Is Human Papillomavirus Viral Load a Clinically Useful Predictive Marker? A Longitudinal Study

Christothea Constandinou-Williams¹, Stuart I. Collins², Sally Roberts¹, Lawrence S. Young¹, Ciaran B.J. Woodman¹, and Paul G. Murray¹

Abstract

Background: It has been suggested that in women who test positive for high-risk human papillomavirus (HPV) types, viral load can distinguish women who are at increased risk of cervical neoplasia from those who are not.

Methods: Quantitative PCR (qPCR) was used to measure HPV copy number in serial samples taken from 60 and 58 young women previously found to have incident cervical HPV16 or HPV18 infections, respectively, using GP5+/GP6+ primers; women provided at least three samples for qPCR testing, at least one of which was positive.

Results: A 10-fold increase in HPV16 or HPV18 copy number was associated with a modestly increased risk of acquiring a cytologic abnormality [HPV16: hazards ratio, 1.76 (95% confidence interval, 1.38-2.25); HPV18: hazards ratio, 1.59 (95% confidence interval, 1.25-2.03)]. However, in most women, copy number increased during follow-up, before decreasing again. In women with a HPV16 infection, the median copy number per 1,000 cells was 7.7 in their first qPCR HPV-positive sample, 1,237 in the sample yielding the maximum copy number, and 7.8 in their last qPCR HPV-positive sample; corresponding copy numbers for women with HPV18 infection were 2.3, 87, and 2.4. Maximum HPV16 and HPV18 copy number did not differ significantly between women who acquired an incident cervical cytologic abnormality and those who did not.

Conclusion: Whereas large relative increases in copy number are associated with an increased risk of abnormality, a single measurement of viral load made at an indeterminate point during the natural history of HPV infection does not reliably predict the risk of acquiring cervical neoplasia. Therefore, a single measure of HPV viral load cannot be considered a clinically useful biomarker. Cancer Epidemiol Biomarkers Prev; 19(3); 832–7. ©2010 AACR.

Background

The identification of high-risk human papillomavirus (HPV) types as a necessary cause of cervical cancer offers not only the prospect of effective primary prevention but also the possibility that testing for the presence of high-risk HPV types could improve the efficiency of cervical screening programs. However, it is now clear that there is a compelling need for additional biomarkers that allow us to distinguish between those women who test positive for high-risk HPV and are likely to acquire cervical neoplasia and those women who test positive for high-risk HPV and are at no increased risk (1). HPV viral load is an attractive candidate. Among women who test positive for high-risk HPV types, cytologic abnormality is consistently found to be more common in those with a high viral load than in those with a low viral load (reviewed in ref. 2). However, it is now clear that the relationship

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Note: Supplementary data for this article are available at Cancer Epidemiology Biomarkers and Prevention Online (http://cebp.aacrjournals.org/).

C. Constandinou-Williams and S.I. Collins contributed equally to this work. C. Constandinou-Williams designed the study, analyzed and interpreted the data, drafted the article, revised the article critically for important intellectual content, and approved the final version to be published. S.I. Collins designed the study, interpreted the data, drafted the article, revised the article critically for important intellectual content, and approved the final version to be published. L.S. Young conceived the study, interpreted the data, revised the article critically for important intellectual content, and approved the final version to be published. P.G. Murray designed the study, analyzed and interpreted the data, drafted the article, revised the article critically for important intellectual content, and approved the final version to be published.

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between the viral load of cervical HPV infections and cervical neoplasia is more complex than was previously thought. When viral load is measured using real-time quantitative PCR (qPCR) and normalized for specimen cellularity, longitudinal studies fail to reveal a consistent association between the viral load of cervical HPV infections and the risk of acquiring an epithelial abnormality of the cervix (1-4). Program trials have concluded that a measurement of viral load provides little or no additional useful clinical information beyond that provided by cytologic examination or testing for the presence of high-risk HPV (1-5). However, in almost all of these studies, viral load is measured at a single point in time. The effect of changes in viral load after this time on the risk of acquiring cervical neoplasia remains undefined. We have addressed this issue by measuring the viral load of HPV16 and HPV18, the types most frequently detected in cervical cancers, in serial samples taken during the follow-up of a cohort of young women who were recruited soon after they first had sexual intercourse.

**Materials and Methods**

**Study Population**

As previously described, a cohort of 2,011 women ages 15 to 19 y, who visited one Birmingham Brook Advisory Centre (a family planning clinic) in Birmingham, United Kingdom, were recruited between 1988 and 1992 and asked to re-attend at intervals of 6 mo; follow-up ended on August 31, 1997 (6). At each visit, one cervical sample was taken for cytologic examination, following which a second sample was taken and stored for subsequent virologic examination. All women in whom a cytologic abnormality was identified were immediately referred to a dedicated research clinic, irrespective of the severity of that abnormality. In this clinic, a sample of any colposcopically abnormal epithelium was removed for histologic examination. Colposcopic and cytologic surveillance was maintained in these women; treatment was postponed until there was histologic evidence of high-grade CIN (CIN2 or CIN3), at which point women left the study. After all clinical follow-up had ended, cervical samples were tested for the presence of HPV DNA using a general primer (GP5+/GP6+)-mediated PCR, and further PCR tests were done with type-specific primers on samples that were HPV positive (6). Where appropriate, to avoid confusion, we subsequently refer to this testing strategy as the “GP5+/GP6+ system.” A 2-μL aliquot was taken from the stored sample and DNA was extracted using guanidinium thiocyanate acid; 100 ng of sample DNA were then used in a 50-μL PCR reaction, as previously described (7). The study was approved by the appropriate ethical committee and informed oral consent was obtained from all women. The study population for this analysis comprises the subset of all women who were cytologically normal and HPV negative at study entry and who first tested positive during follow-up for HPV16 or HPV18, or both, using the GP5+/GP6+ system.

**Sample Preparation and Measurement of Viral Load**

For this analysis, DNA was isolated from study samples using proteinase K digestion and phenol/chloroform extraction, according to methods previously described (7). HPV viral load was measured using a modified singleplex qPCR assay (ABI 7700 Applied Biosystems). In brief, sequence-specific primers and fluorescein-labeled probes (Eurofins MWG Operon) were designed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HPV16 E6, and HPV18 E7 (Supplementary Table S1). Genomic DNA (50 ng) and standards (10-fold plasmid dilutions between 10⁸ and 10² copies of GAPDH, HPV16, and HPV18) were amplified using TaqMan Universal PCR Master Mix (Applied Biosystems) with 0.4 μmol/L of appropriate primer mix. Amplifications were done using the ABI 7700 sequence detection system; the cycle conditions for GAPDH, HPV16 E6, and HPV18 E7 were 50°C for 2 min, 95°C for 12 min, followed by 50 cycles of 95°C for 15 s and 55°C for 30 s. The HPV16-positive cervical carcinoma cell line SiHa and the HPV18-positive cell line HeLa were used as positive controls. Standard curves using HPV16 or HPV18 plasmids and GAPDH plasmids were used to generate measurements of viral load normalized for cellular DNA content.

**Statistical Analysis**

Analyses of the association between viral load and the acquisition of cervical cytologic abnormality were undertaken using methods appropriate for interval-censored time-to-event data. Time to acquisition of cytologic abnormality was measured from the date of the first qPCR-evaluable sample until the interval between the date of the first detection of cytologic abnormality and the date of the immediately preceding cytologically normal smear; censoring occurred on the earliest of the date of the last qPCR-evaluable sample or the date of the last smear. Estimates of hazards ratios (HR) were obtained using a semiparametric method for modeling interval-censored time-to-event data as a generalized linear model (8), with the logarithm-to-base 10 of the maximum viral load observed prior to and including a given point in time treated as a time-varying covariate (note that because the logarithm-to-base 10 of this estimate of viral load was entered into the model as a continuous variable, a change in value of 1 corresponds to a 10-fold increase in viral load). 95% confidence intervals (95% CI) were constructed from parameter estimates and their SEs. Tests of hypotheses were undertaken using likelihood ratio tests. All tests of statistical significance were conducted at the 5% two-sided significance level.

**Descriptive Analysis of Changes over Time in Viral Load**

Modeling viral load kinetics proved impossible because of the statistically intractable nature of the data.
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Measurement of Viral Load as a Time-Varying Covariate

Each woman had repeated measurements of viral load made at intervals of approximately 6 mo. For illustrative purposes only, these repeat measurements can be considered as a series of consecutive pairs of visits. For any given pair of consecutive visits, there are two viral load measurements available: that taken in the baseline sample (the first sample of the pair) and that taken in the follow-up sample (the second sample). The current viral load for a woman will be that measured in the second sample of the relevant pair; viral load status “lagged” by one measurement will be that measured in the first sample of the relevant pair. The sample containing the first detection of cytologic abnormality will always be the last follow-up sample for a woman (because observations are terminated at this point). Therefore, when analyzing the association between viral load and the acquisition of cytologic abnormality, an analysis based on the first sample is preferred to that based on the second sample because the former allows us to describe more precisely the temporal relationship between the detection of HPV DNA and the first acquisition of cytologic abnormality. In this article, we analyzed the association between the maximum viral load observed before a given point in time and the risk of acquiring an incident cervical cytologic abnormality. This was a longitudinal analysis in which different women were compared against each other, rather than making within-woman comparisons.

Note that in this case, although HPV viral load is measured as a time-varying covariate, only a single measurement of viral load is used as a risk factor. For example, consider two women, woman A and woman B, who are both cytologically normal in their cervical sample taken at study entry. Suppose that these women both made visits at time 0 (study entry) and then at 6, 12, and 18 mo after study entry (this is an artificial example because visits were made in continuous time, and thus it is highly unlikely that two women would have exactly the same visit pattern despite the study design). Suppose further that for both women, the first detection of a cervical cytologic abnormality occurred at the 18 mo visit. For woman A, suppose that the observed measurements of HPV16 viral load were 0, 10,202, 8,529, and 568 copies per 1,000 cells at times 0, 6, 12, and 18 mo, respectively (i.e., an increase in the magnitude of HPV16 viral load over time, followed by a decrease). For woman B, suppose that the observed measurements of HPV16 viral load were 0, 435, 1,095, and 1,872 copies per 1,000 cells at times 0, 6, 12, and 18 mo, respectively (i.e., an increasing HPV16 viral load over time for the first 18 mo of follow-up). Examination of Supplementary Fig. S1 reveals that study numbers 69 (for HPV16) and 240 (HPV16 and HPV18) have similar patterns in viral load of the relevant type(s) over varying periods of time for woman A. A similar pattern is seen for study numbers 147 (HPV16) and 1367 (HPV18) for woman B. When the first detection of a cervical cytologic abnormality occurs at the 18 mo visit, the maximum viral load observed before and including that point in time (18 mo) is 10,202 copies per 1,000 cells for woman A and 1,095 copies per 1,000 cells for woman B (compared with woman B, woman A has a 10,202/1,095 = 9.3-fold greater HPV16 viral load).

Results

Before investigating the relationship between viral load and the risk of acquiring an incident cervical cytologic abnormality, we first describe how cervical HPV viral load changes over time in women infected with HPV16 or HPV18 (illustrative examples are provided in Supplementary Fig. S1).

Viral Load Waxes and Wanes during Follow-up

For this analysis, the study population was restricted to 60 and 58 women who had had an incident HPV16 or HPV18 infection, respectively, detected using the GP5+/GP6+ system, and who provided at least three evaluable samples for qPCR testing during follow-up, at least one of which was positive.

Sixty women were tested for HPV16 using qPCR in three or more samples: 41 tested positive for HPV16 in three or more samples; 10 in two; and 9 in one. Fifty-eight women were tested for HPV18 using qPCR in three or more samples: 39 tested positive for HPV18 in three or more samples; 5 in two; and 14 in one. In 60 women with a HPV16 infection, the median copy number (viral load) per 1,000 cells was 7.7 in their first qPCR HPV-positive sample, 1,237 in the sample yielding the maximum viral load, and 7.8 in their last HPV-positive sample; the corresponding copy numbers for the 58 women with HPV18 infection were 2.3, 87, and 2.4 per 1,000 cells.

Viral load seemed to wax and wane during follow-up. Supplementary Tables S2 and S3 present the viral loads of consecutive samples tested for HPV16 and HPV18 by qPCR, respectively. The maximum viral load observed during follow-up was greater than that detected in the first qPCR-positive sample in 43 women with a HPV16 infection and in 35 women with a HPV18 infection, and was greater than that detected in the last qPCR-positive sample in 37 of these women with a HPV16 infection and in 32 of those with a HPV18 infection (Table 1). A similar trend was seen when the median viral load for the first and last qPCR-positive samples and that for the sample containing the maximum viral load were compared.
### Table 1. Changes in HPV viral load in cervical samples taken during follow-up according to HPV type

<table>
<thead>
<tr>
<th>HPV type</th>
<th>No. of women in the analysis</th>
<th>No. of samples in the analysis</th>
<th>Maximum viral load compared with viral load in the first qPCR-positive sample</th>
<th>Viral load in the last qPCR-positive sample compared with that in the sample containing the maximum viral load</th>
<th>No. of women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Median (IQR) viral load* in the first qPCR-positive sample</td>
<td>Median (IQR) viral load* in the sample containing the maximum viral load</td>
<td>Median (IQR) viral load* in the last qPCR-positive sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>in the sample containing the maximum viral load</td>
<td>Same as the maximum viral load</td>
<td>Same as the maximum viral load</td>
</tr>
<tr>
<td>HPV16</td>
<td>60</td>
<td>429</td>
<td>7 (1-72)</td>
<td>25,559 (748-337,398)</td>
<td>Increased 25,559 (748-337,398)</td>
</tr>
<tr>
<td></td>
<td>4 (1-33)</td>
<td>1,310 (279-69,135)</td>
<td>Increased 1,310 (279-69,135)</td>
<td>1,310 (279,1-69,135)</td>
<td>Same 6</td>
</tr>
<tr>
<td></td>
<td>371 (22-5,924)</td>
<td>371 (22-5,924)</td>
<td>Same 371 (22-5,924)</td>
<td>1,351 (0.2-24,536)</td>
<td>Decreased 1,351 (0.2-24,536)</td>
</tr>
<tr>
<td>HPV18</td>
<td>58</td>
<td>386</td>
<td>2 (1-34)</td>
<td>758 (42-11,749)</td>
<td>Increased 758 (42-11,749)</td>
</tr>
<tr>
<td></td>
<td>0.2 (0.009-3)</td>
<td>1,351 (0.2-24,536)</td>
<td>Increased 1,351 (0.2-24,536)</td>
<td>1,351 (0.2-24,536)</td>
<td>Same 8</td>
</tr>
<tr>
<td></td>
<td>659 (11-28,588)</td>
<td>659 (11-28,588)</td>
<td>Same 659 (11-28,588)</td>
<td>1,351 (0.2-24,536)</td>
<td>Decreased 1,351 (0.2-24,536)</td>
</tr>
<tr>
<td></td>
<td>1 (0.05-9)</td>
<td>1 (0.05-9)</td>
<td>Same 1 (0.05-9)</td>
<td>1 (0.05-9)</td>
<td>Same 3</td>
</tr>
</tbody>
</table>

**NOTE:** The study population was restricted to women who provided at least three evaluable cervical samples for qPCR testing for HPV16 or HPV18, or both, during follow-up, at least one of which was qPCR positive. For ease of presentation, as few decimal places as possible were used to complete the table. Abbreviation: IQR, interquartile range.

*Copy number per 1,000 cells.
was diagnosed. Our use of serial sampling allowed us to
was observed in the period before cytologic abnormality
the change in viral load contributing to this increased risk
both, during follow-up. It is important to emphasize that
women with cervical HPV16 or HPV18 infections, or
acquiring an incident cervical cytologic abnormality in
load is associated with a significantly increased risk of
modeling as a log10-transformed continuous covariate,
\( P = 0.42 \) for HPV18). In contrast, when viral load was
controlling for whether or not a woman had ever tested
acquired an incident cytologic abnormality and those
who did not (Wilcoxon rank-sum test with continuity
correction: \( W = 426, P = 0.60 \) for HPV16 and \( W = 432, P = 0.42 \) for HPV18). In contrast, when viral load was
modeled as a log10-transformed continuous covariate,
controlling for whether or not a woman had ever tested
positive for the relevant type using qPCR, a 10-fold in-
crease in either HPV16 or HPV18 viral load was associ-
ated with a significantly increased risk of acquiring a
cervical cytologic abnormality [HR, 1.76 (95% CI, 1.38-
2.25) for HPV16; HR, 1.59 (1.25-2.03) for HPV18; Table 2].
Further controlling for the detection of HPV types other
than types 16 and 18, using the GP5+/GP6+ system,
had a negligible effect on the estimated HRs (data not
shown).

### Table 2. The association between the logarithm-to-base 10 of the greatest cervical HPV viral load observed before the detection of cervical cytologic abnormality and the risk of acquiring an incident cervical cytologic abnormality

<table>
<thead>
<tr>
<th>HPV type</th>
<th>No. of women in the analysis</th>
<th>No. with incident cervical cytologic abnormalities</th>
<th>HR per 10-fold increase in viral load (95% CI)</th>
<th>Likelihood ratio test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV16</td>
<td>62</td>
<td>37</td>
<td>1.76 (1.38-2.25)</td>
<td>21.8; 1; ( P &lt; 0.001 )</td>
</tr>
<tr>
<td>HPV18</td>
<td>56</td>
<td>32</td>
<td>1.59 (1.25-2.03)</td>
<td>12.1; 1; ( P &lt; 0.001 )</td>
</tr>
</tbody>
</table>

*\( \chi^2 \) statistic; degrees of freedom; \( P \) value

### Increasing Viral Load Is Associated with an Increased Risk of Acquiring an Incident Cervical Cytologic Abnormality

When analyzing the association between viral load and the acquisition of cervical cytologic abnormality, the only restriction placed on the study population was that viral load had to have been measured at study entry. Of the 62 women with a HPV16 infection contributing to this analysis, 37 first had an abnormal smear during follow-up; this was reported as containing borderline nuclear abnormalities in 19 and mild dyskaryosis in 18. Of 56 women with a HPV18 infection who contributed to this analysis, 32 first had an abnormal smear during follow-up; this was reported as borderline nuclear abnormalities in 19 and mild dyskaryosis in 13.

Neither the maximum HPV16 viral load nor the maximum HPV18 viral load observed during follow-up differed significantly between women who subsequently acquired an incident cytologic abnormality and those who did not (Wilcoxon rank-sum test with continuity correction: \( W = 426, P = 0.60 \) for HPV16 and \( W = 432, P = 0.42 \) for HPV18). In contrast, when viral load was modeled as a log10-transformed continuous covariate, controlling for whether or not a woman had ever tested positive for the relevant type using qPCR, a 10-fold increase in either HPV16 or HPV18 viral load was associated with a significantly increased risk of acquiring a cervical cytologic abnormality [HR, 1.76 (95% CI, 1.38-2.25) for HPV16; HR, 1.59 (1.25-2.03) for HPV18; Table 2]. Further controlling for the detection of HPV types other than types 16 and 18, using the GP5+/GP6+ system, had a negligible effect on the estimated HRs (data not shown).

### Discussion

We have shown that a 10-fold increase in HPV viral load is associated with a significantly increased risk of acquiring an incident cervical cytologic abnormality in women with cervical HPV16 or HPV18 infections, or both, during follow-up. It is important to emphasize that the change in viral load contributing to this increased risk was observed in the period before cytologic abnormality was diagnosed. Our use of serial sampling allowed us to overcome some of the methodologic limitations of previous studies that have reported an association between changes in HPV viral load and the acquisition or progression of cervical neoplasia. In these studies, viral load was measured at only two time points, with the sample taken at the time of diagnosis of cervical neoplasia used to provide the second of the two samples necessary to define the change in viral load (1, 9). Observations on viral load made at or after the time of diagnosis of an event are uninformative with respect to determining the change in viral load necessary for that event to occur; an outcome cannot be attributed to a given level of exposure until that period of exposure has been completed (2).

However, our observations also help to explain why the measurement of HPV viral load might be less informative than was once hoped. We have shown that the HPV viral load in cervical samples waxes and wanes during the course of an infection. Therefore, it is impossible to predict from a single measurement, made at an indeterminate point during the natural history of that infection, what the viral load kinetics of that infection will be: An initially low viral load may be followed by a high viral load, and vice versa. This may explain, in part, the discrepant results reported by longitudinal studies, which relate the acquisition or the progression of cervical neoplasia to a single measurement of viral load made in a baseline sample (14). Although our repeated observations allowed us to define the maximum HPV viral load attained during an episode of cervical HPV infection, even this single measurement was insufficient to distinguish those women who acquired an incident cervical cytologic abnormality from those who did not. The substantial overlap in the range of maximum HPV viral loads for these two groups, one that is reprinted in other studies, illustrates why a clinically useful cutoff for HPV viral load has yet to be defined (10).

Real-time quantitative PCR, as used in this study, is now probably the method of choice when measuring viral load, insofar as it provides a type-specific assay that allows for normalization for cellular content and has, as we have found, a dynamic range of at least seven logs (10^8-10^7 copies). However, comparisons made using this assay may still be confounded by differences in the proportion of infected and uninfected cells present in a given...
sample. Sampling variation or heterogeneity within cervical lesions may also distort comparisons: for example, viral load is reported to vary with the endocervical cell content of the cytologic sample (11) and to be higher in women with high-grade CIN when low-grade CIN is also present than when it is not present (12). Of course, it is possible that serial measurements of viral load might yet be shown to provide clinically useful information. However, the interpretation of such changes in viral load over time may not be a simple matter because of our imperfect understanding of the natural history of cervical HPV infections. For example, counterintuitively, a decrease in HPV viral load may be associated with other aspects of viral infection that are themselves associated with disease progression. For example, we have shown using samples taken from this cohort that disruption of the HPV E2 gene is a common and early event in the natural history of incident cervical HPV16 and HPV18 infections and is associated with a substantial reduction in viral load (7). However, in no longitudinal survey linking viral load to a disease outcome have changes in integration status over time been defined. Finally, it must be remembered that viral replication is not necessary for maintaining the malignant phenotype. In situ assays on cervical cancer cell lines have found that not all HPV copies are transcriptionally active; in women with incident HPV16 infections, HPV E7 mRNA levels, but not viral load, are associated with an increased risk of developing squamous intraepithelial lesions (13, 14). Robust measurements of type-specific viral load in samples in which integration status and the expression of viral oncogenes are also defined will continue to provide useful insights into the pathogenesis of HPV-associated disease. However, unless and until other longitudinal studies report an increased risk of cervical neoplasia associated with high HPV viral load in women with normal cervical smears, the clinical value of a single measurement of HPV viral load must be considered unproven.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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