Epstein-Barr virus, but not human cytomegalovirus, is associated with a high grade human papillomavirus-associated cervical lesions among women in North Carolina

Tamina Kienka[1], **Matthew G. Varga**[2,3], **Josie Caves**[2], **Jennifer S. Smith**[2,3], and **Vijay Sivaraman**[1]

[1]Department of Biological and Biomedical Sciences, North Carolina Central University, Durham, NC, USA

[2]Department of Epidemiology, UNC Gillings School of Global Public Health, University of North Carolina Chapel Hill, NC, USA

[3]Lineberger Comprehensive Cancer Center, University of North Carolina Chapel Hill, NC, USA

Abstract

Statement of the Problem.—Human papillomavirus (HPV) infection is known to contribute to cervical carcinogenesis, yet other co-factors that may contribute to oncogenesis are poorly understood. Herein, we examine whether Epstein-Barr virus (EBV) and human cytomegalovirus (CMV), two onco-modulatory viruses, are associated with HPV-mediated cervical neoplastic progression.

Methods of Study.—Sixty patient cervical brush samples from a study of North Carolina women were obtained. HPV RNA positivity was determined by Aptima testing (Hologic Corporation). The level of viral transcripts for EBV and CMV were quantified (RT-PCR analysis), and co-infection status with HPV was then compared to the patient's cervical cytology grade.

Results: Over one third (38.3%) of the study population was CMV-positive, while 43.3% was EBV-positive. When sample data were stratified by cytology grade, 36.5% (19/52) of normal patients, 75% (3/4) of low-grade squamous intraepithelial lesions (LSIL) and 100% (4/4) of patients with high-grade SIL (HSIL) were EBV positive. Conversely, 35.2% (18/52) of normal patients, 25% (1/4) of patients with LSIL and 50% (2/4) of patients with HSIL were CMV positive. When examining only HPV-positive associated HSIL,100% (4/4) were positive for both HPV and EBV detection. This suggests co-viral detection with HPV and EBV is associated with more advanced HSIL cervical lesions, while CMV displayed no clear association with higher grade of cervical cytology.

Correspondence to: Vijay Sivaraman, PhD, Assistant Professor, Department of Biological and Biomedical Sciences, North Carolina Central University, 1801 Fayetteville St, MTSC 2104, Durham, NC 27717.

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Conclusions: Co-viral detection with EBV may increase the oncogenicity and/or serve as a viral marker of progression to HPV-associated high-grade cervical dysplasia.

Keywords

Cervical cancer; HPV; human papilloma virus; EBV; Epstein-Barr virus; CMV; cytomegalovirus

INTRODUCTION

Cervical cancer is the fourth most common malignancy among women in the world, and accounts for 7.5% of female cancer deaths worldwide[1]. In the United States, approximately 12,000 cases of cervical cancer are diagnosed and 4,000 women die from this disease each year[2]. Nearly all cervical cancers arise in the context of long-term persistence from its viral etiological agent, human papillomavirus (HPV) infection[3,4]. However, not all HPV types are oncogenic, and infection with high-risk types of HPV does not always lead to cancer[5,6]. Many HPV infections can spontaneously resolve within months, while only a small proportion of infections result in other disease manifestations of high-grade cancer-precursor lesions or invasive cervical cancer. In most cases, the immune response will eradicate HPV naturally, demonstrating that while HPV is necessary for cervical cancer, it is not sufficient[7]. Therefore, there is an open possibility that another cofactor may contribute to the HPV-dependent carcinogenesis.

Epstein-Barr virus (EBV) is a near ubiquitous human pathogen that causes mononucleosis and, in some cases, harbor oncogenic potential[8–10]. EBV is a member of the *Herpesviridae* family and has been well characterized for its role in cancers including nasopharyngeal carcinoma and Burkitt lymphoma, and possibly Hodgkin lymphoma and gastric carcinoma[9,11,12]. While the role of EBV has been established in the aforementioned cancers, there is conflicting evidence regarding the role of EBV in cervical cancer[9,11–13]. The prevalence of EBV infection was significantly higher in women with relatively higher grades of cervical disease among studies in India and Africa[14,15]. Furthermore, both EBV encoding regions (EBERs) and EBV latent oncoproteins have been found in cervical tumor tissue[14,16]. Taken together, these data suggest that EBV may be a marker for, or cofactor in, the etiology of high-grade cervical pre-cancer. Therefore, testing for EBV may be an effective modality as a triage marker to improve the specificity of HPV testing for the detection of high-grade cervical intraepithelial neoplasia (CIN 2+).

Similar to EBV, human cytomegalovirus (CMV) is another near ubiquitous and potentially onco-modulatory pathogen[17]. In some cancer types, such as colorectal, cervical or prostatic cancers, there exists evidence suggesting the presence of both genetic and proteinaceous remnants of CMV within these tumors[18]. However, unlike EBV, CMV has not been shown to be a direct etiologic agent of cancer. Rather, studies have explored the concept of "onco-modulation", whereby CMV expression may enhance the development of tumor malignancy by expressing the oncogenic immediate early proteins (IE1 and IE2)[19]. Research on infected tumor cell lines suggest that CMV expression may interfere with important signaling pathways, consequently promoting cell survival and angiogenesis, as well as alterations in cell motility and adhesion[20]. CMV-activated gene transcription may thus promote malignant

transformation by dysregulating various normal physiological processes that control the cell cycle. CMV may also be involved in cell invasion and modulation of the host immune system[18,20,21]. For example, the CMV protein UL16 may promote resistance against cell lysis mediated by NK cells and T cells[18]. To date, the role of CMV as a cofactor in HPV cervical carcinogenesis has not been thoroughly investigated. A high prevalence of CMV was found in an array of cervical sample types (normal tissue, LSIL, HSIL and CIS (carcinoma *in situ*) or ICC (invasive cervical cancer), with increased prevalence in CIS/ICC cases with Portuguese women[22]. However, it remains unclear if CMV is an opportunistic infection in HPV-infected cases, or if it promotes an immunosuppressive environment that will favor HPV-associated carcinogenesis[19]. These data, in conjunction with the onco-modulation capabilities demonstrated by CMV, suggest that testing for this pathogen may also assist in further our understanding of risk biomarkers for HPV-associated high-risk disease.

This study aims to investigate both EBV and/or CMV co-detection with HPV to better understand how these viruses may be associated with the grade of cervical neoplasia. We used semi-quantitative RT-PCR for detection of EBV and CMV, in conjunction with high risk HPV (HrHPV+) detection (Aptima, Hologic USA) and cytology data collected from a subset of patient samples obtained from higher risk, infrequently screened women in North Carolina patients enrolled in the My Body My Test 2 (MBMT2) study (*Zhao Y et al*, *Submitted*). Our primary aim was to identify whether either oncogenic virus is associated with more advanced pre-cancerous cervical lesions within HPV-positive patients. In contrast to previous studies which have examined viral prevalence of either EBV or CMV detection in relation to disease severity in HPV-positive patients, this study examines the associated contibution of the three viruses in cervical carcinogenesis within an infrequently screened population of women. Findings are important for improving knowledge on onco-viral contribution to HPV mediated cervical carcinogenesis, as well as informing effective clinical screening strategies, particularly in terms of HPV triage to improve the detection of CIN2+ in cervical cancer screening programs.

METHODS

Patient Samples.

Clinical collected cervical brush samples were collected during the MBMT2 study as described elsewhere (*Zhao, Y et al, submitted*). MBMT2 was designed to characterize the performance of self-collected sampling compared to clinician-collected samples using cervical brushes for HPV testing in a population of low income, underscreened women. Briefly, female residents in North Carolina were recruited if they lived within an eligible county, were 30–65 years of age, had not received a pap smear in 4 years or more, reported being "not pregnant", had not received a hysterectomy, had neither health insurance nor Medicaid, and did not live above 250% of the poverty line. Cervical brush and cytology samples were then collected from eligible participants by a clinician during the same clinic visit. In the current study, all HPV positive samples (n=17) from the MBMT2 were included, as well as a random sample of HPV negative samples (n=43).

All enrollees provided informed consent. All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki. The primary study protocol was approved by IRB of the University of North Carolina, and was conducted in accordance with all applicable ethical standards for research on human subjects.

DNA Purification for EBV and CMV laboratory testing.

Clinician-collected cervical brush samples were suspended in Aptima buffer (Hologic, Marlborough, MA, USA) and DNA was extracted via ethanol purification. For DNA purification, a 2x volume (volume: volume) of 100% ethanol was added to each patient sample, gently mixed, then centrifuged at 12,000 revolutions per minute (rpm) at 4°C for 13 minutes. Supernatants were discarded and pellets were resuspended in 500μL of 70% ethanol. The suspension was centrifuged again at 15,000 rpm, 4°C for 10 minutes. The supernatant was then discarded and DNA pellets were subsequently dried and resuspended in nuclease-free water. Optical densities ($OD_{260/280}$) of samples were subsequently quantified by UV spectrometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA) to determine concentration and purity. All work was performed within a UV irradiated BSL2 Tissue Culture cabinet, using DNASE and RNASE-free reagents. Filtered pipet tips were utilized to mitigate aerosolized contamination.

Real-Time PCR for EBV and CMV detection.

To determine which samples were either EBV[+] or CMV[+], we used Real Time-PCR and previously published primer sequences[23,24]. All reactions were run in a 96-well plate in an Applied Biosystems 7500 Thermocycler (Foster City, CA, USA) under the following conditions: 92°C for 5 minutes, followed by 40 cycles of amplification at 92°C for 15 seconds, 54°C for 30 seconds, and 72°C for 32 seconds, and then by a final extension at 72°C for 5 minutes. All reactions, including no-template controls, were performed in triplicate. Each RT-PCR reaction contained 2 μL of purified DNA (50 ng/μL), 6.5 μL of 2x iTaq SyBr Green iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA USA), 3.5 μL of nuclease-free water an 1.5 μL each of 10 nM forward and reverse primers: EBNA1 for EBV (Forward: 5'-TACAGGACCTGGAAATGGCC-3'; Reverse: 5'-TCTTTGAGGTCCACTGCCG-3'), UL55 for CMV (Forward: 5'-GCGGTGGTTGCCCAACAGGA-3'; Reverse: 5'-ACGACCCGTGGTCATCTTTA-3'), or human beta-actin (Forward: 5'-AGAGCTACGAGCTGCCTGAC-3'; Reverse: 5'-AGCACTGTGTTGGCGTACAG-3'). Semi-quantitative analysis performed to calculate Fold Change (over control), and positive viral identification was determined as greater than 0.5-fold gene activation over controls (DNA isolated from lysed A293T cells (ATCC)). All sample preparation was performed within a HEPA-Filtered BSL-2 cabinet, with all laboratory processes to mitigate laboratory contamination.

HPV Detection.

Positivity to high-risk HPV types (including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 59, 66, and 68) was determined using Aptima HPV mRNA Detection Assay according to the manufacturer's protocol (Hologic, Marlborough, MA, USA). Analyses were conducted

blinded of EBV, CMV and cytology results at Hologic Corporation testing laboratory, San Diego, California.

Cytology Assessment.

Cytology samples were assessed at UNC for cytological status according to Bethesda classification[25]. For the purpose of this study, an N=3 had a score of ASCUS and were scored as normal, compared histological assessment of LSIL (N=4) and HSIL (N=4). All samples were identified for HPV+ status, with one patient in the cohort associated with both HPV and ASCUS.

Study Design and Statistical Analysis

We conducted a cross-sectional study with selection on HPV expression status to evaluate the associations of interest. We first generated descriptive statistics to evaluate the distributions of cofactors overall and by viral exposure. We then explored the distributions of viral co-infection subgroups by cytology status. Next, the associations between EBV and HPV, as well as CMV and HPV, were determined using unadjusted and age-adjusted logistic regression to generate odds ratios (ORs) and 95% confidence intervals (95% CI). Age was dichotomized at < or 45 years. We similarly used logistic regression to determine whether EBV or CMV viral detection was associated with higher cytology disease grade, however the adjusted models included both age and HPV status, as the sample was selected according to HPV status. All analyses were performed using SAS 9.4 software (SAS, USA).

RESULTS

For this study, we examined 60 patient samples aquired from the MBMT-2 Cohort. Overall, 28% of the sample population were HPV-RNA positive, 38% were positive for CMV detection within samples, and 43% were positive for EBV detection within samples. Patient ages ranged from 32 to 64 years old (median: 46, interquartile range: 42–53.5) (Table 1). Within this cohort, 66% of the sample population reported being white, 28% as black, and 6% as other, and most patients (79%) had a high-school education or higher. Single viral detection was similar in percentages between race and education, with lower numbers HPV+ patients distributed simlarly (Table 1).

When examining co-detection status, 20% (n=12) carried both HPV and EBV, 12% (n=7) tested positive for both HPV and CMV, 28% (n=17) were positive for both CMV and EBV, and 10% (n=6) tested positive for HPV, EBV, and CMV (Table 2). Most of the sample population (87%, n=52) had a normal cytology result, while low-grade squamous intraepithelial lesions (LSIL, n=4) and 4 high-grade squamous intraepithelial lesions (HSIL, n=4) accounted for 6% each (Table 2). 3 samples were scored as AS-CUS (n=3), and were scored as" normal" for this study. When sample data were stratified by cytology grade, HPV positivity was 21% (11/52) among normal patients, 75% (3/4) of LSIL and 100% (4/4) of patients with HSIL. EBV positivity was detected in 37% (19/52) of normal patients, 75% (3/4) of low-grade squamous intraepithelial lesions (LSIL) and 100% (4/4) of patients with high-grade SIL (HSIL). In contrast, CMV positivity was detected in 35% (18/52) of normal patients, 25% (1/4) of patients with LSIL and 50% (2/4) of patients with HSIL. When

examining HPV-positive LSIL and HSIL, 100% (2/2 and 4/4, respectively) were positive for EBV detection, , and 0% (0/2) and 50% (2/4) were positive for CMV detection, respectively.

We found statistically significant unadjusted associations between cross-sectional EBV- and HPV positive status (OR: 5.0, 95% CI: 1.5, 16.9; Table 3) and between EBV and abnormal cytology (OR: 12.2, 95% CI: 1.4, 106.5). A statistically significant association between EBV and HPV positive status was sustained after adjustment, but was lost after adjusting for age and HPV status in the association between EBV and cytology (OR: 7.6, 95% CI: 0.8, 74.7; Table 3). There was no statistically significant association between cross-sectional CMV and HPV status, and between CMV and cytology.

Finally, HPV-positivity and EBV-positivity closely aligned with both disease severity and viral co-expression (Figure 1), though data were not statistically significant due to the relatively low numbers of LSIL and HSIL samples. This alignment in disease severity and viral co-detection did not exist for HPV-positive and CMV-positive patient samples.

DISCUSSION

Our findings suggest that co-detection with EBV, more so than CMV, may be associated with a greater risk of high-grade cervical lesions. Compared to CMV, EBV was more often detected in high-grade cervical precancer than normal or low-grade diagnoses in this sample of underscreened women in North Carolina. Though this study reveals a relatively low prevalence for cervical co-detection with these various viruses, results improve understanding of the burden of EBV and CMV detection in the cervix.

We observed a fairly high EBV prevalence in cervical exfoliated cell samples, which when combined with HPV positivity, resulted in higher cervical disease burden. EBV, a member of *Herpesviridae* family, is an understudied genital infection. Several studies have demonstrated a clear role for EBV in cancers such as nasopharyngeal carcinoma and Burkitt lymphoma, and possibly Hodgkin lymphoma and gastric carcinoma[9,11,12]. In contrast, the role of EBV as a marker of HPV-mediated cervical carcinogenesis remains relatively underexplored. EBV has been shown to be sexually transmitted, and replicates in cervical epithelial cells with eventual shedding[14,26]. However, some studies have shown that EBV does not reside in the cervical epithelium but rather within infiltrating lymphoid cells adjacent to the tumor epithelium, potentially influencing the microenvironment[27,28]. Although cellular localization of EBV remains contested, previous reports are in agreement that EBV can infect cervical tissues and potentially influence the risk of progression to high-grade cervical lesions and cervical cancer[15,16,29,30].

Our present findings confirm previous studies including a meta-analysis which found EBV infection was significantly and positively associated with lesion grade in cervical epithelia and was more prevalent in malignant lesions[14,18,24,31]. This meta-analysis also demonstrated an association with EBV proteins and integration of high risk-HPV DNA, though further studies were suggested to be performed before a firm link between EBV and cervical carcinoma can be established. Recent work following our cohort of Kenyan women also confirmed an association between EBV and a higher risk of high-grade abnormal cervical

cytology, particularly among HIV-infected women[32]. Another study conducted among women in a periurban community in India also found that women with CIN1 or greater were almost 4 times more likely to be EBV positive than women without disease[14]. Though EBV latency is common amongst the population, active viral shedding may be enhanced within CIN2 cervical lesions due to the localized inflammation and immune activation that can ensue. We believe that this present North Carolina study provides further evidence on a possible role of EBV as an etiological factor or marker of greater cervical disease risk.

Several studies have shown that CMV is shed from cervical tissue and correlates with greater disease severity[18,20,22]. CMV detection, in contrast, in our North Carolina study was not associated with higher grade of cervical disease. Our results are consistent with a study conducted among women in Andhra Pradesh, India, where a relatively high level of CMV viral load was not correlated with a higher grade cervical precancerous lesions[14]. In contrast, a study of Portugese women found that while CMV was present at low frequency in the sample population, CMV viral expression was associated with an increased risk of CIS (carcinoma *in situ*) or ICC (invasive cervical cancer) (22.2%), and of low-grade lesions (9.5%), as compared with normal cytology (4.5%)[16].

Within the MBMT2 cohort, a relatively small number of participants had abnormal cytology (4 LSIL, 4 HSIL), limiting the power for analyses of associations between cytology and viral co-detection. Further, this cohort of infrequently screened women is likely not adequately representative of the general population, as the number is ASCUS+ samples were quite small (n=3), with only one found to be HPV+. However, our study has a number of strengths. First, the MBMT2 cohort is comprised of low-income, underscreened or never screened women from North Carolina, who are at a higher risk of disease due to infrequent (or absent) screening histories. Additionally, this is the first U.S. study, to our knowledge, to quantify the prevalence of EBV and CMV viruses with onco-modulatory potential, in conjunction with HPV, the primary cause of cervical cancer, in relation to cytology among women at high risk of cervical cancer.

The recent advent and implementation of prophylactic HPV vaccines will have a dramatic effect on HPV-induced carcinogenesis in the future[33]. However, screening programs will be needed for several decades before the impact of HPV vaccination will be seen on the population-level, and screening-age eligible females will remain unvaccinated or age ineligible since only adolescent girls/young women are currently vaccination-eligible[8]. For cervical cancer screening programs, our data suggest that EBV may be a marker or etiological cofactor for high-grade cervical precancer risk, and that the triage of HPV-positive women to additional screening based on testing for EBV co-detection may have the potential to improve the specificity for the detection of CIN2+ lesions (Table 2). This would reduce the number of HPV-positive women referred to additional screening and thus reducing the burden on patients and on the healthcare system. Reducing follow-up screening is especially important for women with limited resources. At the same time, the question remains as to whether and how these viral expressions interact, and further studies will be required to better understanding HPV and EBV interactions. Importantly, these findings suggest a potential relevance of EBV viral detection as a potential triage method among HPV-positive women for colposcopy referral, particularly those who are medically

underserved. Future research with a larger number of high-grade cervical precancer cases is needed to determine whether the triage of HPV-positive women to additional screening based on testing for EBV co-detection will to improve the specificity of screening for CIN2+ detection.

Acknowledgements:

Abbreviations:

CIN	cervical intraepithelial neoplasia
HPV	human papillomavirus
EBV	Epstein-Barr virus
CMV	cytomegalovirus
PCR	polymerase chain reaction
MBMT	My Body My Test

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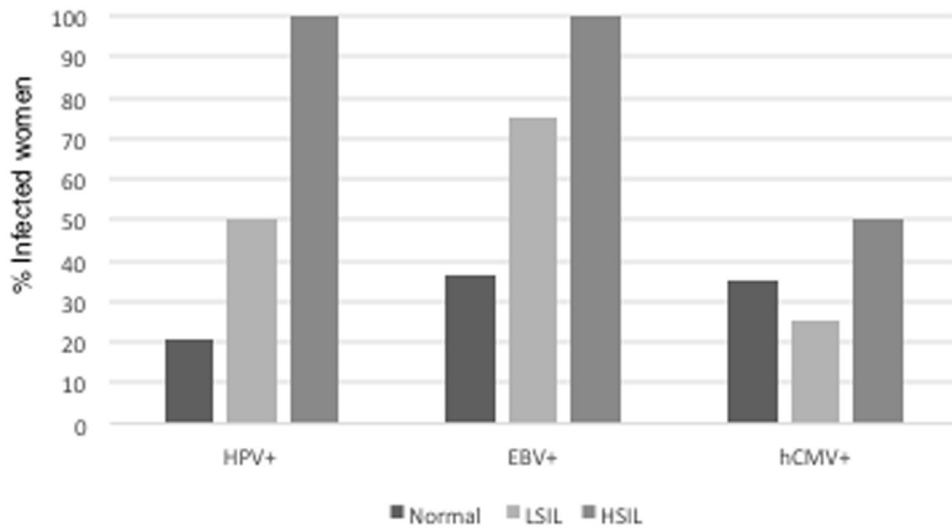
Figure 1.
Viral Prevalence, by Disease Grade Stratified

Table 1:

Socio-demographic and Viral Infection Status of 60 Low-income Underscreened Women Participating in the MBMT2 Study

	Total Sample N=60	HPV positive * n=17 (28%)	EBV positive * n=26 (43%)	CMV positive * n=23 (38%)
Age median (IQR)	46 (32–64)	43 (38–53)	46.5 (41–52)	43 (40–52)
Race				
White	40 (66%)	15 (88%)	18 (69%)	15 (65%)
Black	17 (28%)	1 (6%)	6 (23%)	6 (26%)
Other	3 (6%)	1 (6%)	2 (8%)	2 (9%)
High School/above [1]	27 (78%)	9 (53%)	15 (60%)	11 (50%)

HPV = human papillomavirus, CMV = human cytomegalovirus, EBV = Epstein-Barr virus, MBMT2=My Body My Test –2. Study, IQR=interquartile range *Other* includes Asian (n=1), or American Indian and "Mixed" (n=2).

*
categories are not mutually exclusive.

[1] from reported data, excluding unreported status.

Table 2:

Viral Detection of EBV and CMV, Stratified by Cytology Result and HPV-DNA Status

Cytology	HPV-/EBV-/CMV- n=24 (40%)	HPV-/EBV+/CMV- n=3 (5%)	HPV-/EBV-/CMV+ n=5 (8%)	HPV-/EBV+/CMV+ n=11 (18%)	HPV+/EBV-/CMV- n=4 (7%)	HPV+/EBV+/CMV- n=6 (10%)	HPV+/EBV-/CMV+ n=1 (2%)	HPV+/EBV+/CMV+ 6 (10%)
Normal n=52	23 (96%)	3 (100%)	5 (100%)	10 (91%)	4 (100%)	2 (33%)	1 (100%)	4 (67%)
LSIL n=4	1 (4%)	0 (0%)	0 (0%)	1 (10%)	0 (0%)	2 (33%)	0 (0%)	0 (0%)
HSIL n=4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (33%)	0 (0%)	2 (33%)

HPV= human papillomavirus, CMV= human cytomegalovirus, EBV= Epstein-Barr virus.

LSIL= low-grade squamous intraepithelial lesion (LSIL), HSIL= high-grade squamous intraepithelial lesion),

Table 3:

EBV and CMV as risk factors for HPV and HPV Associated-Outcomes

	EBV			CMV		
	N (% EBV+)	Unadjusted OR	Adjusted OR[1]	N (% CMV+)	Unadjusted OR	Adjusted OR[1]
Total HPV Negative	43 (32.6)	1 (ref)	1 (ref)	43 (37.2)	1 (ref)	1 (ref)
Total HPV Positive	17 (70.6)	5.0 (1.5, 16.9)	4.9 (1.4, 16.9)	17 (41.2)	1.2 (0.4, 3.7)	1.1 (0.3, 3.5)
Normal cytology	52 (36.5)	1 (ref)	1 (ref)	52 (38.5)	1 (ref)	1 (ref)
Abnormal cytology	8 (87.5)	12.2 (1.4, 106.5)	7.6 (0.8, 74.7)	8 (37.5)	1.0 (0.2, 4.5)	0.9 (0.2, 4.6)
Non-HSIL	56 (39.3)	1 (ref)	1 (ref)	56 (37.5)	1 (ref)	1 (ref)
HSIL	4 (100)	NA	NA	4 (50.0)	1.7 (0.2, 12.7)	1.3 (0.1, 13.9)

[1] Estimates are adjusted for age (<= 45 years versus > 45 years) and cytology models were also adjusted for HPV status

LSIL= low-grade squamous intraepithelial lesion or ASCUS, HSIL= high-grade squamous intraepithelial lesion, HPV= human papillomavirus, CMV= human cytomegalovirus, EBV= Epstein-Barr virus, ccOR= odds ratio, ref= reference. There are 0 observations for EBV-/HSIL, hence not applicable (NA).