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# Type-dependent association between risk of cervical intraepithelial neoplasia and viral load of oncogenic human papillomavirus types other than types 16 and 18

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## Abstract

Studies of the clinical relevance of human papillomavirus (HPV) DNA load have focused mainly on HPV16 and HPV18. Data on other oncogenic types are rare. Study subjects were women enrolled in the atypical squamous cells of undetermined significance (ASC-US) and low-grade squamous intraepithelial lesion (LSIL) triage study who had 1 of 11 non-HPV16/18 oncogenic types detected during a 2-year follow-up at 6-month intervals. Viral load measurements were performed on the first type-specific HPV-positive specimens. The association of cervical intraepithelial neoplasia grades 2–3 (CIN2/3) with type-specific HPV DNA load was assessed with discrete-time Cox regression. Overall, the increase in the cumulative risk of CIN2/3 per 1 unit increase in $\log_{10}$-transformed viral load was statistically significant for four types within species 9 including HPV31 (adjusted hazard ratio [$HR_{adjusted}$] = 1.32: 95% confidence interval [CI], 1.14–1.52), HPV35 ($HR_{adjusted}$ = 1.47; 95% CI, 1.23–1.76), HPV52 ($HR_{adjusted}$ = 1.14; 95% CI, 1.01–1.30) and HPV58 ($HR_{adjusted}$ = 1.49; 95% CI, 1.23–1.82). The association was marginally significant for HPV33 (species 9) and HPV45 (species 7) and was not appreciable for other types. The per 1 $\log_{10}$-unit increase in viral load of a group of species 9 non-HPV16 oncogenic types was statistically significantly associated with risk of CIN2/3 for women with a cytologic diagnosis of within normal limits, ASC-US, or LSIL at the first HPV-positive visit but not for those with high-

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grade SIL. Findings suggest that the viral load-associated risk of CIN2/3 is type-dependent, and mainly restricted to the species of HPV types related to HPV16, which shares this association.

### Keywords

human papillomavirus; viral load; cervical intraepithelial neoplasia; risk association

## Introduction

Human papillomaviruses (HPV) belong to Papillomaviridae family that are taxonomically classified as genus, species, and type according to degrees of the genome homology.[1] To date, >200 types have been identified and ~170 types have been fully characterized.[2] Twelve of them phylogenetically clustered in four species groups of the alpha genus are epidemiologically defined as oncogenic types,[3] including HPV51 in species 5, HPV56 in species 6, HPV types 18, 39, 45, and 59 in species 7 and HPV types 16, 31, 33, 35, 52, and 58 in species 9. Genital infection with oncogenic HPVs is a precondition for the development of cervical cancer and its precursor, cervical intraepithelial neoplasia grades 2–3 (CIN2/3),[4–7] particularly, the types in species 7 and 9 that account for almost 90% of all cases of cervical cancers worldwide.[8]

For a given type of oncogenic HPV, the etiologic role of viral persistence in carcinogenesis is well documented,[9] but the role of viral load is inconclusive. Studies of the clinical relevance of viral load have focused mainly on HPV16 and HPV18.[10–21] Data in favor of viral load being informative for underlying histopathology are mainly from studies of HPV16 infections. HPV18 DNA load, however, appears less significant[17] or even lower among women with, compared to without, a diagnosis of CIN2/3.[21] Currently, data on the clinical relevance of viral load of oncogenic types other than HPV16 and HPV18 (non-HPV16/18 oncogenic types) are rare and inconsistent.[22–34] It remains largely undetermined whether phylogenetically closely related oncogenic types behave similarly in the viral load-associated risk of CIN2/3.

The present study analyzed type-specific viral load of non-HPV16/18 oncogenic types using the first HPV-positive cervical swab samples from longitudinal visits in a large-scale clinical trial. With a quantitative measure for individual types, we were able to ascertain relationships between risk of CIN2/3 and HPV DNA load across the type and species spectrum and find out whether, and to what extent, the viral load-associated risk of CIN2/3 differs by cervical cytology at the first type-specific HPV-positive visit where the viral load was measured.

## Methods

### Subjects and study design

Study subjects were women enrolled in the Atypical Squamous Cells of Undetermined Significance (ASC-US) and Low-Grade Squamous Intraepithelial Lesion (LSIL) Triage Study (ALTS), a clinical trial designed to evaluate three strategies for management of women with equivocal or mildly abnormal cervical cytology. Women in ALTS were

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followed every 6 months for 2 years for detection of HPV and cervical lesions. A detailed description of the ALTS design and study population is available elsewhere.[35]

An ALTS participant was eligible for the present study if she had 1 of 10 non-HPV16/18 oncogenic types plus HPV68 (a possible oncogenic type of HPV in species 7) detected in her cervical swab sample(s) by polymerase chain reaction (PCR)-based reverse-line blot assay at any time during the trial. In total, 3,002 eligible women with 5,558 non-HPV16/18 oncogenic type-specific infections were identified from the ALTS database. The first HPV-positive cervical swab sample for each infection was chosen for viral load measurement. Two hundred and thirty-five infections were excluded due to a lack of remaining sample for viral load testing. We additionally excluded four infections because of a negative β-actin result (a reference for viral load normalization), leaving 5,319 type-specific infections (2,902 women) in analysis. Proportions of infections excluded from the study were comparable across HPV species (data not shown). Infections excluded from the study compared to those included were more likely to be detected in women with histologic CIN2/3, cytologic HSIL, and coinfection by other oncogenic types at the first positive visit; there were no appreciable differences in age, race, smoking status, and number of lifetime sex partners (data not shown).

The primary endpoint used in this study was the first episode of HPV type-related CIN2/3 histologically confirmed by the panel of expert pathologists. In ALTS, HPV testing was performed only on cervical swab samples. Thus, for samples positive for multiple types, the causal role of a particular type of HPV could not be assigned definitely. To estimate the likely contribution of an HPV infection, types detected in a swab sample at the screening visit immediately preceding the colposcopic visit yielding a histologic diagnosis of CIN2/3 were considered to be related to the lesion identified. For each type of infection, only the first episode of type-related CIN2/3 was counted. One episode of CIN2/3 could be related to one type of HPV and another episode to other types. If 2 types were concurrently detected at the time of CIN2/3 diagnosis, the lesion was considered to be related to multiple types.

Data on HPV genotyping results, diagnoses of cervical lesions, and characteristics of study subjects were obtained from the ALTS database. The study protocol was approved by the institutional review board at University of Washington.

## Quantification of type-specific HPV DNA load

DNAs were extracted and purified with QIAamp DNA mini kit (Qiagen, Valentia, CA) from an aliquot of 50–100 μl cervical swab sample in Specimen Transport Medium (Qiagen) and then resuspended in 25 μl AE buffer. Type-specific HPV DNA copy number and cellular DNA amount (determined by testing for β-actin gene) were measured by real-time PCR. Primers and probes for the assay and construction and calibration of the standards for absolute quantification were reported previously.[36] The assay was set up with the TaqMan Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA) in a final reaction volume of 5 μl containing 0.5 μl DNA sample input; each sample was tested in triplicate on the same plate. Amplification was performed on Applied Biosystems 7900 HT Sequence Detection System with a cycling program of holding at 50°C for 2 min and then at 95°C for 10 min followed by a two-step cycle of 10 sec at 95°C and 1 min at 60°C for 40 cycles. Two

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log-phase five-point standard curves were implemented in each set of the assay for absolute quantification, one for HPV and the other for cellular DNA. The number of viral copies was normalized according to the input amount of cellular DNA and $\log_{10}$-transformed. The mean value of three measures (expressed as $\log_{10}$ [HPV copy number per 1 nanogram of cellular DNA]) was used for analysis.

Type-specific HPV DNA was undetectable by real-time PCR for 698 (13.1%) of 5,319 infections that were previously detected by PCR-based reverse-line blot assay. The negative result was not explained by a lack of sufficient sample input or presence of potential inhibitors as the amount of cellular DNA between samples with and without detectable HPV DNA by real-time PCR was comparable (data not shown). Also, repeated testing of these samples with double the amount of sample input did not yield a positive signal (data not shown). Considering that the "negativity" might result from a tiny amount of HPV DNA, a value of one viral copy per 1 nanogram of cellular DNA was arbitrarily assigned to each of these samples. Results remained similar when these samples were excluded from the analysis; thus, for simplicity, results with these samples excluded were not presented.

## Statistical analyses

The main exposure of interest was viral load of 11 non-HPV16/18 oncogenic types at the first positive visit. The analysis was performed at the level of individual infections. Thus, women positive for  2 types of interest, whether concurrently at the same visit or not, would be counted multiple times. For analysis of viral load of a group of oncogenic types, robust variance estimates were used to account for correlation within subjects. In addition, a variable indicating types within the group (*i.e.*, HPV types 31, 33, 35, 52, and 58 in a group of species 9; HPV types 39, 45, 59, and 68 in a group of species 7) was always included as a covariate for estimation of adjusted statistics.

A discrete-time Cox regression model[37] was used to estimate hazard ratios (HR) and their 95% confidence intervals (CI) for the association of cumulative risk of CIN2/3 with viral load of non-HPV16/18 oncogenic types at the first positive visit. The length of follow-up was the time from the first HPV-positive visit to the visit where the first episode of type-related CIN2/3 was diagnosed or was censored at the last available visit for women who did not have a diagnosis of CIN2/3 during the study period. Factors potentially related to CIN2/3 occurrence but presumably not an intermediary step in the viral load-mediated causal pathway (including age, race, current smoking status, lifetime number of male sex partners, use of condoms or birth control pills, number of Pap tests in the past 5 years, type of HPV, time of the first positive detection, and coinfection by other oncogenic types) were initially examined by backward stepwise regression with $p < 0.20$ (Wald test) as the criterion for entering and removing variables. Covariates included in the final model were race (Caucasian versus non-Caucasian), current smoking status (yes versus no), time of the first HPV-positive detection (enrollment versus follow-up), and a time-dependent variable of coinfection by other oncogenic types (yes versus no for all oncogenic types except for the one evaluated). This analysis was repeated with CIN3 as the endpoint.

A one-way analysis of variance was used to assess type-specific viral load by concurrent cervical cytology; Scheffe's method was used for multiple pair-wise comparisons for each

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type of HPV. A linear regression model[38] was used to compare type-specific viral load at the first positive visit between women with and without a concurrent diagnosis of CIN2/3 while controlling for age at enrollment (18–24 versus 25) and time of the first HPV-positive detection, a set of covariates selected by backward stepwise linear regression with $P<0.20$ as the criterion. All statistical analyses were performed using STATA version 11 (StataCorp, College Station, TX); all tests were at the 5% two-sided significance level.

## Results

The present study included 2,902 women with 5,319 non-HPV16/18 oncogenic type-specific infections initially detected at enrollment ($n = 2{,}996$) or during follow-up ($n = 2{,}323$). These women reported a mean age of 25.3 (standard deviation, 9.6) years at enrollment and a median lifetime number of 5 (interquartile range [IR], 7) male sex partners. Approximately 38% of them were current smokers. The self-reported race information was available from 2,884 women (including 1,738 Caucasian, 993 African-American, 87 Asian/Pacific Islander, and 66 American-Indian/Alaskan women). The median time of follow-up from the first positive detection was 19.3 months (IR, 15.6). Irrespective of type of HPV, the viral load at the first positive visit was significantly higher for women with a concurrent cytologic diagnosis of ASC-US, LSIL, or HSIL as compared to those with normal cytology (Table 1). The viral load of HPV types 31, 39, 45, 56, 59, and 68 appeared slightly lower for women with HSIL as compared to those with LSIL, although a statistically significant difference was seen only for HPV59 ($p = 0.04$) and a group of species 7 non-HPV18 oncogenic types ($p = <0.001$).

CIN2/3 was histologically confirmed in 565 women with 872 type-related CIN2/3 episodes initially detected at the first HPV-positive visit ($n = 603$) or thereafter ($n = 269$). After adjusting for age at enrollment and time of the first HPV-positive detection, the viral load at the first positive visit among women with, compared to without, a concurrent diagnosis of CIN2/3 was statistically significantly higher for HPV types 31, 35, 58, and a group of species 9 non-HPV16 oncogenic types but not for others (Table 2). Coexistence of other oncogenic types and any HPV types at the first positive visit was common, detected in 58.1 and 80.1% of the infections, respectively. When the analysis was restricted to women without a coinfection by other oncogenic types, results remained similar with the exception that the difference in HPV58 DNA load became non-statistically significant ($p_{\text{adjusted}} = 0.17$). To further rule out the influence of other HPV types, the analysis was additionally restricted to single type infections. Because of a limited sample size, this analysis was performed only for groups of types. The viral load among women with, compared to without, a concurrent diagnosis of CIN2/3 was statistically significantly higher for single type infection by a group of species 9 non-HPV16 oncogenic types ($p_{\text{adjusted}} = 0.001$) but not for single type infection by a group of species 7 non-HPV18 oncogenic types ($p_{\text{adjusted}} = 0.43$).

In this dataset, CIN2/3 occurrence was correlated with race, current smoking status, time of the first HPV-positive detection, and coinfection by other oncogenic types (data not shown). With the adjustment for these variables, the association of cumulative risk of CIN2/3 with per 1 unit increase in $\log_{10}$-transformed viral load at the first positive visit was statistically

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significant for HPV31 ($HR_{adjusted}$ = 1.32; 95% CI, 1.14–1.52), HPV35 ($HR_{adjusted}$ = 1.47; 95% CI, 1.23–1.76), HPV52 ($HR_{adjusted}$ = 1.14; 95% CI, 1.01–1.30), HPV58 ($HR_{adjusted}$ = 1.49; 95% CI, 1.23–1.82), and a group of species 9 non-HPV16 oncogenic types ($HR_{adjusted}$ = 1.28; 95% CI, 1.20–1.38). The association was marginally significant for HPV33, HPV45 and a group of species 7 non-HPV18 oncogenic types and was not appreciable for other types (Table 3). Results remained similar when all cases of CIN2/3 whatever the lesion was type-related or not were censored at the time of initial diagnosis (data not shown). When the analysis was restricted to visits without a coinfection by other oncogenic types, the estimates remained similar except for minor changes for HPV52 ($HR_{adjusted}$ = 1.27; 95% CI, 0.99–1.63), HPV33 ($HR_{adjusted}$ = 1.29; 95% CI, 0.92–1.81) and a group of species 7 non-HPV18 oncogenic types ($HR_{adjusted}$ = 1.18; 95% CI, 0.94–1.49). Further restricting the analysis to visits without a coinfection by any HPVs yielded an adjusted HR of 1.37 (95% CI, 1.20–1.58) for infections with a group of species 9 non-HPV16 oncogenic types and 1.24 (95% CI, 0.81–1.90) for infections with a group of species 7 non-HPV18 oncogenic types.

CIN3 was histologically confirmed in 296 women who had 453 non-HPV16/18 oncogenic type-specific infections detected at the time of histologic diagnosis. With CIN3 as the endpoint, the increase in the cumulative risk per 1 unit increase in $\log_{10}$-transformed viral load remained statistically significant for HPV31 ($HR_{adjusted}$ = 1.21; 95% CI, 1.00–1.45), HPV35 ($HR_{adjusted}$ = 1.48; 95% CI, 1.17–1.88), HPV58 ($HR_{adjusted}$ = 1.56; 95% CI, 1.19–2.05), and a group of species 9 non-HPV16 oncogenic types ($HR_{adjusted}$ = 1.26; 95% CI, 1.15–1.37). No appreciable association was seen for other types except for a marginal association for HPV33 ($HR_{adjusted}$ = 1.24; 95% CI, 0.99–1.56) and a group of species 7 non-HPV18 oncogenic types ($HR_{adjusted}$ = 1.09; 95% CI, 0.99–1.21).

To determine the impact of cervical cytology at the first HPV-positive visit on the viral load-associated risk of CIN2/3, the analysis was performed only for a group of species 9 non-HPV16 oncogenic types. After adjusting for race, current smoking status, time of the first HPV-positive detection, coinfection by other oncogenic types, and type within species 9, the association of cumulative risk of CIN2/3 with per 1 $\log_{10}$-unit increase in viral load was statistically significant for women with a cytologic diagnosis of within normal limits, ASC-US, or LSIL at the first HPV-positive visit, but not for those with HSIL (Table 4). When the analysis was restricted to visits without a coinfection by other oncogenic types, results remained similar with the exception that the association was no longer statistically significant for women with LSIL at the first positive visit ($HR_{adjusted}$ = 1.08; 95% CI, 0.88–1.32). When the analysis was restricted to the first positive visit, the estimates of the cytology-stratified, viral load-associated risk of concurrent diagnoses of CIN2/3 remained similar to those presented in Table 4 (data not shown).

## Discussion

The present study is an extension of our previous analyses of the clinical relevance of HPV16/18 DNA load. Our previous reports have demonstrated that the increase in baseline HPV16 DNA load was significantly correlated to higher risk of concurrent and cumulative diagnoses of CIN2/3[20] whereas HPV18 DNA load appeared lower among women with, compared to without, a diagnosis of CIN2/3.[21] In this study of viral load of 11 non-

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HPV16/18 oncogenic types, we observed a significantly higher viral load at the first positive visit among women with, compared to without, a concurrent diagnosis of CIN2/3 for HPV types phylogenetically close to HPV16. In addition, a statistically significant association of cumulative risk of CIN2/3 with per 1 $\log_{10}$-unit increase in viral load was seen for four species 9 non-HPV16 oncogenic types but not others except for a marginal association for HPV33 (species 9) and HPV45 (species 7). The type-dependent association was not explained by factors that were correlated to diagnoses of CIN2/3 nor by potential variant-induced PCR errors as all known sequence variations in the primer/probe binding region were taken into account in our assay design.[36] In this well-controlled trial setting, the diagnosis of cervical lesions was extensively reviewed by the panel of expert pathologists, testing for viral load was performed without knowledge of any clinical information, and the majority of participants were examined, followed, and diagnosed according to the standardized protocol. Thus, a potential bias in ascertainment of exposure and outcome was minimized.

The higher viral load of species 9 non-HPV16 oncogenic types in cases of CIN2/3 should not be considered to be from the lesion itself as CIN2/3, particularly CIN3, cells usually featured with clonal expansion may contain fewer viral copes as compared to proliferating cells featured with productive viral replication. Also, cervical specimens obtained by scraping favor the collection of maturing cells from broad areas of cervical epithelium and CIN2/3 lesions, even if present, contribute only a small fraction of cells removed by scraping.[39]

More likely, the increase in viral load of species 9 non-HPV16 oncogenic types may signal an elevated risk of the underlying CIN2/3. HPV begins its life cycle by infecting basal cells of the epithelium and utilizes cellular DNA machinery for replication which is tightly linked to cellular differentiation. The viral load that reflects cytologic changes of exfoliative cells in samples is a function of the number of infected cells and viral copies in individual cells. As shown in a study of women with CIN3, HPV DNA load was closely correlated with the number of ASC-US and LSIL cells present in cervical samples.[40] Cervical neoplasia is usually initiated from a single (or few) cell(s) and then developed through clonal expansions. Accordingly, the association of CIN2/3 with viral load of species 9 non-HPV16 oncogenic types could be in part explained by a possibility that a likelihood of having cells with transforming infections increases as increasing numbers of infected cells and/or increasing numbers of viral copies in individual cells.

Similar to what was observed among HPV16-positive women,[20] the cumulative risk of CIN2/3 associated with viral load of a group of species 9 non-HPV16 oncogenic types differed by cervical cytology at the first positive visit with a statistically significant association seen for women with a cytologic diagnosis of within normal limits, ASC-US, or LSIL but not for those with HSIL. As interpreted previously,[20] this could be due to a possibility that the number of abnormal epithelial cells in samples increases as increasing severity of cervical cytology and the increased number of these cells makes viral load contributed by CIN2/3 and its surrounding areas be less apparent, thereby leading to a lack of the association for women with HSIL.

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Importantly, by linking HPV types to their evolutionary grouping ("clade"), we have demonstrated here for the first time that oncogenic types phylogenetically close to HPV16 behaved more like HPV16 in the viral load-associated risk of CIN2/3 whereas those close to HPV18 behaved like HPV18, although not as extreme as HPV18. This finding is somewhat consistent with a previous report of the carcinogenicity of HPV types and their phylogenetic grouping.[41] Factors that cause apparent differences in the viral load-associated risk of CIN2/3 between types in species 9 and those in other species groups are currently unknown. The lack of association could not be simply attributable to insufficient statistical power as sample sizes did not differ substantially across types except for HPV52.

One concern is whether the type-dependent association was in part a result of overstatement of the causal role for types evaluated. As described in the method section, the likely contribution of an HPV to the development of CIN2/3 was determined based on results of HPV-testing on preceding cervical swab samples. Obviously, not all detectable types that were defined as CIN2/3-related in our analyses played a causal role because as shown by HPV-testing on microdissected tissue samples,[42,43] most CIN2/3 cases infected with multiple types had only one type detected in the case-defining high-grade lesion. Had the overstatement of the causal role of an HPV occurred differentially by types, the viral load-associated risk of CIN2/3 might have been seen for some types but not all. Arguing against this, however, is the fact that results remained similar when the analysis was restricted to visits without a coinfection by other oncogenic types. Particularly, the cumulative risk of CIN2/3 remained associated with viral load of a group of species 9 non-HPV16 oncogenic types but not a group of species 7 non-HPV18 oncogenic types when visits with a detectable coinfection by any HPVs were removed from analyses.

As noted, in contrast to a step-wise increase in viral load of species 9 non-HPV16 oncogenic types (except for HPV31) with increasing severity of cervical cytology, the viral load of other types appeared slightly lower among women with HSIL compared to those with LSIL. Intuitively, this may lead to an attenuation of differences in viral loads between women with and without CIN2/3, as HSIL relative to other cytomorphologic abnormalities is the best cytologic correlate of histologic diagnoses of CIN2/3. Apparently, the interpretation of the viral load-related increase in transforming infections does not work for types that displayed comparable viral loads between women with and without CIN2/3.

It is known that HPV16-related cervical cancers and pre-cancerous lesions tend to develop in squamous epithelial cells whereas those HPV18-related tend to develop in columnar epithelial cells that are relatively difficult to sample with swabs. Also, unlike multi-layers of squamous epithelial cells where the HPV lifecycle is closely linked to epithelial differentiation, the columnar epithelial cells have only a single layer, the environment that may be less permissive for intracellular replication of the virus. If species 9 non-HPV16 oncogenic types, similar to HPV16, preferentially induce neoplastic transformation of squamous epithelial cells; species 7 non-HPV18 oncogenic types, similar to HPV18, preferentially induce transformation of columnar epithelial cells, the type-dependent association could be in part explained by tropism for the host cells, location of the HPV-related neoplasia, and behavior of the virus in these cells.

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Another possibility deserving consideration is whether types that did not differ in viral loads between women with and without CIN2/3 play a causal role through mechanisms not well reflected by viral load. Integration of HPV DNA into the host genome occurs preferentially with disruption of the open reading frames of E1 and E2. While the disruption of the E2 gene has been suggested in the development of precancerous lesions, it might lead to a decrease in viral replication.[44] At the same time, the E2 disruption favors transcriptional activation of the E6 and E7 oncogenes by release of the E2-mediated suppression of the early promoter.[45,46] It has been reported that the genome of HPV45 compared to HPV31 and HPV33 was substantially more often in the integrated state among women with CIN3 or cervical cancer[47] and the increase in severity of histopathologic diagnoses was associated with increasing HPV transcripts but not DNA loads.[48] Further studies are warranted to examine whether the lack of the viral load-associated risk of CIN2/3 is a result of balance between the abnormal cytology-related increase in viral load and the integration-related decrease of viral replication.

Finally, it should be pointed out that despite a recognition of the association between CIN2/3 and viral load of species 9 non-HPV16 oncogenic types, our results do not justify changing the format of clinically available HPV tests because of a substantial overlap of the viral load between women with and without CIN2/3. Nonetheless, the clinical implication of this study is that interpretation of the viral load-associated underlying histopathology differs by type and species of oncogenic HPVs and by cervical cytology at the time of viral load measurement. Clinicians could one day apply this concept to their patient consultation and management. Our findings also imply that estimates of the clinical relevance of HPV DNA load using summary measurements without distinction of individual types are likely to be misleading.

There are several limitations of this study that warrant discussion. Most importantly, although the study included over 5,000 type-specific infections, we still did not have sufficient statistical power to exclude visits positive for non-oncogenic HPV types for analyses of the viral load-associated risk of CIN2/3 for individual types. However, risks contributed by these types are likely to be minimal. Second, ~4% of type-specific infections were excluded from the study due to a lack of remaining sample for viral load testing. This somewhat decreased statistical power. However, no evidence suggests that it might differentially affect the estimates of the type-dependent association as proportions of infections excluded from the study were comparable across HPV species. Third, our findings pertain to women who had a cytologic diagnosis of ASC-US or LSIL within 6 months prior to enrollment into ALTS. Thus, a diagnosis of normal cytology, ASC-US/LSIL, and HSIL at the first HPV-positive visit, particularly for infections initially detected at enrollment, can be viewed as cytologic regression, persistent mild abnormality, and cytologic progression, respectively. As a result, the viral load detected in a particular cytologic category might not be generalizable to that in the same category of general populations. However, no evidence suggests that this lack of generalizability would affect the validity for assessment of the viral load-associated risk of CIN2/3. Lastly, measuring viral load on the first positive samples still gave estimates at an indeterminate time point in the course of the infection because women in ALTS were followed every 6 months. Naturally, viral loads fluctuate overtime; an initially

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low viral load could be followed by a high viral load, and vice versa. The clinical relevance of changes of HPV DNA load remains undetermined.

Taken together, it is now clear that the relationship between HPV DNA load and risk of CIN is much more complex than what thought previously. The viral load-associated risk of CIN2/3 was type-dependent, with phylogenetically closely related types appearing to behave more similarly. The concordance of the viral load-associated risk of CIN2/3 with phylogenetic relatedness of the types suggests that oncogenic types within the same species may possess some common genetic makeup to define phenotypic traits of the virus. These data support further investigation of the genomic determinants of and underlying mechanisms for the type-dependent association to deepen our understanding of HPV-induced pathogenesis of cervical lesion.

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## Abbreviations used

| | |
|---|---|
| **HPV** | human papillomavirus |
| **CIN** | cervical intraepithelial neoplasia |
| **ASC-US** | atypical squamous cells of undetermined significance |
| **LSIL** | low-grade squamous intraepithelial lesion |
| **HSIL** | high-grade squamous intraepithelial lesion |
| **PCR** | polymerase chain reaction |

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### What's new?

Genital infection with oncogenic human papillomavirus (HPVs) is a precondition for the development of cervical cancer. Here, the authors conducted an innovative study of the relationship between viral load-associated risk of CIN2/3 and phylogenetic relatedness of HPV types using samples from a large-scale clinical trial. The findings suggest that the viral load-associated risk of CIN2/3 is type-dependent, and is mainly restricted to the species of HPV types related to HPV16, which shares this association. These data support further investigation of the genomic determinants of the type-dependent association and underlying mechanisms to deepen our understanding of HPV-induced pathogenesis of cervical lesion.

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## Table 1

Type-specific HPV DNA load at the first positive visit by concurrent diagnoses of cervical cytology

| Alpha HPV | | | | | | | |
|---|---|---|---|---|---|---|---|
| Species | Type | Cervical Cytology | No. of Women[1] | Mean (SD) of $log_{10}$-transformed HPV copy number per 1 Nanogram of cellular DNA | *p* values[2] | | |
| 5 | 51 | Within normal limits | 165 | 2.22 (±1.60) | Referent | | |
| | | ASC-US | 167 | 2.86 (±1.69) | 0.01 | Referent | |
| | | LSIL | 267 | 4.14 (±1.74) | <0.001 | <0.001 | Referent |
| | | HSIL | 49 | 4.19 (±1.82) | <0.001 | <0.001 | 1.00 |
| 6 | 56 | Within normal limits | 125 | 2.45 (±1.51) | Referent | | |
| | | ASC-US | 98 | 3.07 (±1.37) | 0.04 | Referent | |
| | | LSIL | 213 | 3.96 (±1.63) | <0.001 | <0.001 | Referent |
| | | HSIL | 19 | 3.27 (±1.62) | 0.21 | 0.96 | 0.32 |
| 7 | 39 | Within normal limits | 183 | 1.96 (±1.46) | Referent | | |
| | | ASC-US | 134 | 2.85 (±1.44) | <0.001 | Referent | |
| | | LSIL | 200 | 3.60 (±1.77) | <0.001 | 0.001 | Referent |
| | | HSIL | 25 | 3.31 (±1.42) | 0.001 | 0.63 | 0.86 |
| 7 | 45 | Within normal limits | 157 | 1.55 (±1.42) | Referent | | |
| | | ASC-US | 117 | 2.57 (±1.50) | <0.001 | Referent | |
| | | LSIL | 112 | 3.29 (±1.63) | <0.001 | 0.01 | Referent |
| | | HSIL | 26 | 2.95 (±1.35) | <0.001 | 0.72 | 0.78 |
| 7 | 59 | Within normal limits | 170 | 1.91 (±1.66) | Referent | | |
| | | ASC-US | 129 | 2.75 (±1.75) | 0.001 | Referent | |
| | | LSIL | 158 | 3.65 (±1.75) | <0.001 | <0.001 | Referent |
| | | HSIL | 24 | 2.55 (±1.69) | 0.40 | 0.97 | 0.04 |
| 7 | 68 | Within normal limits | 126 | 1.68 (±1.48) | Referent | | |
| | | ASC-US | 83 | 2.51 (±1.60) | 0.002 | Referent | |
| | | LSIL | 90 | 3.62 (±1.47) | <0.001 | <0.001 | Referent |
| | | HSIL | 19 | 2.72 (±1.45) | 0.05 | 0.96 | 0.13 |
| 7 | non-HPV18 | Within normal limits | 636 | 1.79 (±1.52) | Referent | | |
| | | ASC-US | 463 | 2.69 (±1.57) | <0.001 | Referent | |

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| Alpha HPV | | | | | | | |
|---|---|---|---|---|---|---|---|
| Species | Type | Cervical Cytology | No. of Women[1] | Mean (SD) of $\log_{10}$-transformed HPV copy number per 1 Nanogram of cellular DNA | $p$ values[2] | | |
| | | LSIL | 560 | 3.56 (±1.69) | <0.001 | <0.001 | Referent |
| | | HSIL | 94 | 2.90 (±1.49) | <0.001 | 0.22 | <0.001 |
| 9 | 31 | Within normal limits | 173 | 2.55 (±1.38) | Referent | | |
| | | ASC-US | 153 | 3.33 (±1.45) | <0.001 | Referent | |
| | | LSIL | 151 | 3.96 (±1.47) | <0.001 | 0.002 | Referent |
| | | HSIL | 51 | 3.75 (±1.25) | <0.001 | 0.35 | 0.84 |
| 9 | 33 | Within normal limits | 81 | 2.03 (±1.68) | Referent | | |
| | | ASC-US | 55 | 2.46 (±1.52) | 0.49 | Referent | |
| | | LSIL | 80 | 3.40 (±1.65) | <0.001 | 0.01 | Referent |
| | | HSIL | 42 | 3.79 (±1.28) | <0.001 | 0.001 | 0.63 |
| 9 | 35 | Within normal limits | 152 | 2.30 (±1.53) | Referent | | |
| | | ASC-US | 120 | 2.99 (±1.37) | 0.001 | Referent | |
| | | LSIL | 125 | 3.84 (±1.28) | <0.001 | <0.001 | Referent |
| | | HSIL | 31 | 4.14 (±1.18) | <0.001 | 0.001 | 0.77 |
| 9 | 52 | Within normal limits | 295 | 1.80 (±1.48) | Referent | | |
| | | ASC-US | 230 | 2.53 (±1.51) | <0.001 | Referent | |
| | | LSIL | 219 | 3.17 (±1.52) | <0.001 | <0.001 | Referent |
| | | HSIL | 57 | 3.25 (±1.48) | <0.001 | 0.01 | 0.99 |
| 9 | 58 | Within normal limits | 144 | 1.87 (±1.52) | Referent | | |
| | | ASC-US | 134 | 3.25 (±1.54) | <0.001 | Referent | |
| | | LSIL | 126 | 3.87 (±1.40) | <0.001 | 0.01 | Referent |
| | | HSIL | 36 | 4.09 (±0.94) | <0.001 | 0.02 | 0.88 |
| 9 | non-HPV16 | Within normal limits | 845 | 2.08 (±1.52) | Referent | | |
| | | ASC-US | 692 | 2.92 (±1.52) | <0.001 | Referent | |
| | | LSIL | 701 | 3.61 (±1.50) | <0.001 | <0.001 | Referent |
| | | HSIL | 217 | 3.74 (±1.30) | <0.001 | <0.001 | 0.25 |

[1] A woman was counted multiple times if she was positive for 2 non-HPV16/18 oncogenic types. Excluded were eight infections from women who had a Pap smear insufficient for cytologic diagnosis at the first positive visit.

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[2]Estimated by one-way ANOVA with Scheffe's method for pair-wise multiple comparisons. The *p* values were adjusted for type within the species and robust variance estimates were used for analysis of viral load of a group of types.

HPV = human papillomavirus, non-HPV18 = a group of species 7 oncogenic types other than HPV18, non-HPV16 = a group of species 9 oncogenic types other than HPV16, SD = standard deviation, ASC-US = atypical squamous cells of undetermined significance, LSIL = low-grade squamous intraepithelial lesion, HSIL = high-grade squamous intraepithelial lesion.

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## Table 2

Type-specific HPV DNA load at the first positive visit between women with and without a concurrent diagnosis of CIN2/3

| Alpha HPV | | $Log_{10}$-transformed HPV copy number per 1 nanogram of cellular DNA at the first positive visit among women | | | | | |
|---|---|---|---|---|---|---|---|
| | | Without CIN2/3 | | With CIN2/3 | | | |
| Species | Type | No.[1] | Mean (SD) | No.[1] | Mean (SD) | *p* values | *p* values[2] |
| 5 | 51 | 573 | 3.27 (±1.90) | 75 | 3.74 (±1.76) | 0.04 | 0.69 |
| 6 | 56 | 419 | 3.29 (±1.68) | 36 | 3.72 (±1.44) | 0.13 | 0.84 |
| 7 | 39 | 490 | 2.79 (±1.70) | 52 | 3.38 (±1.79) | 0.02 | 0.63 |
| 7 | 45 | 369 | 2.37 (±1.67) | 43 | 2.69 (±1.49) | 0.23 | 0.70 |
| 7 | 59 | 440 | 2.70 (±1.87) | 42 | 3.15 (±1.65) | 0.13 | 0.74 |
| 7 | 68 | 291 | 2.48 (±1.72) | 28 | 2.74 (±1.43) | 0.44 | 0.91 |
| 7 | non-HPV18 | 1590 | 2.61 (±1.75) | 165 | 3.03 (±1.63) | 0.003 | 0.77 |
| 9 | 31 | 442 | 3.17 (±1.54) | 88 | 3.89 (±1.27) | <0.001 | 0.001 |
| 9 | 33 | 216 | 2.71 (±1.73) | 42 | 3.49 (±1.49) | 0.01 | 0.17 |
| 9 | 35 | 374 | 2.97 (±1.57) | 54 | 3.82 (±1.09) | <0.001 | 0.003 |
| 9 | 52 | 710 | 2.41 (±1.62) | 95 | 3.03 (±1.44) | <0.001 | 0.16 |
| 9 | 58 | 392 | 2.92 (±1.71) | 48 | 4.10 (±1.06) | <0.001 | 0.001 |
| 9 | non-HPV16 | 2134 | 2.79 (±1.65) | 327 | 3.61 (±1.35) | <0.001 | <0.001 |

[1] A woman was counted multiple times if she was positive for 2 non-HPV16/18 oncogenic types.

[2] Adjusted for age at enrollment (18–24 or 25) and time of the first HPV-positive detection (enrollment or follow-up). The *p* values were additionally adjusted for type within the species and robust variance estimates were used for analysis of viral load of a group of types.

HPV = human papillomavirus, non-HPV18 = a group of species 7 oncogenic types other than HPV18, non-HPV16 = a group of species 9 oncogenic types other than HPV16, SD = standard deviation, CIN = cervical intraepithelial neoplasia.

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## Table 3

Hazard ratios (HR) for the association of per 1 unit increase in $\log_{10}$-transformed type-specific HPV DNA load at the first positive visit with 2-year cumulative risk of CIN2/3

| Alpha HPV | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Species** | **Type** | **No. of women[1]** | **Mean (SD) of $\log_{10}$-transformed HPV copy number per 1 nanogram of cellular DNA** | **No. of person-visits at risk** | **No. of CIN2/3 (per 100 person-visits)** | **HR $_{crude}$ (95% CI)** | **HR $_{adjusted}$ (95% CI)[2]** |
| 5 | 51 | 648 | 3.33 (±1.89) | 2006 | 101 (5.0) | 1.12 (1.00–1.26) | 1.06 (0.94–1.20) |
| 6 | 56 | 455 | 3.32 (±1.67) | 1448 | 47 (3.3) | 1.16 (0.96–1.41) | 1.09 (0.88–1.34) |
| 7 | 39 | 542 | 2.85 (±1.72) | 1652 | 79 (4.8) | 1.10 (0.96–1.27) | 1.04 (0.90–1.21) |
| 7 | 45 | 412 | 2.40 (±1.66) | 1194 | 67 (5.6) | 1.23 (1.05–1.44) | 1.17 (0.98–1.39) |
| 7 | 59 | 482 | 2.74 (±1.86) | 1470 | 54 (3.7) | 1.13 (0.97–1.31) | 1.10 (0.93–1.31) |
| 7 | 68 | 319 | 2.51 (±1.69) | 966 | 36 (3.7) | 1.06 (0.87–1.30) | 0.97 (0.78–1.20) |
| 7 | non-HPV18 | 1755 | 2.65 (±1.75) | 5282 | 236 (4.5) | 1.13 (1.05–1.22) | 1.08 (0.99–1.17) |
| 9 | 31 | 530 | 3.29 (±1.52) | 1540 | 132 (8.6) | 1.32 (1.15–1.51) | 1.32 (1.14–1.52) |
| 9 | 33 | 258 | 2.83 (±1.71) | 749 | 60 (8.0) | 1.26 (1.06–1.50) | 1.19 (0.99–1.44) |
| 9 | 35 | 428 | 3.08 (±1.54) | 1243 | 87 (7.0) | 1.49 (1.25–1.78) | 1.47 (1.23–1.76) |
| 9 | 52 | 805 | 2.49 (±1.61) | 2442 | 138 (5.7) | 1.21 (1.08–1.36) | 1.14 (1.01–1.30) |
| 9 | 58 | 440 | 3.05 (±1.69) | 1379 | 71 (5.2) | 1.55 (1.29–1.87) | 1.49 (1.23–1.82) |
| 9 | non-HPV16 | 2461 | 2.90 (±1.64) | 7353 | 488 (6.6) | 1.33 (1.24–1.42) | 1.28 (1.20–1.38) |

[1] A woman was counted multiple times if she was positive for 2 non-HPV16/18 oncogenic types.

[2] Adjusted for race (Caucasian or non-Caucasian), current smoking status (yes or no), time of the first HPV-positive detection (enrollment or follow-up), and coinfection with other oncogenic types (yes or no). The HR was additionally adjusted for type within the species and robust variance estimates were used for analysis of viral load of a group of types.

HPV = human papillomavirus, non-HPV18 = a group of species 7 oncogenic types other than HPV18, non-HPV16 = a group of species 9 oncogenic types other than HPV16, CIN = cervical intraepithelial neoplasia, SD = standard deviation, CI = confidence interval.

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**Table 4**

Hazard ratios (HR) for the association of per 1 unit increase in $\log_{10}$-transformed DNA load of a group of species 9 non-HPV16 oncogenic types with 2-year cumulative risk of CIN2/3, stratified by cervical cytology at the first positive visit

| Cervical cytology | No. of women[1] | Mean (SD) of $\log_{10}$-transformed HPV copy number per 1 nanogram of cellular DNA | No. of person-visits at risk | No. of CIN2/3 (per 100 person-visits) | $HR_{crude}$ (95% CI) | $HR_{adjusted}$ (95% CI)[2] |
|---|---|---|---|---|---|---|
| Within normal limits | 845 | 2.08 (±1.52) | 2413 | 68 (2.8) | 1.44 (1.23–1.67) | 1.37 (1.17–1.61) |
| ASC-US | 692 | 2.92 (±1.52) | 2192 | 139 (6.3) | 1.22 (1.08–1.38) | 1.23 (1.07–1.42) |
| LSIL | 701 | 3.61 (±1.50) | 2232 | 156 (7.0) | 1.16 (1.03–1.31) | 1.17 (1.02–1.33) |
| HSIL | 217 | 3.74 (±1.30) | 498 | 124 (24.9) | 0.97 (0.80–1.17) | 1.02 (0.83–1.25) |

[1] A woman was counted multiple times if she was positive for 2 non-HPV16 species 9 oncogenic types. Excluded were 6 infections (18 visits at risk, one linked to a diagnosis of CIN2/3) from women who had a Pap smear insufficient for cytologic diagnosis at the first positive visit.

[2] Adjusted for race (Caucasian or non-Caucasian), current smoking status (yes or no), time of the first HPV-positive detection (enrollment or follow-up), coinfection with other oncogenic types (yes or no), and type within species 9.

HPV = human papillomavirus, CIN = cervical intraepithelial neoplasia, SD = standard deviation, CI = confidence interval, ASC-US = atypical squamous cells of undetermined significance, LSIL = low-grade squamous intraepithelial lesion, HSIL = high-grade squamous intraepithelial lesion.