>17</sup> For quality assurance, known positive controls were run on each ELISA plate throughout the testing period. When comparing all the samples with duplicates, the intra-assay coefficient of variations (CVs) were 5.8%, 7.4% and 8.7% for HPV16, 33 and 45, respectively, while the inter-assay CVs (between different assays plates) were 16.1%, 13.3% and 23.2% for HPV16, 33 and 45, respectively.

Banked baseline serum samples were also tested for antibodies to recombinantly expressed HPV16 E6 and E7 oncoproteins. Antibody testing was performed using ELISAs with a microtiter plate with HPV 16 E6 and E7 GST-fusion proteins expressed in E.coli according to the protocol of Sehr P et al.<sup>18</sup> For E6 and E7, the seropositivity cutpoint was defined as an optical density (OD) over three standard deviations above the mean OD of sera of low-risk control cohort of 93 female US army recruits between the ages of 18 and 35 after excluding positive outliers. Younger females were considered an adequate control population given their very low risk for HPV16 E6 and E7 seropositivity.<sup>13</sup> We additionally considered a more stringent cutpoint defined as an OD over five standard deviations above the mean OD in the control cohort. When duplicates were compared, the intra-assay CV was 9.3% for E6 and 6.9% for E7.

Oral rinse samples were collected at up to seven semi-annual visits through a 30 second rinse and gargle with Scope™ mouthwash. DNA was isolated from these samples using a magnetic bead-based automated platform (QIA Symphony SP, Qiagen),<sup>19</sup> and then tested for

37 HPV types utilizing the Roche linear array with PGMY09/11 PCR primer pools and reverse line blot hybridization, as previously described.<sup>1,19</sup>

### Statistical methods

To compare baseline HPV16 L1 seroprevalence by each risk factor, we utilized Chi-square tests for categorical variables and Mann-Whitney tests for continuous variables. We calculated prevalence ratios (PRs) and 95% confidence intervals (95% CIs) using Poisson regression with robust variance to analyze risk factors associated with baseline HPV16 L1 seroprevalence.

To evaluate the association of baseline L1 antibodies with subsequent risk of infection with the same HPV type, we excluded prevalent oral HPV infections and restricted outcomes to incidently detected infections. We calculated incidence rates, and utilized unadjusted and adjusted Wei-Lin-Weissfeld (WLW) modeling to evaluate the impact of seropositivity on oral HPV incidence. Seropositivity was also examined by antibody titer level, as titers were a priori categorized into tertiles to match the technique of a previous study.<sup>4</sup>

For the HPV16 E6/E7 antibody analysis, logistic regression was utilized to examine whether prevalent oral HPV16 infection was associated with E6 and/or E7 seropositivity at the same visit. Models were adjusted for variables that have been associated with prevalent or incident oral HPV infection.<sup>1,20</sup> In different sensitivity analyses for HPV L1, we stratified by gender and HIV-status and required two negative tests before classifying an infection as “incident”. We also examined the results when restricting to persistent infection (requiring two consecutive positive HPV tests) for both the HPV16 L1 and E6/E7 analyses. All statistical tests were two sided and considered significant using an  $\alpha=0.05$  level. All analyses were performed by STATA-MP Version 12.0.

## Results

### HPV L1 Seropositivity

Among the 756 eligible participants, there were 167 (22%) who were HPV16 L1 seropositive at baseline. HPV16 L1 seropositivity was similar by age and gender ( $p$ -values $>0.20$ , Table 1). However, never smoking cigarettes, increased number of recent oral sex partners, and HIV-status were all associated with increased HPV16 L1 seroprevalence, even after adjustment for other risk factors (Table 1, all  $p<0.05$ ).

Baseline HPV16 L1 seroreactivity did not reduce the subsequent risk of oral HPV16 infection in either unadjusted (hazard ratio (HR)=1.4, 95%CI=0.59–3.3) or adjusted analyses (aHR=1.1, 95%CI=0.41–3.0, Table 2). Results were similar when restricted to HIV-infected (aHR=1.0, 95%CI=0.30–3.5) or HIV-uninfected (aHR=1.4, 95%CI=0.27–6.9) individuals, or among only individuals who reported having sex during the study. Results were also similar when requiring two consecutive negative tests before the first positive for an infection to be considered incident and when restricting to HPV16 persistent infection as an outcome (data not shown). While we were underpowered to examine effect modification, we cannot exclude the possibility of an effect of seropositivity on subsequent oral HPV16 infection in certain subgroups such as females (aHR, 0.63; 95%CI, 0.13–3.1), particularly

considering seropositive women had a higher HPV16 titer level than did seropositive MSM (OD=0.51 vs. 0.41,  $P=0.02$ ).

When stratifying the 167 HPV16 L1 seropositive individuals into antibody titer tertiles, the 55 individuals within the highest tertile had a non-significantly lower risk of oral HPV16 infection compared to the L1 seronegative group (aHR=0.44, 95%CI=0.05–3.7, Supplemental Table 1). Results also appeared to differ among other HPV types, as HPV33 L1 seropositivity was associated with reduced risk of subsequent oral HPV33 infection (aHR=0.11, 95%CI= 0.01–0.78, Table 2), while HPV45 L1 seropositivity was associated with a *higher* risk of subsequent oral HPV45 infection (aHR=3.6, 95%CI=1.1–11.8, Table 2).

### HPV16 E6 and E7 seropositivity

Among 195 HIV-infected and 69 at-risk HIV-uninfected participants evaluated, 7.6% ( $n=20$ ) were positive for antibodies to the HPV16 E6 oncoprotein, while 3.4% ( $n=9$ ) were positive for antibodies to the HPV16 E7 oncoprotein. E6 or E7 seropositivity was similar by gender (males vs. females: 8.2% vs. 8.5%,  $p=0.94$ ), but was doubled among HIV-infected compared to HIV-uninfected individuals, although the difference was not statistically significant (9.7% vs. 4.4%,  $p=0.16$ ).

The prevalence of both HPV16 E6 seroreactivity and HPV16 E7 seroreactivity were similar among individuals with an oral HPV16 infection detected during the POPS compared to those who never had an oral HPV16 infection (Table 3, E6: 10.2% vs. 6.3%,  $p=0.25$ ; E7: 4.6% vs. 2.8%,  $p=0.47$ ). After adjustment, the odds of E6 and E7 seroreactivity did not statistically differ when comparing those with and without an oral HPV16 DNA detected during POPS (Table 3, E6: aOR=2.0, 95%CI=0.55–6.9; E7: aOR=2.0, 95%CI=0.52–7.9). Results were also similar and non-significant when restricting to oral HPV16 infections detectable at baseline of this study (i.e. prevalent,  $p=0.78$ ), and when restricting to oral HPV16 infections persisting at least six months ( $p$ -value=0.58).

When a more stringent seropositivity cutpoint was utilized (an OD that was five standard deviations above the mean OD in the control cohort), the number of E6 positive individuals declined from 20 to 6 individuals (prevalence=2.3%), while the number of E7 positive individuals declined from 9 to 3 individuals (prevalence=1.1%). However, there was still no association for either E6 or E7 seropositivity with HPV16 DNA (E6: OR=1.2, 95%CI=0.22, 6.88; E7: OR=1.2, 95%CI=0.11–13.6).

### Discussion

This study found that HPV16 L1 seropositivity from natural infection did not reduce the subsequent risk of oral HPV16 infection. One potential explanation is that natural immunity may not protect against oral HPV16 infection. Additionally, HPV16 E6/E7 seropositivity was not more common among individuals with concurrent oral HPV16 DNA suggesting, like in cervical cancer, these E6/E7 antibodies may not be induced early in the carcinogenesis process and may not be suitable biomarkers for oral HPV16 infection in populations without HPV-related cancer.

Assessing the existence of naturally acquired (L1) immunity against oral HPV can be useful to evaluate the possible benefit of vaccinating sexually active individuals for HPV.<sup>21</sup> While we did not find evidence that HPV16 L1 seropositivity protects against subsequent oral HPV16 infection, it is unclear whether it may have a differential impact on infection risk at different anatomical sites. Several studies have suggested that HPV16 L1 seropositivity may partially protect against subsequent cervical HPV infection;<sup>4-6</sup> but it is unclear if that protection is conferred against HPV at other anatomical sites as two other recent studies observed no protection for penile<sup>22,23</sup> or anal HPV acquisition in men.<sup>23</sup> However, there are several caveats to our finding that HPV16 L1 seropositivity may not impact risk of oral HPV16 infection that need to be considered as the results may differ by population, HPV type, titer level, or by assay.

While our results were similar by HIV-status, results in this population may differ from other populations. The incident infections detected in this study may include many re-activated infections as well as some newly acquired infections, as HPV latency has been suggested especially among immunosuppressed and older individuals.<sup>24,25</sup> In addition, we cannot exclude the possibility that HPV16 L1 seropositivity has a different effect on oral HPV16 infection in women than in MSM. While we had limited power in this study, particularly to examine potential effect modification; differences by gender should be further examined as a genital HPV study suggested HPV16 L1 antibodies had no protective effect in men.<sup>22</sup>

We also cannot preclude the possibility that high titers of HPV16 L1 antibodies may have a protective effect against oral HPV16 infection. A few cervical HPV studies have suggested that protection from naturally acquired antibodies may be stronger among those with higher antibody titers,<sup>4,6</sup> and the protective ability of the considerably higher antibody titers induced by the HPV vaccine<sup>26</sup> supports this notion. One recent study has suggested that the high titers from the L1-based HPV vaccine may protect against subsequent prevalent oral HPV16, but further examination is needed.<sup>27</sup>

While HPV16 L1 seropositivity did not protect against oral HPV16 infection, results differed for the other HPV types examined. Indeed, HPV33 L1 seropositivity was associated with reduced oral HPV33 incidence while HPV45 L1 seropositivity was paradoxically associated with increased HPV45 incidence in this study. Explanations from these incongruities include: potential limitations in the assays, residual or unmeasured confounding, or possible actual differences. A previous cervical HPV study also found differences in natural protection by HPV type (protection for HPV16, but no protection for other types such as HPV33 and HPV45).<sup>5</sup> Similar to other serologic assays, the VLP-ELISAs used in this study are limited as there are no standard reference serum samples and the assay has been suggested to detect an antibody response for only 50–60% of women who previously had detectable cervical HPV DNA.<sup>16,28,29</sup> The VLP-ELISA utilized in this study measured the total type-specific binding IgG antibodies which include both neutralizing and non-neutralizing antibodies. Further research is needed to determine if these results may differ across other serologic assays, particularly those that restrict to neutralizing antibodies.<sup>30</sup>



This study also observed that HPV16 E6/E7 seropositivity was not associated with concurrent oral HPV16 DNA. While oral HPV16 DNA and HPV16 E6/E7 seropositivity have both been strongly associated with oropharyngeal cancer,<sup>12</sup> this study suggests the implications of oral HPV16 DNA and E6/E7 seropositivity may be less clear in populations without a diagnosed HPV-related cancer. Although it is unknown whether any participants had undetected oral pre-malignancies, the lack of an association between E6/E7 seropositivity and persistent oral HPV16 infection supports previous evidence from the cervical cancer field that these oncogenes are not normally expressed until late in the carcinogenesis process.<sup>11</sup>

Another recent study detected HPV16 E6 antibodies in some oropharyngeal cancer cases more than ten years prior to their cancer diagnoses,<sup>13</sup> conflicting with what has been seen for cervical cancer.<sup>11</sup> While E6 antibodies have been suggested to have a high specificity for HPV16-positive oropharyngeal cancer,<sup>13,31</sup> our ELISA detected HPV16 E6 seroreactivity in 6.3% of our cancer-free participants who did not have an oral HPV16 infection, albeit our population is a higher-risk group. While specificity may vary depending on the assay, the specificity of a potential HPV16 E6 serologic biomarker in high risk groups such as HIV-infected individuals would need to be considered in any screening modality.

To our knowledge, this is the first longitudinal study to examine the relationship between HPV16 L1, E6, and E7 serostatus and oral HPV16 infection. This study, coupled with cervical HPV literature,<sup>4-6</sup> raises the question of whether the relationship between natural HPV seropositivity and HPV infections at different anatomical sites may vary.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Table 1**

Characteristics of the 756 POPS participants by HPV16 L1 serostatus in unadjusted and adjusted modeling

Characteristics of POPS participants	% HPV16 L1 Seropositive <sup>#</sup>		HPV16 L1 Serology <sup>~</sup>	
	n=756	n=167	Unadjusted (PR)	Adjusted (PR) <sup>^</sup>
<b>Age</b>				
Younger than 45	217	27%	REF	REF
45–55	318	21%	0.79 (0.58–1.1)	0.81 (0.59–1.1)
55 or older	220	20%	0.76 (0.54–1.1)	0.78 (0.54–1.1)
continuous p-trend			0.42	0.62
<b>Gender</b>				
Female (WIHS)	387	21% <sup>#</sup>	REF	REF
Male (MACS)	369	24% <sup>#</sup>	1.1 (0.87–1.5)	1.2 (0.71–2.0)
p-value			0.34	0.53
<b>Cigarette Smoker</b>				
Never	241	28%	REF	REF
Former	211	18%	0.65 (0.46–0.91)	0.67 (0.47–0.95)
Current	297	20%	0.69 (0.51–0.94)	0.68 (0.48–0.97)
p-value			0.01	0.03
<b>Recent* oral sex partners</b>				
0	403	18%	REF	REF
1	165	25%	1.4 (0.99–2.0)	1.4 (0.97–2.0)
2 or more	179	30%	1.7 (1.2–2.3)	1.7 (1.1–2.5)
p-trend			0.001	0.01
<b>Lifetime number of oral sex partners</b>				
0–4	260	20%	REF	REF
5 to 99	307	22%	1.1 (0.80–1.5)	1.0 (0.71–1.4)
100 or more	176	26%	1.3 (0.90–1.8)	1.0 (0.60–1.6)
p-trend			0.18	0.91
<b>HIV-infection</b>				
No	293	20%	REF	REF
Yes	463	24%	1.2 (0.90–1.6)	1.4 (1.0–1.9)
p-value			0.23	0.02
<b>HIV-status + CD4 T cell count</b>				
Negative	293	20%	REF	REF
Positive CD4>500 cells/μL	253	24%	1.2 (0.87–1.6)	1.3 (0.97–1.9)
Positive CD4 200–499 cells/μL	169	22%	1.1 (0.77–1.6)	1.4 (0.95–2.0)
Positive CD4<200 cells/μL	38	32%	1.6 (0.95–2.7)	2.2 (1.3–3.8)
CD4 p-trend in HIV-positives			0.53	0.22

<sup>^</sup> Adjusted for age, gender, smoking status, number of recent and lifetime oral sex partners, HIV/CD4 status, study site, alcohol use, and frequency of recent toothbrushing

\* Recent defined as within the last six months

~ Risk factors for HPV16 L1 seropositivity were calculated with Poisson Regression with robust variance

# Optical density (OD) of seropositive individuals was similar across risk factors, except HPV16 seropositive women had a higher OD than seropositive MSM (OD=0.51 vs. 0.41, p=0.02)

Table 2

Baseline HPV seropositivity and subsequent risk of corresponding oral HPV infection for HPV types 16, 33, and 45.

Measure*	Oral HPV incidence					
	Participants	Oral HPV Infections	Person-time (person-years)	Incidence Rate (infs/100 p-ys)	Unadjusted HR#	Adjusted HR^#
<b>HPV 16 L1 seropositive</b>						
No	606	16	1459	1.10	REF	REF
Yes	183	7	427	1.64	1.4 (0.59–3.3)	1.1 (0.41–3.0)
<b>HPV 33 L1 seropositive</b>						
No	510	10	1286	0.78	REF	REF
Yes	279	1	706	0.14	0.18 (0.02–1.4)	0.11 (0.01–0.78)
<b>HPV 45 L1 seropositive</b>						
No	558	8	1422	0.56	REF	REF
Yes	232	11	578	1.90	3.4 (1.3–8.4)	3.6 (1.1–11.8)

^ Adjusted for age, gender, smoking status, number of recent and lifetime oral sex partners, HIV/CD4 status, study site, and frequency of recent toothbrushing

\* Seropositivity measured via the ELISAs optical density values

# Seropositivity's effect on subsequent hazard of corresponding oral HPV infection was measured through the Wei-Lin-Weissfeld method

**Table 3**  
 Oral HPV16 DNA and Baseline Prevalence of HPV16 E6 and E7 Seropositivity in the Persistence Oral Papillomavirus Study

	n=264	HPV16 E6	Unadjusted OR (95%CI)#	Adjusted OR (95% CI)^#	HPV16 E7	Unadjusted OR (95%CI)#	Adjusted OR (95% CI)^#
Overall	264	7.6% (n=20)	---	---	3.4% (n=9)	---	---
Oral HPV16 DNA- throughout study	176	6.3%	REF	REF	2.8%	REF	REF
Oral HPV16 DNA+ in study	88	10.2%	1.7 (0.68-4.3)	2.0 (0.55-6.9)	4.6%	1.6 (0.43-6.2)	2.0 (0.52-7.9)

^ Adjusted for age, gender, smoking status, number of recent and lifetime oral sex partners, HIV/CD4 status, and study site

~ Adjusted only for age, smoking status, HIV/CD4 status as other covariates were collinear

# Oral HPV16 DNA's association with HPV16 E6 and E7 seropositivity was measured through logistic regression

^ When seropositivity was defined as an OD five standard deviations above the mean OD in the control cohort, there were six E6+ individuals (prevalence=2.3%), and three E7+ individuals (prevalence=1.1%). However was still no association for either E6 or E7 seropositivity with HPV16