

Determinants of Human Papillomavirus Load among Women with Histological Cervical Intraepithelial Neoplasia 3: Dominant Impact of Surrounding Low-Grade Lesions¹

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Abstract

Measurement of human papillomavirus (HPV) DNA load has been suggested as a means for improving the positive predictive value of HPV testing for detecting cervical intraepithelial neoplasia 3 and cancer (CIN3+). We hypothesized that lesions surrounding CIN3+ (especially the extent of associated CIN1) are important determinants of load, which limit its clinical use. To test this hypothesis, we reviewed the pathology of women at enrollment in the Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion Triage Study who were managed at enrollment with oncogenic HPV testing using Hybrid Capture 2 (Digene Corp., Gaithersburg, MD) or colposcopy. We focused our analysis on 133 cases of CIN3+ that were identified, stratified by the number of HPV types detected using a PCR-based method (62 single, 71 multiple infections). Infection with multiple types ($P < 0.01$) and identification of CIN1 or CIN2 in tissues surrounding CIN3 ($P = 0.01$) were each significantly associated with higher load (two-way ANOVA). Higher load was associated with more extensive CIN1 ($P < 0.01$), especially when associated with multiple HPV types. Intensive partial rescreening of enrollment thin-layer slides obtained from women with histopathological CIN3+ revealed that ASC-US/LSIL cells were more numerous than ASC-H/HSIL cells. ASC-US/LSIL cell counts were significantly correlated with load ($r = 0.31$; $P < 0.01$), whereas counts of ASC-H/HSIL cells were uncorrelated. We conclude that the extent of histopathological CIN1, number of HPV types, and the number of ASC-US/LSIL cells in

exfoliative cervical samples vary greatly among cases of CIN3+ and strongly affect HPV load. These factors severely limit the clinical use of load measurements irrespective of HPV testing methodology.

Introduction

Oncogenic types of HPV³ are the causal agents of virtually every cervical cancer worldwide (1, 2). Accordingly, patients whose exfoliative cervical samples test negative for HPV DNA are unlikely to have cervical cancer or its precursor, CIN3 (3, 4). Furthermore, a negative HPV test also indicates that a patient's future risk of developing CIN3 or cancer (CIN3+) is low for several subsequent years (5). The negative predictive value of HPV testing for CIN3+ is high among women of all ages (reflecting high sensitivity). However, the positive predictive value of HPV testing for CIN3+ is low among young women, a group in which transient, innocuous infections (and their pathological manifestations, cytological LSIL and histological CIN1) are very common (6–10). Measuring HPV load has been considered as a means for improving the positive predictive value of HPV testing based on the assumption that higher load values are more strongly associated with severe disease (e.g., CIN3+; Refs. 10–31).

Despite multiple investigations, which have used various study designs and methodologies, the clinical use of measuring HPV load remains unclear. Investigations have established that higher HPV load levels are more strongly associated with prevalent histopathological CIN, as compared with clinically occult infections (only detectable with DNA testing). However, it is uncertain whether high HPV load is specifically associated with severe grades of CIN, especially when measured using Hybrid Capture 2 (Digene, Gaithersburg, MD), a clinical test that uses a probe mixture to detect 13 oncogenic HPV types. Determination of HPV load using Hybrid Capture 2 has been shown to be linear over a broad dynamic range and has correlated well with quantitative PCR measurements (10, 18). Although Hybrid Capture 2 does not adjust load determinations for specimen cellularity and cannot distinguish between infections with single or multiple types, the assay automatically provides load data, and the test is widely used clinically in the United States. Accordingly, clinical laboratories must grapple with issues of reporting and interpreting these data. Researchers have also developed more sensitive, specific, and quantitative laboratory techniques for measuring HPV load (32). However, the clinical promise of improved methods remains contingent

Received 3/21/03; revised 6/11/03; accepted 7/14/03.

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¹ This work was performed as an approved ancillary study to the ASCUS/LSIL Triage Study.

³ The abbreviations used are: HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; LSIL, low-grade squamous intraepithelial lesion; ASCUS, atypical squamous cells of undetermined significance; ALTS, ASCUS/LSIL Triage Study; HSIL, high-grade squamous intraepithelial lesion; LEEP, loop electrosurgical excision procedure; AIS, adenocarcinoma in-situ.

upon the existence of a specific relationship between HPV load as determined using exfoliative cervical specimens and the underlying cervical pathology, which is the source of these cells. This association has not been fully explored.

We investigated the relationship between HPV load, measured using Hybrid Capture 2, and the underlying pathology at enrollment in the ASCUS/LSIL triage study (ALTS). We hypothesized that two inherent features of HPV load determinations would limit its clinical use for identifying women with ASCUS or LSIL cytology who have an underlying histologically confirmed CIN3: (a) variability in the severity, extent, and HPV DNA content of lower grade lesions surrounding CIN3 lesions; and (b) the intrinsic characteristics of exfoliated specimens obtained by scraping, which necessarily consist mainly of surface epithelium.

Materials and Methods

Study Population. ALTS was a randomized clinical trial that enrolled eligible volunteers who had received community Pap smear results of ASCUS ($n = 3488$) or LSIL ($n = 1572$) in four regions of the United States (November 1996 to December 1998). Ethical review boards at the National Cancer Institute and participating institutions approved the study. At enrollment, women were randomized to one of three immediate management protocols: (a) colposcopy referral for cytology of HSIL based on a liquid-based cytology specimen collected at enrollment (~8 weeks after the community smear of ASCUS or LSIL); (b) colposcopy referral for a positive HPV DNA test for oncogenic viruses (or cytology of HSIL if the HPV test was negative, which almost never occurred); and (c) immediate colposcopy. Women with colposcopically directed biopsies or endocervical curettages diagnosed as CIN2 or worse (including adenocarcinoma *in situ*) were treated with LEEP. Details of the study design and key enrollment results are presented elsewhere (33–35).

The current analysis is limited to enrollment data for women randomized to either the HPV testing or the immediate colposcopy arms. We limited our analysis to enrollment data because results from both Hybrid Capture 2 and PCR-based HPV testing were available. We used Hybrid Capture 2 results to estimate HPV load and PCR testing to assess the number of HPV types present (single *versus* multiple, restricted to the 13 oncogenic types also targeted by Hybrid Capture 2). We excluded women randomized to colposcopy referral for repeat cytology of HSIL because this arm was relatively insensitive in detecting CIN3 at enrollment compared with the other two sensitive arms (34, 35). We also excluded subjects enrolled at one of the four clinical centers, which was unable to release pathology slides for the current investigation (see below), and women with histopathological CIN3+ who were not treated with a LEEP as part of ALTS. Finally, we excluded the relatively small percentage of cases in which HPV was detected by either Hybrid Capture 2 or the PCR method but not by both methods.

In the resultant study group, there were 146 women with histopathological CIN3+, 164 with histopathological CIN2, and 1077 with histopathological CIN1 or oncogenic HPV detection associated with either negative histopathology or a negative colposcopic examination without tissue sampling (referred to collectively as \leq CIN1).

Pathology Review. The ALTS pathology review has been described in detail elsewhere (36, 37). Briefly, a pathology quality control panel reviewed the referral smears (initially interpreted at community laboratories as ASCUS or LSIL), the

enrollment thin-layer slides (prepared, screened, and interpreted at the ALTS clinical centers), histopathology slides from all specimens collected throughout the trial, and most cytology slides obtained during 2 years of follow-up. The clinical center pathology reviews were used primarily to decide patient management; the pathology quality control group reviews were performed to establish the trial end point (histopathological CIN3+) and for subject safety. After the trial was completed, a single pathologist (M. E. S.) performed a poststudy review of all available histopathological slides from all enrollment specimens (biopsies, curettages, and LEEPs) obtained from women diagnosed by the pathology control group with histopathological CIN3+ at enrollment. The goal of the poststudy review was to characterize the size and appearance of the CIN3 lesions (including seven CIN3 lesions associated with invasive carcinoma) and to identify all tissue blocks containing CIN1 (including blocks that were originally classified as CIN2 or more severe by the pathology quality control group and recorded as such in the main ALTS database; Ref. 38).

For the current analysis, we performed an additional poststudy cytological review, masked to preceding interpretations and HPV data of 129 available thin-layer slides prepared at enrollment that had been obtained from women who had received an enrollment histopathological diagnoses of CIN3+. Briefly, a template was prepared by photocopying one of the special thin-layer microscopic slides, which are imprinted with a circle, indicating the area of cellular deposition. We drew two diameters across the center of the photocopied image of this circle. Before screening, the reviewer (M. E. S.) aligned the circle of the template with the slide and marked the ends of the two diameters. The slides were carefully screened microscopically at $\times \sim 400$ (instead of $\times 100$ as usual) along each diameter (~75 microscopic fields) using a mechanical stage to ensure that all fields across both diameters were examined. Finally, we counted cells interpreted as (a) ASC-US⁴ or LSIL and as (b) ASC-H³ or HSIL using criteria proposed in the Bethesda System. We combined categories because our goal was to determine which exfoliated cells (those suggestive of productive HPV infection or those suggestive of high-grade lesions) were correlated with load measurements.

HPV Testing. After preparation of a thin-layer slide, a 4-ml aliquot of PreservCyt (Cytoc Corp., Boxborough, MA) was taken for oncogenic HPV testing (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68) at a detection threshold of 1.0 pg/ml HPV DNA (~5000 viral copies) using Hybrid Capture 2 (Digene Corp.; Refs. 33–39). Testing was performed in a masked fashion at each clinical center. Briefly, alkali denaturation was performed to create single-stranded DNA that was then mixed in microplate wells with a mixture of 13 different type-specific full-length RNA probes. In positive samples, hybrids composed of HPV DNA and RNA probes were captured in sample wells coated with antibodies directed against hybrids composed of DNA and RNA. Subsequently, the presence of HPV DNA was detected by applying a second, fluorescently tagged antibody directed against these hybrids. The light emission from each specimen was compared with a standard containing 1.0 pg/ml HPV-16 DNA (~5000 viral copies) in triplicate. Signal strengths were linearly related to the quantity of

⁴ In Bethesda 2001, the newly created category of atypical squamous cells is qualified as “of undetermined significance (ASC-US)” and “cannot exclude HSIL (ASC-H).”

Table 1 HPV load stratified by histopathological diagnosis and number of HPV types detected

Restricted to women with positive HPV test results by Hybrid Capture 2 and PCR-based method, restricted to 13 types targeted by Hybrid Capture 2. Excludes 31 women with missing or negative HC2 results (1 CIN3 and 30 \leq CIN1); 264 with missing or negative PCR results (4 CIN3+; 15 CIN2; 245 \leq CIN1); and 74 with both results missing or negative (8 CIN3+; 15 CIN2; 51 \leq CIN1).

Histopathological Diagnosis HPV types	No.	HPV Load (pg HPV DNA/ml)		Comparison of load Single versus multiple infections ^a
		Mean \pm SE	Median	
CIN3+ ^b				
Single	62	313.2 \pm 59.4	132.6	$P < 0.01$
Multiple	71	615.9 \pm 63.1	522.0	
CIN2				
Single	71	446.8 \pm 59.5	197.1	$P = 0.02$
Multiple	63	620.5 \pm 66.3	492.9	
\leq CIN1				
Single	451	440.1 \pm 24.8	191.0	$P < 0.01$
Multiple	300	583.8 \pm 33.0	384.6	

^a Wilcoxon two-sample test.

^b CIN3+, CIN3 or invasive carcinoma; \leq CIN1, histopathological CIN1 or HPV infection associated with either negative histopathology or normal colposcopic examination.

HPV DNA (and number of HPV copies) in the sample and are presented as pg/ml of HPV DNA (10, 18).

PCR-based testing was performed on 100–150- μ l aliquots of standard transport medium (Digene Corp.) collected on the same day as the PreservCyt specimen using a biotinylated MY-09-MY11-HMB01 L1 consensus primer set as described previously (35, 40). Biotinylated GH20 and PC04 primers were used to amplify β -globin DNA and confirm specimen adequacy. Genotyping was performed using a reverse-line blot hybridization system (41).

Analysis. The initial analysis compared HPV load for different grades of CIN, stratified by the number of HPV types detected. We used the pathology quality control group histopathological diagnoses for this analysis rather than the poststudy review because the latter did not include cases of CIN2 or \leq CIN1. Of the 146 cases of histopathological CIN3+ diagnosed at enrollment by the pathology quality control group, we excluded 13 cases that did not test positive with both Hybrid Capture 2 and PCR testing for oncogenic HPV types. We excluded these cases because we could not assess load stratified by the number of HPV types present (single versus multiple; presented in Table 1). We used data from the poststudy review for the remaining analyses, which were limited to cases of histopathological CIN3+.

For analyses based on the poststudy histological review of CIN3+ cases, we excluded an additional 12 cases: 5 in which CIN3 was unconfirmed; 1 of AIS without associated CIN3; and 6 for which the LEEP specimen was not available, leaving 121 total cases for analysis (Tables 2–4). Finally, 2 cases were excluded from comparisons of cell counts in thin-layer slides and viral load because of missing values for the volume of fluid required for slide preparation (Table 5).

Given that the goal of HPV load assessment is to increase the positive predictive value of HPV testing for identifying women with CIN3+, we designed the remaining analyses to understand the pathological factors that contributed to load determinations in these cases. We stratified all analyses by the number of oncogenic HPV types detected by PCR (1 or 2+), restricted to the 13 types targeted by Hybrid Capture 2. First, we compared load among CIN3+ cases stratified according to the presence or absence of CIN1 or CIN2 elsewhere in the cervix. For improved statistical power, we combined the rare cases of CIN3+ associated with CIN2 (without CIN1) and the more numerous cases of pure CIN3+. We then examined the relationship between the number

Table 2 HPV load associated with histopathological CIN3+ stratified surrounding lesions

Excludes 12 cases based on the poststudy review: unconfirmed histopathological CIN3 ($n = 5$); AIS unassociated with CIN3 ($n = 1$); and LEEP specimen unavailable for review ($n = 6$).

No. HPV types	Histopathologic findings	No.	HPV load (pg HPV DNA/ml)	
			Mean \pm SE	Median
Single				
	CIN3+ only	14	153.4 \pm 79.6	47.2
	CIN3+/CIN2	2	150.3 \pm 49.5	150.3
	CIN3+/CIN1	15	289.9 \pm 108.6	135.7
	CIN3+/CIN2/(1) Block CIN1	9	251.5 \pm 92.5	189.3
	CIN3+/CIN2/> (1) Block CIN1	18	564.4 \pm 153.7	319.8
Multiple				
	CIN3+ only	8	459.2 \pm 172.7	380.8
	CIN3+/CIN2	2	614.3 \pm 580.7	614.3
	CIN3+/CIN1	23	622.2 \pm 120.4	522.0
	CIN3+/CIN2/(1) Block CIN1	8	432.5 \pm 148.5	360.5
	CIN3+/CIN2/> (1) Block CIN1	22	762.0 \pm 112.1	780.6

of blocks containing CIN1 in LEEP specimens (based on examination of all slides, irrespective of pathology quality control group diagnosis) and HPV load.

HPV testing in ALTS was performed on cervical scrapes, as in most research and clinical practice settings. Exfoliative samples may not contain the same types and relative numbers of cells that are present in women's cervixes. Accordingly, we focused additional analyses on the cytology of the PreservCyt collections that had been used in ALTS for both liquid-based cytology and Hybrid Capture 2 HPV testing. Specifically, we assessed the relationship between concurrent cytopathological interpretations of thin-layer slides and HPV load. Finally, recognizing that the thin-layer cytological specimens obtained from women with histopathological CIN3+ may contain cells showing a spectrum of abnormalities (although classified based on the most severe appearing cells), we compared the counts of ASC-US/LSIL and ASC-H/HSIL cells derived from intensive partial rescreening of thin-layer slides to HPV load. Although a standard 4.0-ml aliquot was used for Hybrid Capture 2 testing, the volume of fluid (sip volume) used to prepare each thin-layer slide varied considerably among specimens. Therefore, we ad-

Table 3 HPV load in cases of histological CIN3+ stratified by the number of blocks containing CIN1

Excludes 12 cases based on the poststudy review: unconfirmed histopathological CIN3 ($n = 5$); AIS unassociated with CIN3 ($n = 1$); and LEEP specimen unavailable for review ($n = 6$). Blocks containing histopathological CIN1 were not identified in 26 cases.

No. HPV types No. of blocks containing CIN1	No.	HPV load (pg HPV DNA/ml)	
		Mean \pm SE	Median
Single			
One	16	281.4 \pm 80.9	194.1
Two	10	412.5 \pm 165.8	124.2
Three	9	135.6 \pm 46.6	65.3
Four Or More	7	989.0 \pm 297.0	791.3
Multiple			
One	19	361.6 \pm 99.6	202.3
Two	8	630.0 \pm 169.0	524.6
Three	14	840.7 \pm 160.0	952.1
Four Or More	12	904.5 \pm 138.2	1066.5

justed the counts of abnormal cells (ASC-US/LSIL or ASC-H/HSIL) to the standard 4.0-ml volume used for HPV testing [(4.0 ml/specimen sip volume) \times (number of abnormal cells counted)]. This correction was performed to improve our approximation of the number of abnormal cells in each 4.0-ml PreservCyt sample that was tested using Hybrid Capture 2 (but not examined microscopically), not to standardize overall specimen cellularity. We then compared cell counts to HPV load, stratified by the number of HPV types detected.

Values for HPV load were presented as means with SE and as medians. Log-transformed data were compared using ANOVA. The Wilcoxon rank test was used for two-way comparisons of nonparametric data and the Kruskal-Wallis test was used for three-way comparisons. The ordinal relationship between the number of blocks of CIN among cases of CIN3+ and HPV load was assessed using linear regression. The relationship between log-transformed cell counts on thin-layer slides, and HPV load was assessed using Pearson's correlation coefficient.

Results

HPV Load and Grade of CIN. Multiple HPV infections were identified in 71 (53.4%) of 133 women with histopathological CIN3+, 63 (47.0%) of 134 with CIN2, and 300 (39.9%) of 751 women with \leq CIN1 (Table 1). Among women infected with single HPV types, those with histopathological CIN3+ had the lowest load. In each of these three diagnostic strata, HPV load was significantly higher among women with multiple HPV infections as compared with those with single infections. However, none of the analyses comparing grade of CIN and HPV load was statistically significant, including those restricted to infections with single or multiple types (Kruskal Wallis, n.s.). Neither mean nor median HPV load was associated with extent of histopathological CIN3 based on previous size assessments (data not shown; Ref. 38). Although types previously shown to cross-react with those targeted by Hybrid Capture 2 were identified frequently in this analysis using PCR testing, exclusion of cases associated with these types (or inclusion of extra cases in which only cross-reactive types were detected) had minor effects on HPV load by category and on patterns of load across categories (data not shown; Ref. 42).

HPV Load in Cases of CIN3+ Stratified by Lesions in Surrounding Cervical Mucosa and Number of HPV Types Detected. Both the histopathological findings and the number of HPV types detected were significantly associated with HPV

Table 4 HPV load and classification of thin-layer cytology among women with histological CIN3+

Excludes 12 cases based on the poststudy review: unconfirmed histopathological CIN3 ($n = 5$); AIS unassociated with CIN3 ($n = 1$); and LEEP specimen unavailable for review ($n = 6$). CC, clinical center cytopathology result.

No. HPV types Thin-layer (CC)	No.	HPV load (pg HPV DNA/ml)	
		Mean \pm SE	Median
Single			
Normal	6	50.8 \pm 26.0	23.0
ASC-US	9	429.2 \pm 227.4	187.0
LSIL	12	425.5 \pm 154.8	167.8
ASC-H	2	537.9 \pm 253.4	537.9
HSIL	29	305.9 \pm 80.4	161.6
Multiple			
Normal	9	319.2 \pm 151.1	84.8
ASC-US	5	454.9 \pm 193.6	356.5
LSIL	21	964.4 \pm 111.3	1134.5
ASC-H	4	232.3 \pm 92.2	231.1
HSIL	24	546.2 \pm 102.3	383.9

load (Table 2; two-way ANOVA, $P = 0.01$ for histopathological findings; $P < 0.01$ for number of types). Among women infected with a single HPV type, cases of CIN3+ only (lower grades of CIN not identified) had the lowest median and the second lowest mean HPV load levels. Irrespective of the number of HPV types detected, CIN3+ lesions associated with both CIN2 (any number of blocks) and multiple blocks of CIN1 had the highest levels.

To explore the relationship between the extent of CIN1 surrounding CIN3 lesions and HPV load, we developed a linear regression model stratifying CIN3+ cases by the number of associated blocks containing CIN1 and the number of HPV types detected (Table 3). Both the number of blocks of CIN1 ($P < 0.01$) and the number of HPV types detected ($P = 0.04$) were significant predictors of HPV load. CIN3+ cases associated with extensive CIN1 had the highest mean and median HPV loads, irrespective of whether one or multiple HPV types were detected. For cases associated with multiple HPV types but not for those associated with a single HPV type, HPV load increased progressively with the number of blocks containing histological CIN1.

HPV Load among Women with CIN3+, Stratified by Cytological Interpretations. Although eligibility for the trial was based on a smear interpreted in the community as ASCUS or LSIL, HSIL was the most common clinical center cytopathological interpretation of enrollment thin-layer slides among women with CIN3+, reported in 53 (43.8%) of 121 cases (Table 4). Overall, both the clinical center cytopathological interpretations and the number of HPV types detected were significantly associated with HPV load (two-way ANOVA, $P < 0.01$ for both), although the patterns were not entirely consistent. Load did not differ significantly between cytopathological categories among cases associated with single HPV types, whereas significant differences between categories were found when multiple types were identified (Kruskal-Wallis, $P < 0.01$). Among cases associated with multiple types, cytological LSIL was associated with a much higher mean and median HPV load than other categories. HPV load was significantly higher among cases of cytological LSIL (Wilcoxon, $P = 0.02$) or HSIL ($P = 0.03$) when multiple types as opposed to a single type were detected. Analysis of load based upon the pathology quality control group review of the same slides was similar (data not shown).

Table 5 Comparison of HPV load and cell counts in ThinPreps adjusted for Sip volume

Excludes 12 cases based on the poststudy review: unconfirmed histopathological CIN3 ($n = 5$); AIS unassociated with CIN3 ($n = 1$); and LEEP specimen unavailable for review ($n = 6$). Note: all cell counts for ASC-H/HSIL in quartile 1 = 0.

Cell counts (Quartiles)	HPV load (mean \pm SE)/median		
	All	Single HPV type	Multiple HPV types
ASC-US/LSIL			
Q1 ($n = 32$)	214.9 \pm 44.5/143.6	164.2 \pm 50.9/89.0	280.1 \pm 76.6/169.4
Q2 ($n = 29$)	325.4 \pm 79.5/95.3	209.5 \pm 91.8/52.3	468.0 \pm 129.9/356.5
Q3 ($n = 30$)	767.6 \pm 110.2/604.7	580.6 \pm 178.7/281.0	910.6 \pm 132.5/960.1
Q4 ($n = 28$)	653.7 \pm 106.7/455.5	487.7 \pm 176.8/275.4	761.1 \pm 130.9/596.4
ASC-H/HSIL			
Q1 ($n = 54$)	450.6 \pm 71.1/188.1	292.6 \pm 76.6/164.2	633.8 \pm 116.5/550.2
Q2 ($n = 10$)	621.7 \pm 185.8/561.7	100.5 \pm 35.2/100.5	751.9 \pm 208.2/600.4
Q3 ($n = 26$)	537.0 \pm 107.7/379.9	442.1 \pm 168.0/199.0	631.8 \pm 136.4/522.0
Q4 ($n = 29$)	453.0 \pm 93.2/267.0	341.8 \pm 140.0/52.3	556.7 \pm 122.5/423.6

HPV Load and Cell Counts in Exfoliative Cervical Specimens among Women with CIN3+. Thin-layer preparations associated with infections by multiple HPV types contained more abnormal cells than those associated with a single HPV type based on systematic rescreening of a portion of each slide (data not shown). ASC-US/LSIL cells were numerically predominant; however, the number of HPV types detected was not significantly associated with the number of ASC-US/LSIL or ASC-H/HSIL cells counted (data not shown). Cases associated with multiple HPV types had higher viral loads than those associated with single types (Table 5). HPV load increased from the first to the third quartile for number of ASC-US/LSIL cells and then decreased slightly in the highest quartile; we did not identify a consistent pattern for load and counts of ASC-H/HSIL cells.

Overall, counts of ASC-US/LSIL cells were significantly associated with HPV load ($r = 0.31$, $P = 0.001$), with little variation between single and multiple infections. In contrast, ASC-H/HSIL cell counts were not associated with HPV load ($r = 0.02$, $P = 0.87$), irrespective of number of HPV types identified.

Discussion

This analysis demonstrated two fundamental reasons for null cross-sectional associations between HPV load and a patient's most severe underlying cervical histopathology. First, the extent of lower grade lesions surrounding CIN3 varied greatly among cases and cells shed from these lesions were important determinants of HPV load. Second, specimens obtained by cervical scraping favored the collection of maturing cells (cytologically appearing as ASC-US/LSIL), even when histopathological CIN3+ was present. These issues are particularly important when HPV testing is performed to determine management of ASC-US cytology because the great majority of CIN3 lesions in these women are small and therefore contribute only fractionally to the cells removed by scraping (38). Our findings indicate that the inherent heterogeneity of cervical pathology and the nature of exfoliative cervical samples make it unlikely that technical improvements in measuring HPV load will greatly improve its clinical use for detecting prevalent CIN3+.

Approximately 50% of CIN3 and CIN2 lesions in our study were infected with multiple oncogenic HPV types. Sensitive PCR methods have found that infections with multiple types are frequent among HPV-infected women with normal cytology and those with histological CIN (7, 43–45). Within any specific grade of CIN, infections with multiple oncogenic types were associated

with significantly higher HPV loads using Hybrid Capture 2 than infections with single types. However, the mean and median HPV loads associated with different grades of CIN, stratified by the number of HPV types detected, were relatively similar, ranged widely, and overlapped. Therefore, even after adjusting for number of HPV types detected, HPV load would not have specifically identified patients with CIN3+. Interestingly, HPV load was similar in cases of CIN2 and \leq CIN1 associated with single-type infections. This may indicate that many cases of CIN2 represent productive infections akin to CIN1 rather than true cancer precursors, but this requires further study.

In well-screened populations, cytology of ASC-US or LSIL may lead to the detection of nearly two-thirds of histological CIN2 or worse among women < 40 years of age and over half of such cases among older women; most women with histological CIN2 or worse do not present with HSIL cytology (46). This result reflects the tendency of cytology to underestimate the severity of small CIN3 lesions (38). Commonly, histopathological CIN1 and CIN2 are more extensive than CIN3 in these cases, although patients receive clinical diagnoses of CIN3, reflecting the most severe pathology present. In this analysis, cases of CIN3 in which CIN1 or CIN2 were not found in the surrounding mucosa were associated with the lowest median HPV loads, whereas increased load was related to the presence of extensive histopathological CIN1 in the surrounding mucosa. These data suggest that diffuse-productive infections (LSIL/CIN1) account for the higher HPV load found among infections with multiple types. Size of the CIN3 lesions was not associated with HPV load, although most lesions were quite small (38).

The important effect of CIN1 on HPV load determinations is consistent with *in situ* hybridization studies, demonstrating that maturing dysplastic squamous cells, especially koilocytes, contain more viral DNA/cell than CIN3 cells (47, 48). Studies of keratinocyte cultures infected with HPV-type 16 have found that small cells contain \sim 100 episomal copies of HPV, whereas large cells contain 3500 copies on average (49). The pattern of results for HPV-18 and HPV-31 were similar, although absolute load values were lower. If findings in culture mirror clinical HPV infections, HPV load determinations may be strongly effected by the extent to which subpopulations of heavily infected cells are sampled.

Among women with underlying histopathological CIN3+, mean and median HPV load among those with LSIL cytology was similar or higher than those with HSIL. On the basis of intensive partial rescreening of thin-layer slides, ASC-US/LSIL cells outnumbered ASC-H/HSIL cells among women with histopatholog-

ical CIN3+, especially when multiple infections were present. These cytological data are consistent with the relatively large extent of CIN1 as compared with CIN3 in these cases and the tendency of scraping to preferentially dislodge maturing dysplastic cells from the epithelial surface. Although cellular maturation in histological CIN3 is limited to the most superficial third of the mucosa, CIN of all grades may contain koilocytes at the surface.

In contrast to ALTS, which used a liquid-based cytological collection for both cytology and HPV testing, other studies have performed HPV testing on a second scrape, which theoretically could sample deeper epithelial layers and yield different HPV load assessments. Although some studies have adjusted load values for total genomic DNA to correct for the variable cellularity of samples, the value of this procedure has been debated (27, 28). Gravitt *et al.* (18) found excellent correlation between determinations of HPV-16 copy number that were adjusted for total cellular DNA and those that had not been adjusted. (Spearman's $\rho = 0.89$).

The number of ASC-US/LSIL cells, even as roughly estimated in this study, was significantly correlated with HPV load, albeit weakly, whereas the number of ASC-H/HSIL cells was uncorrelated. We probably under-estimated the strength of association between ASC-US/LSIL cell counts and HPV load because of our inability to obtain exact cell counts. Therefore, both our histological and cytological data indicated that the pathology in the mucosa surrounding CIN3 lesions was the strongest determinant of HPV load among women with prevalent histopathological CIN3+ in ALTS. In a colposcopic analysis, larger lesion size was associated with higher HPV load, but the contribution of lesions of different grades to total lesion size was not assessed (25). Unlike this study, ALTS did not include women who presented initially with cytology of HSIL (all women had smears interpreted as ASC-US or LSIL in the community before the enrollment liquid-based cytology sample was obtained). However, women with cytological HSIL are automatically referred for colposcopy in the United States, therefore, HPV testing and load determinations are irrelevant for management of these women.

Women with CIN3+ in ALTS were younger than those included in some other studies of HPV load (mean, 25 years; median, 23 years). However, ALTS is representative of women with ASC-US in the United States who are frequently tested clinically. Identification of another marker that stratified HPV-infected women according to risk would be clinically useful, especially given the high prevalence of transient infections in this age group.

Studies have suggested that elevated HPV-16 load among cytologically normal women predicted the future development of high-grade CIN (28, 30). These results would assume added importance if HPV testing were used for primary screening. Interpretation of progression studies raises two important issues: (a) missed prevalent lesions when misclassified as incident inflate the value of baseline load measurements; and (b) the clinical relevance of load is restricted to women with positive HPV tests. Inclusion of women with negative HPV tests in this type of analysis blurs the distinction between the value of a positive HPV test (at any load) and that of high viral load given a positive HPV test. Fluctuation in HPV load over short time intervals (weeks to months) also raises questions about the reliability of point prevalence measurements (50), although analyses of serial measurement using recently developed technology are needed. On the basis of the average of two initial measurements of load using a quantitative PCR method with adjustment for genomic DNA, Schlecht *et al.* (51) found that HPV load of >100 copies/cell (compared with <1 copy/cell) was associated with a relative risk = 2.2 (0.9–5.8) for

cytology of HSIL (CIN3) over 8 years of follow-up. The clinical potential of serial measurements deserves further study, but the variability of values within disease states may limit utility. The value of serial measurements to understand natural history and immunological responses remains of interest.

Although we have found consistent evidence that the cervical mucosa surrounding a CIN3 lesion is an important determinant of HPV load in exfoliative cellular samples, we acknowledge that pathological descriptions and interpretations are not perfectly reproducible and that assessments in other study populations or by other pathologists might yield somewhat different data. Furthermore, we think that a study in which cells were microdissected, lysed, and HPV tested would represent a more direct method for determining the association between cellular composition of a sample and load. However, the consistency of our data suggests that conclusions from such a study would likely be confirmatory.

HPV testing has been designated as the preferred method for managing women with ASC-US when testing can be performed on a sample collected at the initial visit (52) and interest in using HPV testing for primary screening is growing (4). Increased use of HPV testing has created a critical need to identify other markers that distinguish transient infections from those that pose an important cancer risk. Lacking such a secondary test increases the risk of excessive management of women with innocuous HPV infections. However, our data indicate that measurement of HPV load does not fulfill this requirement.

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