Published in final edited form as:
J Clin Virol. 2011 May ; 51(1): . doi:10.1016/j.jcv.2011.01.011.

Kinetics of DNA load predict HPV 16 viral clearance

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Abstract

Introduction—While high HPV 16 viral load measured at a single time point is associated with cervical disease outcomes, few studies have assessed changes in HPV 16 viral load on viral clearance.

Objective—To measure the association between changes in HPV 16 viral load and viral clearance in a cohort of Thai women infected with HPV 16.

Study design—Fifty women (n = 50) between the ages of 18–35 years enrolled in a prospective cohort study were followed up every three months for two years. Women positive for HPV 16 DNA by multiplex TaqMan© assay at two or more study visits were selected for viral load quantitation using a type-specific TaqMan© based real-time PCR assay. The strength of the association of change in viral load between two visits and viral clearance at the subsequent visit was assessed using a GEE model for binary outcomes.

Results—At study entry, HPV 16 viral load did not vary by infection outcome. A >2 log decline in viral load across two study visits was found to be strongly associated with viral clearance (AOR: 5.5, 95% CI: 1.4–21.3). HPV 16 viral load measured at a single time point was not associated with viral clearance.

Conclusions—These results demonstrate that repeated measurement of HPV 16 viral load may be a useful predictor in determining the outcome of early endpoints of viral infection.

Keywords

HPV; DNA; Viral load; Epidemiology; Thailand

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5. Conflict of interest statement

SG – employee of Merck Research Laboratories who funded research; KLL – employee of Merck, Sharp and Dohme, which manufactures the quadrivalent HPV vaccine, owns Merck stocks and options; AT – employee of Merck.

1. Background and objectives

Human papillomavirus (HPV) infections of the female anogen-ital tract are the established cause of cervical cancer. Infection with HPV type 16 is responsible for >50% of cervical cancer cases worldwide.[1,2] Additionally, among HPV 16 infected women, elevated DNA viral load measured at a single time-point using either semi- or fully quantitative methods is positively associated with a cross-sectional diagnosis of cervical squamous intraepithelial lesions (SIL) or cervical intraepithelial neoplasia (CIN).[3–27] Higher HPV 16 viral load measured has also been shown prospectively to be associated with development of high-grade cervical pre-cancer (CIN 2+),[28] carcinoma in situ,[29–31] and cervical carcinoma.[32]

In a long term prospective study conducted among women in Colombia, an increased risk of viral clearance was observed in women with lower peak viral load over the course of an incident infection.[33] Risk of clearance by changes in viral load over time was not reported. The impact of viral load change has been assessed in only a few studies.[28,34] An investigation of a hospitalized population of HPV 16 positive, cytologically normal women demonstrated that increases in HPV 16 viral load measured at six month intervals were associated with progression to CIN2/3+, while women who remain cytologically normal were more likely to have decreasing viral load over time.[34] Changes in viral load and their associations with disease risk may have implications on understanding the complex interaction of HPV with the human host as well as potentially serving as an additional predictive marker for outcomes of infection.

Currently, there are no studies to our knowledge that have assessed changes in HPV 16 viral load on early endpoints of natural history such as viral clearance. We compared the association of HPV 16 viral load either at a single time point or repeatedly every three months for 2 years on viral clearance in a cohort of young women from Thailand.

2. Study design

Women attending family planning clinics throughout the Northern (Chiang Mai), Northeastern (Khon Kaen), Central (Bangkok) and Southern (HatYai) regions in Thailand between 2002 and 2003 were recruited into a prospective study to assess the natural history of HPV and CIN 2/3, and were between 18 and 35 years of age. These women were originally enrolled in a study designed to evaluate the effects of hormonal contraceptive use on HIV acquisition (HC-HIV). Selection criteria are described in detail elsewhere.[35] Inclusion criteria for enrollment in the HC-HIV study included: (1) HIV negative; (2) not pregnant; (3) intact uterus; (4) used some form of modern contraception within 3 months prior to enrollment; and (5) willing to adhere to self-selected contraceptive method for at least one consecutive year during a two year follow-up. At enrollment, women self-selected combined oral contraception (ethyl estradiol and levongestrel, COC), progesterone only contraception (depomedroxyprogesterone acetate, DMPA), or non-hormonal contraceptive methods (NHC) for at least 1 year. The study protocols were reviewed and approved by the committees on human subject research at Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, Merck Research Laboratories, West Point, PA, and the Institutional Review Board of the Thailand Ministry of Health (MOH), Thailand, and by the seven collaborating hospitals.

At enrollment and at each follow-up visit, information on sociodemographic characteristics, sexual risk behavior, partner sexual behavior, reproductive and contraceptive history, current contraceptive use status, and self-reported medical history were collected at each study site by trained interviewers using a standardized questionnaire (Fig. 1).

Endo/ecto cervical swab specimens were collected and stored in specimen transport medium at −20 °C until time of HPV 16 genotyping as well as gonorrhea (GC)/chlamydia (CT) detection. A Thin Prep Papanicolaou smear was performed at the enrollment, 12 month and 24 month study visits and was classified as normal, inflammation, atypical squamous cells of unknown significance (ASC-US), low grade squamous intraepithelial lesions (LSIL), or high grade intraepithelial lesions (HSIL). Repeat Pap smears were performed after six months for women with abnormal cytology >ASC-US (Fig. 1). Women with an abnormal cytological diagnosis were referred for colposcopy to confirm the presence of pre-cancerous lesions. Women with a diagnosis of cervical intraepithelial neoplasia 2+ (CIN 2+) were referred for treatment.

At the end of follow-up of this initial study, women were invited to enroll into an observational study for an additional 18-months of follow-up with 6 month sampling. At the enrollment visit in this follow-up study all women received a Pap smear and were referred for colposcopy and treatment as stated above. Thirty-six women (72%) included in this analysis were enrolled in this follow-up study with an average total follow-up of 17.6 months (SD: 2.3).

2.1. HPV 16 DNA detection and viral load quantification

DNA was extracted from cervical swab specimens (Digene© standard transport medium) using the QiaAmp Blood kit (Qiagen, Courtaboeuf, France). Real-time PCR was used to detect the E6, E7 and L1 genes of the HPV 16 genome.[36,37] Specimens were considered positive for HPV 16 if two out of three genes measured were detected, or if the same single gene was detected twice upon repeat testing.

Viral load measurements were performed on all samples determined to be HPV 16 positive by a type-specific TaqMan based real-time PCR assay targeting the E7 ORF of the HPV 16 genome.[38] The assay used in this analysis was shown to have a high level of agreement with another previously described, HPV 16 quantitative viral load assay that targets the E6 ORF.[38] To control for sampling heterogeneity, viral load measures were normalized to 10^4 human cells through quantification of the human β-globin gene.

2.2. Statistical analysis

Women who were HPV 16 DNA positive at enrollment (prevalent infections) or became detectable during follow-up (incident infections) and had at least two subsequent study visits were included in the analysis. The primary outcome of this analysis is the loss of detectability of HPV 16 DNA (i.e., clearance) over two consecutive visits. Intercurrent negative visits (a single visit of HPV 16 DNA negativity flanked by two positive visits) were treated as visits in which HPV 16 DNA load has fallen below the limit of detection for the assay and assigned a value of 1.6 copies/10^4 CE, the lower limit of detection of the RT-PCR assay. HPV 16 viral load was assessed as either (1) a single absolute value at the visit of first detectability of HPV 16 DNA (i.e., study entry or first visit with incident HPV16 infection), or (2) the relative change (i.e., log-fold change) over two consecutive visits (visit-pairs), or (3) the first visit of a given visit pair (i.e., baseline visit) immediately prior to infection outcome assessment. Viral load measures were normalized using log transformation. The absolute measure of viral load at the baseline and index visit was analyzed as a continuous value or categorized as <2000 vs. ≥2000 copies/10^4 cells equating to ~1 pg/ml HPV 16 DNA as previously described.[34]

Contingency tables comparing covariates across infection outcome status were evaluated and the Chi-squared test was used to determine statistical significance. Wilcoxon ranksum test was used to assess differences in median values of viral load across infection outcome

status and covariates. Logistic regression using the generalized estimating equation (GEE) approach was used to estimate adjusted odds ratios (AOR) to assess the strength of the association of HPV 16 viral load measures and infection outcome. A p-value of <0.05 was considered statistically significant.

3. Results

This analysis included 50 women with either incident (n = 16) or prevalent (n = 34) HPV 16 infections detectable over at least two study visits. These women contributed a total of 303 person visits (mean/participant: 6 (SD: 3)) and 253 visit-pairs (mean/participant: 5 (SD: 3.1)) for HPV 16 viral load measures, equating to a total 767 months of follow-up (mean: 15 months (SD: 7.8)). The average time between visits was 2.6 months (SD: 0.9). Twenty-one (n = 21) infections cleared during follow-up with an average duration of detection of 11 months (SD: 5.5).

At enrollment the mean age of the sample was 26.8 years (SD: 4.6) and the majority were cytologically normal with only 4 (8%) having an enrollment pap diagnosis of ASC-US (Table 1). Women who reported use of COC were less likely to clear their infection as compared to non-hormonal contraceptive users and DMPA users (p = 0.024). Conversely, women who reported use of DMPA for >4 years prior to enrollment were more likely to clear their infections as compared to women who reported use of <1 year (p = 0.036).

The median change in viral load between two consecutive visit pairs was −6.9 copies/10,000 cells (IQR: −4672.4, 615.9) (Table 2). Among visit-pairs preceding a cleared vs. a persistent infection, the median change was −612.2 copies/10,000 cells (IQR: −7679.2, 0.07) vs. 18.2 copies/10,000 cells (IQR: −3668.9, 5660.3), respectively (p < 0.05). A ≥2 log fold decline in HPV 16 viral load was found to be significantly associated with viral clearance (Table 3 and Fig. 2) which remained significant after controlling for age, cytological diagnosis at enrollment, contraceptive group, concurrent GC/CT infection and infection type. Conversely, increasing viral loads of ≥2 log fold across a given study visit pair was observed to be non-significantly protective against clearance (i.e., associated with an increased risk of viral persistence; p = 0.291).

A second phase of the initial prospective study was initiated within six to twelve months after the final 24-month study visit for longer term assessment of cervical disease outcomes. A total of 36 out of 50 women with an HPV16 positive test result at the final HC-HIV visit (72%) were re-consented and enrolled into this study (mean time between last virologic measure and second phase study enrollment was 10 months, SD: 7.5) (Table 4). All women who re-enrolled into the extended follow-up were given a Pap smear at the baseline re-enrollment visit. Among these women, 25 (69%) remained detectable for HPV 16 at the enrollment visit of the follow-up study. Additionally, 24 (66.7%) women with follow-up were cytologically normal or had inflammation, 4 (11.1%) were diagnosed with AS-CUS, 3 (8.3%) LSIL, and 5 (13.9%) HSIL. Women with HSIL as compared to women remaining cytologically normal had a higher median HPV 16 viral load at the visit of first detection (3250.8 copies/10^4 cells vs. 87.5 copies/10^4 cells, p = 0.564) but this difference did not reach statistical significance.

4. Discussion

This study demonstrates that in a cohort of women aged 18–35 years from Thailand, a >2 log fold decline in HPV 16 viral load across two consecutive visits is predictive of viral clearance. While a previous study among women in Colombia observed that high HPV viral load was associated with an increased risk of viral clearance,[33] their analysis was based on peak viral loads over the course of incident infection. In our analysis, a higher peak viral

load was non-significantly associated with a reduced risk of viral clearance (OR: 0.29, 95% CI [0.7, 1.13] for a 1 log increase in peak viral load). Replication of this effect is retrospectively possible for epidemiological analysis. However, our observation that the magnitude of change between 2 viral load measures predicts viral clearance offers potential utility of viral load measures in real time. For example, one study of hospitalized women from France did evaluate the impact of viral load changes on disease outcomes. Consistent with our results, they found that declines in HPV 16 viral load were associated with the maintenance of normal cytological outcomes.[3]

The association between declining HPV 16 viral load and HPV 16 clearance may reflect recognition of viral infection by the host immune response. Clearance of HPV is facilitated by a robust CD8+ cytotoxic T-cell response[39] and is characterized by increases in immunologic markers such as IFN-γ and IL-2 in both the periphery and cervical immune environments.[40–42] If it can be confirmed that decreasing viral load correlates with cellular immune responses, serial viral load measurements may have potential utility as early biomarkers of effect in the evaluation of response to therapeutic interventions on HPV infection.

This study has several strengths. The high density of sampling of study participants allows for more precise evaluation of the kinetics of HPV viral load. Second, this study utilized a highly sensitive and specific real-time PCR assay for viral load quantitation normalized to β-globin to minimize differences due to sampling heterogeneity.

This study has some limitations. First, we did not detect and measure viral load of other oncogenic and non-oncogenic HPV types that may be co-infecting women along with HPV 16. Second, the high sampling density in this study made the presence of intercurrent negative visits fairly common with eleven women (22%) having a single HPV 16 DNA negative visit flanked by two positive visits. This could have been the result of laboratory error leading to false negativity or true loss of detection either through suppression of viral replication or complete viral clearance by the host. Women with intercurrent negative visits did not significantly differ by virological (i.e., clearance) and clinical outcomes (i.e., pap status) or demographic factors (i.e., age, study site, hormonal contraceptive group) from those with no intercurrent negative results, and the significance and direction of the association of viral clearance with viral load were not altered after intercurrent negative visits were excluded from the analysis (data not shown). In addition, the sample size was too small to examine the viral load effects among prevalent and incidentally detected infections. The women enrolled in this study were part of a trial assessing the effects of hormonal contraceptive use on HIV acquisition which may limit the generalizablility of study findings to other women in Thailand or other settings worldwide.

Application of HPV 16 viral load as a surrogate endpoint may be valuable in evaluating immunologic response to infection, which may especially have utility in immunotherapeutic studies where it is not possible to wait for lesion regression. Studies are ongoing to determine if viral load changes are correlated with a cellular immune response. If this can be demonstrated, viral load declines in response to therapy may provide an ethically acceptable alternative endpoint for decisions to pursue further clinical trials.

Acknowledgments

This work was funded in part by the NIAID pre-doctoral training fellowship in sexually transmitted infections (5T32AI050056-09). This study was funded by Merck & Co. Inc.

Abbreviations

HPV	human papillomavirus
HR-HPV	high risk human papillomavirus
COC	combined oral contraception
DMPA	depomedroxyprogesterone acetate
GEE	generalized estimating equation

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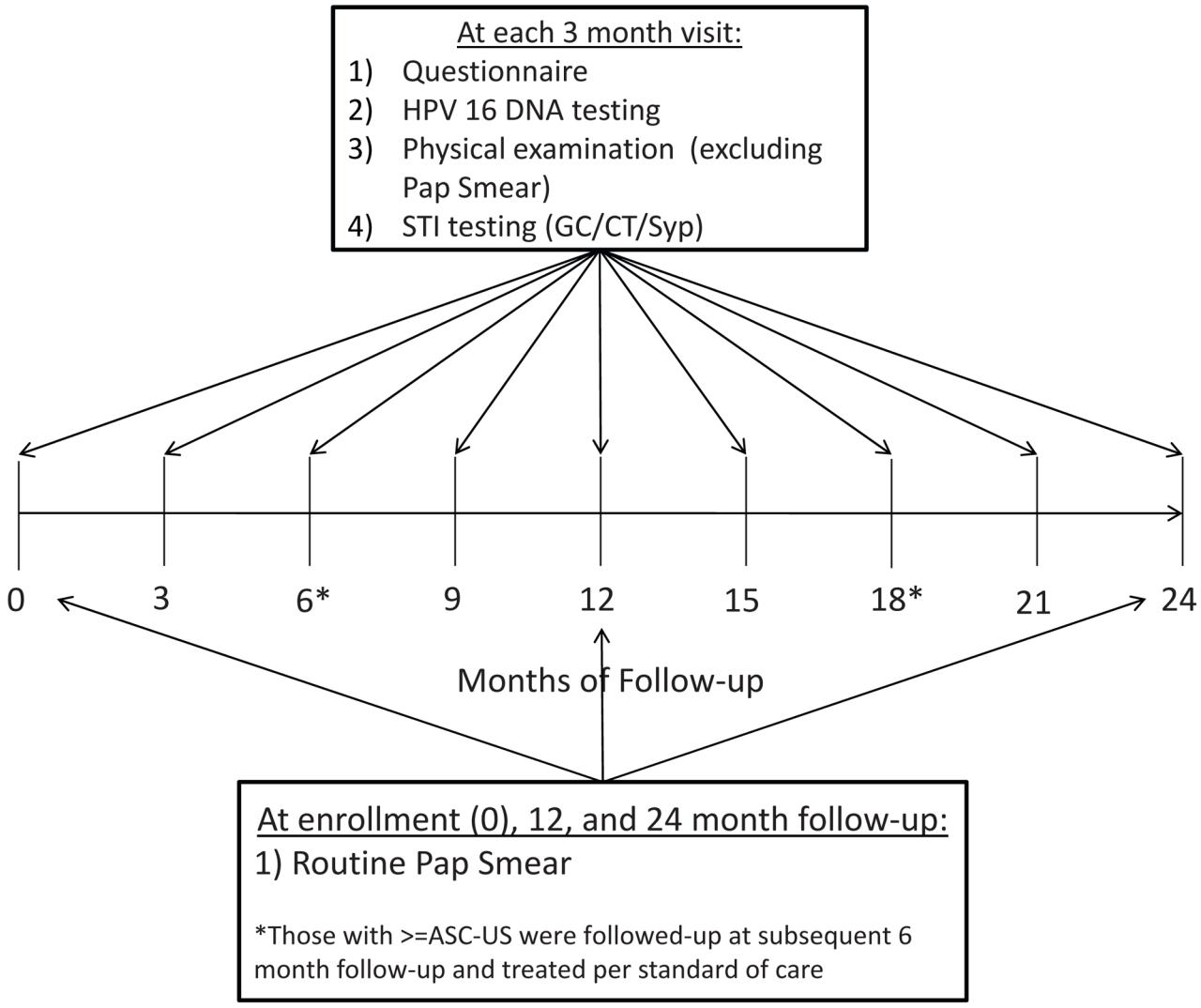

Fig. 1.
Schematic of the timing of data collection including Pap smears in the HC-HIV study.

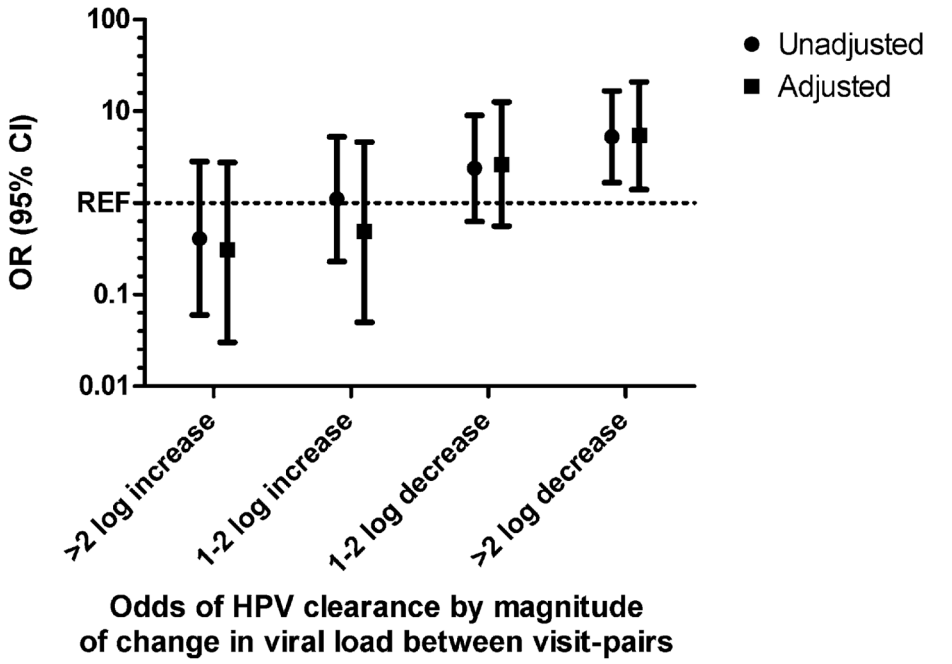

Fig. 2.

The association of change in viral load and risk of HPV 16 viral clearance. Unadjusted and adjusted odds ratios (ORs) are presented. The reference group is a change in HPV 16 viral load <1.0 log in either direction between visits. OR > 1.0 denotes an increased risk of clearance; OR < 1.0 denotes a reduced risk of clearance. ORs were adjusted for age at enrollment, study site, contraceptive use and STI diagnosis at follow-up, and infection type. REF= <1.0 log-fold increase or decrease in viral load.

Table 1

Distribution of the demographic, reproductive, clinical, and STI factors of women with viral load measures at study enrollment.

Variable	Sample (*N* = 50)	Cleared (*n* = 21) *n* (%)	Persisted (*n* = 29) *n* (%)	p-value
Mean age (SD)	26.8 (4.6)	37 (3.8)	26.7 (5.2)	$p = 0.75$
Study site				
N (Chiang-Mai)	13	2 (15.4)	11 (84.6)	
NE (Khon Kaen)	9	6 (66.7)	3 (33.3)	
S (Songhkla-Hat Yai)	14	5 (35.7)	9 (64.3)	
C (Bangkok)	14	8 (57.1)	6 (42.9)	$p = 0.056$
Current contraceptive use				
NHC	13	9 (69.2)	4 (30.8)	
COC	22	5 (22.7)	17 (77.3)	
DMPA	15	7 (46.7)	8 (53.3)	$p = 0.024$
Duration of COC use[a]				
<1 year	4	1 (25)	3 (75)	
4–5 years	8	3 (37.5)	5 (62.5)	
>5 years	10	1 (10)	9 (90)	$p = 0.381$
Duration of DMPA use[a]				
<1 year	5	0 (0)	5 (100)	
4–5 years	6	4 (66.7)	2 (33.3)	
>5 years	4	3 (75)	1 (25)	$p = 0.036$
Lifetime number of partners				
1	24	13 (54.2)	11 (45.8)	
2	12	6 (50)	6 (50)	
3	2	1 (50)	1 (50)	
≥4	12	9 (75)	3 (25)	$p = 0.586$
Number of recent sexual partners[b]				
1	38	21 (55.3)	17 (44.7)	
>1	12	8 (66.7)	4 (33.3)	$p = 0.485$
Cytological diagnosis[c]				
Normal	28	14 (50)	14 (50)	
Inflammation	18	6 (33.3)	12(66.7)	
AS-CUS	4	1 (25)	3 (75)	
≥LSIL	0	0 (0)	0 (0)	$p = 0.414$
Worst pap diagnosis during follow-up				
Normal	17	10 (58.8)	7 (41.2)	
Inflammation	26	9 (34)	17 (65.4)	
AS-CUS	6	2 (33)	4 (66.7)	
LSIL	0	0 (0)	0 (0)	
HSIL	1	0 (0)	1 (100)	$p = 0.529$
STI infection status				

Variable	Sample (N = 50)	Cleared (n = 21) n (%)	Persisted (n = 29) n (%)	p-value
Gonorrhea	1	1 (100)	0 (0)	$p = 0.235$
Chlamydia	9	7 (77.8)	2 (22.2)	$p = 0.735$
HPV 16 infection type				
Prevalent	34	15 (44.1)	19 (55.9)	
Incident	16	6 (37.5)	10 (62.5)	$p = 0.574$

[a] Among COC/DMPA users.

[b] In the last 3 months.

[c] At study enrollment.

Table 2

Differences in median HPV 16 viral load measured at study entry and baseline visit across infection outcome.

Viral load measure	Sample (*N* = 50)	Cleared (*n* = 21)	Persistent (*n* = 29)	*p*-Value
Median at study entry (IQR)[a]	262.2 (15.9, 5414.6)	576.8 (9.9, 18,465.7)	175.4 (39.5, 2,056.6)	$p = 0.78$
≤2000 copies[b]	33	12 (36.4)	21 (63.6)	
>2000 copies	17	9 (52.9)	8 (47.1)	$p = 0.31$
Median at baseline visit (IQR)[a]	1757 (53.4, 14,832.7)	954.2 (36, 24,843)	2135.3 (65.5, 13,971)	$p = 0.56$
≤2000 copies[b]	26	12 (46.2)	14 (53.8)	
>2000 copies	24	9 (37.5)	15 (62.5)	$p = 0.66$

[a] Study entry, visit of first detection of HPV 16; baseline visit, first visit of a visit-pair.

[b] Per 10^4 human cells.

Table 3

Association of viral load measures on HPV 16 viral clearance.

Variable	Unadjusted (OR (95%CI))	Adjusted[a] (AOR (95% CI))
Higher study entry viral load[a]	1.00 (0.88, 1.15)	1.00 (0.84, 1.19)
Index visit viral load		
≤2000 copies/10^4 cells	1.0	1.0
>2000 copies/10^4 cells	1.59 (0.61, 4.1)	1.29 (0.39, 4.22)
Higher baseline visit viral load	0.99 (0.87, 1.1)	0.87 (0.70, 1.1)
Baseline visit viral load		
≤2000 copies/10^4 cells	1.0	1.0
>2000 copies/10^4 cells	0.95 (0.4, 2.3)	0.42 (0.12, 1.4)
Viral load change across visit-pair		
Increase	1.0	1.0
Decrease	3.16 (1.31, 7.65)	5.62 (1.75, 17.9)
>2 log increase	0.41 (0.06,2.83)	0.31 (0.03, 2.77)
1–2 log increase	1.11 (0.23,5.26)	0.49 (0.05, 4.62)
≤1 log change (+/−)	1.0	1.0
1–2 log decrease	2.39 (0.63, 9.02)	2.61 (0.56, 12.6)
>2 log decrease	5.26 (1.66, 16.61)	5.49 (1.40, 20.9)

[a]Adjusted for age, study site, contraceptive use, STI diagnosis during follow-up, cytology during follow-up, infection type.

Table 4

Differences in HPV 16 viral load assessed at the visit of first detection (study entry) by cytological outcomes at six-month post-study follow-up.

Cytological diagnosis at follow-up:	*N*	Median HPV 16 VL (IQR)	*p*-Value
Normal/inflammation	24	87.5 (14.9, 1,749.2)	
AS-CUS	4	107.9 (22.9, 426.9)	
LSIL	3	144.1 (0.43,134.2)	
HSIL	5	3,250 (235.2, 11,156.6)	$p = 0.564$