Human Papillomavirus Infections with Multiple Types and Risk of Cervical Neoplasia

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Abstract

Background: **Besides an established role for certain human papillomavirus (HPV) genotypes in the etiology of cervical cancer, little is known about the influence of multiple-type HPV infections on cervical lesion risk. We studied the association between multiple HPV types and cervical lesions among 2,462 Brazilian women participating in the Ludwig-McGill study group investigation of the natural history of HPVs and cervical neoplasia.**

Methods: **Cervical specimens were typed by a PCR protocol. The cohort's repeated-measurement design permitted the assessment of the relation between the cumulative and concurrent number of HPV types and any-grade squamous intraepithelial lesions (SIL) and high-grade SIL (HSIL).**

Result: **At individual visits, 1.9% to 3.2% of the women were infected with multiple HPVs. Cumulatively during the first year and the first 4 years of follow-up, 12.3% and 22.3% were infected with multiple types, respectively. HSIL risk markedly increased with the number of types [odds ratio (OR), 41.5; 95% confidence interval (95% CI), 5.3-323.2 for single-type infections; OR, 91.7; 95% CI, 11.6-728.1 for two to three types; and OR, 424.0; 95% CI, 31.8-5651.8 for four to six types, relative to women consistently HPV-negative during the first year of follow-up]. The excess risks for multiple-type infections remained after exclusion of women infected with HPV-16, with high-risk HPV types, or persistent infections, particularly for any-grade SIL. Coinfections involving HPV-16 and HPV-58 seemed particularly prone to increase risk.**

Conclusion: **Infections with multiple HPV types seem to act synergistically in cervical carcinogenesis. These findings have implications for the management of cervical lesions and prediction of the outcome of HPV infections.** (Cancer Epidemiol Biomarkers Prev 2006;15(7):1274–80)

Introduction

Genital infection with human papillomaviruses (HPV) is one of the most common sexually transmitted conditions. The central causal role in cervical carcinogenesis of the so-called high oncogenic–risk HPV genotypes (HR-HPV), especially HPV-16, has been clearly established (1-4). Evidence from cohort studies also indicates that risk of cervical neoplasia is greatest among women who develop persistent HR-HPV infections (5-7). On the other hand, the role played by coinfections with multiple HPV types on cervical neoplasia remains a difficult area of investigation.

Coinfection with multiple HPV types has been observed more frequently among younger women and among those with cytologic abnormalities or impaired immune response (8-15). To date, only a few studies have reported on the relationship between multiple HPV infections and cervical neoplasia, and their results are not consistent. Of these, some studies have suggested a possible role for multiple HPV types in the development or progression of cervical neoplasia (8, 11, 15-20), whereas others have shown that the risk of cervical precancerous lesions or invasive cancer in women infected with multiple HPV types was no greater than that in those with single-type infections (9, 12, 21-25). The disparity in results may originate from the fact that many studies have relied on cross-sectional detection of type-specific HPV infections and thereby may have underestimated the cumulative lifetime diversity of exposure to HPV.

With the recent impetus to include HPV testing in cervical cancer screening programs and the prospect that efficacious HPV vaccines will become available in the near future, it becomes imperative to understand the role played by multiple HPV infections. The goal of this study was to use a cohort study with repeated measurements of viral infection and lesion outcomes to investigate the role of multiple HPV types in the development of cervical neoplasia.

Patients and Methods

Subject Recruitment. The women included in this study were enrolled into the Ludwig-McGill cohort, an ongoing longitudinal investigation of the natural history of HPV infection and precursor lesions of cervical cancer. A detailed description of the design and methods of the study has been published previously (26). Briefly, women attending a maternal and child health program catering to a low-income neighborhood in São Paulo (Brazil) were recruited between 1993 and 1997. Women were eligible to participate if they (*a*) were between 18 and 60 years of age, (*b*) were permanent residents of São Paulo, (*c*) were not currently pregnant and had no intention of becoming pregnant during the first year of follow-up, (*d*) had an intact uterus and no current referral for hysterectomy, (*e*) reported no use of vaginal medication in the previous 2 days, and (*f*) had no reported treatment for cervical disease in the previous 6 months. Subjects gave a signed

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informed consent. The study protocol was approved by institutional ethical and research review boards of the participating institutions in Canada and in Brazil.

The study enrolled 2,528 women, corresponding to a 70% response rate. Subsequently, 66 ineligible women were excluded. Follow-up for the remaining 2,462 women consisted of one visit every 4 months for the first year and two visits per year thereafter. Cervical specimens were taken for Papanicolaou cytology and HPV testing at every visit. In additon, for the first four visits and for each annual visit thereafter, subjects answered an interviewer-administrated questionnaire designed to collect information on sociodemographic, lifestyle, sexual, reproductive, and contraceptive characteristics.

Cervical Cell Specimens. An Accelon biosampler (Medscand, Inc., Hollywood, FL) was used to collect ectocervical and endocervical samples for each visit, and a Papanicolaou smear was prepared on a glass slide and fixed in 95% ethanol. The sampler containing the residual exfoliated cells was immersed in a tube containing Tris-EDTA buffer (pH 7.4) and agitated to release the cells. The tubes containing cell suspensions were frozen until testing. Samples were then sent to the Ludwig Institute for storage and testing. Papanicolaou smears were sent to the Sir Mortimer B. Davis-Jewish General Hospital in Canada, one of McGill University's teaching hospitals, for cytology reading (AF). Cytopathology reports were based on the 1992 Bethesda system for cytologic diagnoses (27); the Papanicolaou smears were read ''blinded'' to all other test results for the same sample and for the same woman. Papanicolaou smear findings were classified as normal, atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion (LSIL), or high-grade SIL (HSIL).

HPV DNA Testing. DNA was extracted from all cervical specimens, purified by spin column chromatography, and amplified by a previously described PCR protocol using MY09/11 primers (28, 29), which amplify a highly conserved 450-bp segment in the L1 HPV gene. Typing of the amplified products was done by hybridization with individual oligonucleotide probes and by the RFLP analysis to identify >40 HPV genital types. Amplified products that hybridized only with a generic probe and did not have a discernible pattern in RFLP analysis were classified positive for unclassified type. To verify the specificity of the hybridizations, we included >30 type-specific positive controls in all membranes. To check the integrity of the DNA material extracted from the specimens, assays also included an additional set of primers (GH20 and PC04) to amplify a 268-bp region of the *β-globin* gene (28). Specimens were tested blindly, and standard precautions were taken to prevent contamination. The HPV types tested include HR-HPV types (and probable HR types) 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82 (3), as well as low oncogenic–risk HPV types (LR-HPV) 6/11, 32, 34, 40, 42, 44, 54, 55, 57, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, CP6108, and other unclassified types. Samples that were found to be negative for both HPV and β-globin DNA by PCR were considered inadequate for analysis.

Statistical Analysis. We used several analytic approaches to study the relation between infection with multiple HPV types and cervical lesion risk. We first used a cumulative cross-sectional approach to consider all the HPV testing and cytology results during the first year of follow-up. This analysis included all women with at least three visits of a maximum of four (including the enrollment baseline visit) that were scheduled as part of the first year of follow-up. The same approach was also used in a separate set of analyses to study cumulative viral and lesion events over the first 4 years of follow-up by including all the women with at least seven visits of a maximum of 10 possible scheduled visits during that period. The aim was to assess the association between the cumulative number of HPV types detected, both concurrently at the same visit and sequentially during follow-up, and the worst cytologic evaluation during the same period of observation. Unconditional logistic regression was used to estimate the odd ratios (OR) and their 95% confidence intervals (95% CI) for the association between multiple infections and lesion outcomes. Women who tested positive for LSIL or HSIL at least once throughout the specified follow-up period were defined as cases and were contrasted with women who remained cytologically negative. Analyses were conducted separately for any-grade SIL and for HSIL only. Women infected with one HPV type, two to three types, and four types or more were contrasted with women who were consistently HPV negative (reference group). Analyses were adjusted for age group at enrollment (18-22, 23-29, 30-39, and ≥40 years) and for ethnicity (White versus non-White). *P*s for linear trend in the relation between number of HPV types and risk were obtained by fitting models for HPV-positive women that considered the between-category contrasts as a single ordinal score as follows: one type = 1, two to three types = 2, and four or more types = 3.

We also repeated the same analyses to estimate the ORs of any SIL and HSIL according to the number of HPV types after excluding all women infected with HPV-16 at least once during the specified follow-up period. Because HPV-16 is the most prevalent HPV type and the most strongly associated with cervical cancer (3), this procedure allowed the assessment of the effect of multiple infections that is not attributable to HPV-16. Similarly, we examined the effect of exclusive infection with multiple LR-HPV types after excluding all women infected with at least one HR-HPV or probable HR-HPV types. Moreover, because subjects with persistent HPV infections may have a higher risk of multiple HPV types (30, 31), we also repeated the analyses for women who only experienced transient infections. To achieve that, we removed all women who were positive at least twice for the same HPV type during follow-up, as they were considered to have a persistent infection. For all analyses, we considered consistently HPV-negative women as the reference category, which allows for measuring ORs within the same scale of magnitude.

Although the above set of analyses permitted gauging associations with coinfections quantitatively, they did not allow the assessment of lesion risk originating from coinfections in the context of specific HPV types. Therefore, we conducted additional analyses for each HPV type when detected alone (single infection) and compared its associated risk with that observed when the same type was detected as coinfection with other HPV types in the same woman during the first year and the first 4 years of follow-up. As above, unconditional logistic regression was used using the same cumulative cross-sectional design. The ORs and respective 95% CIs estimated for each specific (index) HPV type were obtained by first contrasting single infections with those consistently negative for any HPVs (reference) and then by contrasting women with multiple types (plus the index type) with women infected only with single infections with the index type (a separate reference category). In the interest of maximizing statistical precision, we computed models only for any-grade SIL as outcome and for HPV types in which there were sufficient numbers of both single and multiple infections to permit an informative analysis.

In a separate set of analyses, we explored the temporal relationship between concurrent coinfections and lesion risk using generalized estimating equation logistic regression models, which take into account the clustering within each individual caused by the repeated-measurements design. In this approach, correlation between outcome events is treated as a nuisance variable allowing for inference based on the coefficients for the covariates in the model. Models

incorporated an exchangeable correlation pattern for the repeated events. Different models were fitted using a moving time window defined by t, corresponding to the exposure time, and $t + n$, at which time outcome was assessed a specific number of months later. We did separate analyses with increasing intervals (n) between exposure (number of HPV types detected at t) and outcome (SIL and HSIL) assessed at the same time (i.e., cross-sectionally) and at 6, 12, and 24 months later. Ps for linear trend in the relation between number of HPV types and risk were also obtained for generalized estimating equation analysis by modeling the number of HPV types using the linear score, as described above.

All analyses were done using Stata 8.2 (Stata Corp., College Station, TX).

Results

A total of 2,113 women provided at least one adequate sample for cytologic evaluation and HPV DNA testing. Altogether, 15,568 visits were evaluable for these subjects. The mean number of visits was 7.4 (SD = 2.4), and the mean follow-up time was 40.0 months (SD = 17.7). The mean age at baseline was 32.9 years (SD = 8.8, median = 32.0, range = 18-59), and most women (65%) were White.

Table 1 shows the distribution of the women according to the number of HPV types detected in individual visits and the cumulative number of HPV types for the first year and the first 4 years of follow-up. At individual visits, 1.9% to 3.2% of the women were infected with multiple HPV types. Cumulatively during the first year and the first 4 years of follow-up, 12.3% and 22.3% were infected with multiple types, respectively. HPV-16 was usually the most prevalent type regardless of whether it was found in isolation or in coinfections with other types: 9.1% to 13.9% of all instances of HPV infections. The equivalent ranges for other common HR-HPVs were HPV-18, 2.0% to 6.7%; HPV-31, 4.0% to 13.6%; HPV-51, 5.3% to 11.8%; and HPV-53, 7.2% to 11.8%. Among the most common types, the proportions of coinfections (concurrent or sequential) during first-year visits were HPV-16, 50.0%; HPV-53, 64.6%; HPV-51, 64.5%; HPV-31, 62.0%; HPV-58, 62.2%; HPV-18, 34.1%; and HPV-52, 74.4%.

Table 2 shows the cumulative frequencies of the worst lesion grade attained by cervical cytology according to the cumulative number of HPV types for the first year and for the first 4 years of follow-up. Frequencies include prevalent cases at enrollment and incident cases during follow-up for HPV infections and lesions (the numbers of prevalent atypical squamous cells of undetermined significance, LSIL, and HSIL at enrollment were 31, 24, and 14, respectively). The proportions of atypical squamous cells of undetermined significance, LSIL, and HSIL diagnosed in the first year and in the first 4 years increased with the number of HPV types detected in the same follow-up period.

Table 3 presents the age and race-adjusted ORs for the association between the cumulative number of HPV types and any-grade SIL or HSIL detected in each of the two follow-up periods. Stronger associations with both lesion outcomes were observed with increasing number of types. The reference category in all analyses included only women with HPV-negative results during the specified follow-up period. Exclusion of women infected with HPV-16 did not appreciably change the magnitude of the associations or produced only slightly lower associations. Further exclusion of women with any HR-HPVs leaving only those infected with LR-HPV types led to a reduction in risk estimates for all SILs. Indeed, the latter maneuver seemed to blur the distinction between associations for single-type and multiple-type LR-HPV infections, particularly in the first 4-year visits. To verify whether the associations reflected the tendency for multiple-type infections to be more persistent than those by single-type infections, we further restricted the analyses to transient infections only. This restriction led to a decrease in the magnitude of the associations. However, the risk of any-grade SIL and HSIL remained greater for women with multiple types than for those with single-type infections in most combinations that yielded estimable associations. Exclusion of women with HPV-16 and other HR-HPV types had little effect on the associations with any-grade SIL. There were no cases of HSIL or a sufficient number of exposed cases to permit estimating risk among women with exclusively transient infections with LR-HPVs. In general, higher ORs were observed for HSIL than for all SILs, and associations seemed stronger for the cumulative cross-sectional analysis of first-year visits than for the longer follow-up period that included the first 4-year visits, although the latter were still strong.

Table 4 shows the analysis of risk of any-grade SIL for each HPV type when detected as a single infection versus in the context of multiple infections. First-year and first 4-year visits are considered as part of the cumulative cross-sectional analyses. In the interest of precision, we included only HPV types that were most common during the latter observation periods. This set of analyses allows a better understanding of the incremental effect of coinfections on risk while controlling for the presence of specific HPV types. For example, the odds of any SILs during the first year-visits was 24.7 (95% CI, 7.9-77.6) times greater for those infected with HPV-16 alone

Table 1. Occurrence of multiple HPV types in individual visits as well as cumulatively during the first year and the first 4 years of follow-up in the Ludwig-McGill cohort

No. HPV types	No. (%) observed by visit number and scheduled follow-up time*										Cumulative	
	1 (enrolment)	2 (4 mo)	3 (8 mo)	4 (1 y)	5 (1.5 y)	6 (2 y)	7 (2.5 y)	8 (3 y)	9 (3.5 y)	10 (4 y)	First year	First 4 y
0	1,704 (83.1)	1,639 (83.0)	1,568 (83.1)	1,552 (84.3)	510 (83.2)	1,495 (85.7)	916 (86.7)	1,383 (87.1)	1,282 (86.3)	956 (88.4)	1,240 (66.3)	822 (51.8)
1	282 (13.8)	282 (14.3)	267 (14.2)	250 (13.6)	86 (14.0)	215 (12.3)	121 (11.5)	172 (10.8)	160 (10.8)	104 (9.6)	402 (21.5)	411 (25.9)
2	52 (2.5)	47 (2.4)	39 (2.1)	32 (1.7)	10 (1.6)	32 (1.8)	15 (1.4)	23 (1.5)	31 (2.1)	16 (1.5)	158 (8.4)	167 (10.5)
3	8 (0.4)	7 (0.4)	10 (0.5)	8 (0.4)	5 (0.8)	2 (0.1)	3 (0.3)	9 (0.6)	9 (0.6)	3 (0.3)	50 (2.7)	96 (6.1)
4	3 (0.2)	1 (0.1)	1 (0.1)		2 (0.3)	1 (0.1)	2 (0.2)	1 (0.1)	3 (0.2)	2 (0.2)	11 (0.6)	47 (3.0)
5	1 (0.1)		1 (0.1)						1 (0.1)	1 (0.1)	9 (0.5)	26 (1.6)
6											1 (0.1)	12 (0.8)
7												6 (0.4)
8												1 (0.1)
Total*	2,050	1,976	1,886	1,842	613 †	1,745	1,057 †	1,588	1,486	1,082	1,871 ‡	1,588§

*Excluded women with missing HPV test results or β-globin-negative specimens and those with a missing cytologic report.
†Visits 5 and 7 were introduced into the study after many women had the opportunity to complete them.
‡Based on women with at least 3 evaluable visits of 4 possible ones.
§Based on women with at least 7 evaluable visits of 10 possible ones.

Table 2. Worst lesion grade observed by cervical cytology according to the cumulative number of HPV types observed during the same follow-up period

Follow-up period	No. HPV types	Most severe cytologic grade attained				
		Normal, *n* (%)	ASCUS, *n* (%)	LSIL, *n* (%)	HSIL, *n* (%)	Total
First year (3-4 visits)	0	1,195 (96.4)	38 (3.1)	6 (0.5)	1 (0.1)	1,240
	1	336 (83.6)	29 (7.2)	26 (6.5)	11 (2.7)	402
	2-3	148 (71.2)	12 (5.8)	38 (18.3)	10 (4.8)	208
	4-6	11 (52.4)	2 (9.5)	6 (28.6)	2 (9.5)	21
	Total	1,690 (90.3)	81 (4.3)	76 (4.1)	24 (1.3)	1,871
First 4 y (7-10 visits)	0	766 (93.2)	48 (5.8)	7 (0.9)	1 (0.1)	822
	1	343 (83.4)	37 (9.0)	22 (5.4)	9 (2.2)	411
	2-3	185 (70.3)	21 (8.0)	44 (16.7)	13 (5.0)	263
	4-8	49 (53.3)	10 (10.9)	27 (29.3)	6 (6.5)	92
	Total	1,343 (84.6)	116 (7.3)	100 (6.3)	29 (1.8)	1,588

Abbreviation: ASCUS, atypical squamous cells of undetermined significance.

than for those consistently HPV-negative, whereas there was a statistically significant incremental risk (OR, 2.9; 95% CI, 1.1-8.5) of such lesions when HPV-16 was present with other type(s), either concurrently or sequentially. We also found a statistically significant incremental risk (OR, 9.9; 95% CI, 1.8-55.0) for coinfections among women infected with HPV-58. Although not statistically significant, there were noteworthy excess risks of any-grade SIL for coinfections detected among women infected with HPV-18 and HPV-51.

Finally, Table 5 shows the results of generalized estimating equation analysis for the association between the number of HPV types at the same visit (concurrent infections) and any SIL after different periods of time had elapsed because the index infection event. The unrestricted analysis shows a strong association between the number of HPV types and lesions especially with relatively short time delays (6 months) between viral and lesion detection. Apart from the loss in precision, the results seemed comparable when we excluded all women infected with HPV-16. Further restriction to LR-HPV types was informative only for the cross-sectional analysis based on any SILs. Those infected with two to three types had a higher risk of such lesions than those with single-type LR-HPV infections at the same time point. Excluding all the women infected with HR types diminished the probability of finding a high

Table 3. Age- and race-adjusted ORs (95% CIs) for the association between cumulative number of HPV types and worst lesion grade detected during the first-year and first 4-year visits

Outcome, duration of follow-up, and HPV coinfection status	All infections (persistent and transient)			Transient infections only		
	All HPVs	All excluding HPV-16*	All excluding any HR-HPVs† (low risk only)	All transient HPVs‡	Transient excluding HPV-16§	Transient excluding any HR-HPVs‖
Any SIL						
First year visits						
0	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
1 type	19.2 (8.5-43.4)	18.5 (8.0-42.4)	9.7 (3.3-28.5)	10.1 (4.1-25.2)	12.0 (4.8-29.8)	8.1 (2.3-28.4)
2-3 types	57.0 (25.1-129.2)	56.9 (24.6-131.5)	18.6 (3.5-98.8)	37.5 (14.6-96.3)	36.0 (13.4-97.1)	39.7 (6.4-245.1)
4-6 types	141.7 (42.2-476.1)	102.0 (23.7-440.1)	x¶	x	x	x
P_{trend}**	<0.001	<0.001	ND	ND	ND	ND
First 4-y visits						
0	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
1 type	8.6 (3.9-19.0)	9.0 (4.0-20.0)	6.3 (2.3-17.3)	5.2 (2.2 -12.5)	5.9 (2.4-14.2)	5.0 (1.6-15.8)
2-3 types	29.2 (13.6-62.8)	27.9 (12.7-61.3)	6.6 (0.8-56.9)	12.0 (4.8-30.3)	11.5 (4.3-30.5)	11.3 (1.2-104.8)
4-8 types	64.1 (27.6-148.9)	53.4 (20.5-139.1)	x	x	x	x
P_{trend}	<0.001	<0.001	ND	ND	ND	ND
HSIL only						
First year visits						
0	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
1 type	41.5 (5.3-323.2)	33.4 (4.1-268.9)	—¶	14.6 (1.5-141.8)	17.3 (1.8-168.0)	—
2-3 types	91.7 (11.6-728.1)	62.4 (7.4-528.4)	—	62.2 (6.3-617.6)	47.9 (4.2-543.5)	—
4-6 types	424.0 (31.8-5651.8)	283.7 (13.2-6111.7)	x	x	x	x
P_{trend}	0.010	0.102	ND	ND	ND	ND
First 4-y visits						
0	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
1 type	20.1 (2.5-159.3)	18.6 (2.3-152.1)	—	3.1 (0.2-49.6)	3.6 (0.2-58.1)	—
2-3 types	51.0 (6.6-395.8)	30.8 (3.6-260.4)	—	23.3 (2.0-269.4)	13.0 (0.8-216.0)	—
4-8 types	90.1 (10.3-787.2)	55.3 (4.6-658.0)	x	x	x	x
P_{trend}	0.005	0.199	ND	ND	ND	ND

NOTE: Cases of any-grade SIL or HSIL were compared with subjects who were consistently HPV negative for the specified follow-up span.
Abbreviation: ND, not determined.
*All women positive at least once for HPV-16 during the specified follow-up period were excluded.
†All women positive at least once for any HR-HPVs (established or probable) during the specified follow-up period were excluded.
‡All women positive at least twice for the same HPV type during the specified follow-up period were excluded.
§All women positive at least twice for the same HPV type or at least once for HPV-16 during the specified follow-up period were excluded.
‖All women positive at least twice for the same HPV type or at least once for any HR-HPVs during the specified follow-up period were excluded.
¶Not estimable: combinations with either very few or no exposed subjects (x) or no lesions (—).
**P for linear trend in the relation between number of types and risk among HPV-positive women only.

number of HPV types for a particular visit, thereby considerably decreasing the number of persons at risk for analysis and thus rendering inconclusive the analysis of some combinations. Interestingly, the risk of HSIL seemed to be considerably greater for women infected with four to six HPV types compared with those with two to three types or with a single infection. Excluding women infected with HPV-16 had somewhat of an effect on the magnitude of the ORs for HSIL. No HSILs were observed among women infected only with LR-HPVs.

Discussion

Although there is consensus that most instances of cervical neoplasia originate from HPV infection, little is known about the additional influence of infections with multiple HPV types on cervical carcinogenesis. The results of the few studies that have reported on this issue are not consistent. At least in part, the heterogeneity in findings may stem from the fact that most studies have been based on prevalence analysis via cross-sectional detection of type-specific HPV infections, which may underestimate the cumulative effects due to exposure to different HPV types over time. Longitudinal, repeated-measurement investigations such as ours have the advantage of permitting the assessment of lesion risk in the context of coinfections detected sequentially over time spans that are relevant to the natural history of cervical carcinogenesis. We conducted a number of complementary analyses to assess the quantitative risk effect of infections with multiple HPV types with and without data restrictions to permit the evaluation of qualitative influences on lesion risk due to individual or grouped types based on their presumed oncogenic potential. In some of the restrictions, we also assessed whether the incremental risk detected with multiple types could have originated from a greater tendency for such infections to persist. Our findings suggest that infections with multiple HPV types may play a role in the development and/or progression of lesions that exceeds that due to infection with a single type. In most combinations, there was evidence that lesion risk increased with the cumulative number of HPV types, and that the associations seemed particularly strong in the short term (i.e., within the first year of follow-up). Even analyses that eliminated the possible effect of persistent HPV infection provided some evidence for the incremental risk effect of infections with multiple HPV types.

Qualitatively speaking, the excess risk contributed by multiple infections seemed to remain even after excluding women who harbored HPV-16, the type to which the greatest carcinogenic potential is most commonly ascribed (3, 32, 33). Moreover, we also found that coinfections with LR-HPV types also influenced incrementally the risk of LSILs. However, having multiple LR-HPV types exclusively did not increase the risk of HSIL.

By focusing on concurrent coinfections, the generalized estimating equation analyses indicated that there was some waning of the risk effects over time, which was particularly more pronounced for any-grade SILs than for HSILs only. The results of the latter analysis and those from the comparison of first-year and 4-year visits provide support for the notion that cytologic abnormalities may be a reflection of viral load contributed by productive infections, many of which may regress over time resulting in less clearly identifiable lesions.

Before we consider the implications of these results, we need to assess the potential limitations of this investigation. First, the actual number of HPV types in a specimen may be greater than

Table 4. Age- and race-adjusted ORs and 95% CIs for the association between HPV infection status with respect to a specified HPV type and any-grade SIL observed cumulatively during the first year and the first 4 years of follow-up

HPV type	Coinfection status (and contrast*)	First year visits		First 4-y visits	
		n (cases/non cases)	OR (95% CI)	*n* (cases/non cases)	OR (95% CI)
	Negative	7/1,195		8/766	
6/11	Single (vs negative)	1/7	30.7 (2.1-323.5)	1/7	18.7 (1.9-181.8)
	Multiple (vs single)	8/18	2.7 (0.3-26.5)	10/32	2.7 (0.3-26.6)
16	Single (vs negative)	6/47	24.7 (7.9-77.6)	4/53	7.1 (2.1-24.4)
	Multiple (vs single)	14/39	2.9 (1.1-8.5)	33/66	7.5 (2.4-23.1)
18	Single (vs negative)	1/22	7.6 (0.9-65.0)	2/14	14.3 (2.7-77.4)
	Multiple (vs single)	3/11	6.9 (0.6-77.8)	9/20	3.2 (0.6-17.5)
31	Single (vs negative)	3/15	42.2 (9.3-192.2)	4/13	39.6 (9.6-163.4)
	Multiple (vs single)	6/21	1.6 (0.3-7.8)	13/23	1.7 (0.4-6.8)
33	Single (vs negative)	1/7	28.1 (2.9-276.1)	1/5	18.0 (1.9-175.5)
	Multiple (vs single)	5/8	3.8 (0.3-44.8)	11/12	5.5 (0.5-58.3)
35	Single (vs negative)	2/7	53.1 (8.8-318.9)	0/10	-
	Multiple (vs single)	5/6	2.8 (0.4-20.9)	12/16	-
39	Single (vs negative)	1/3	143.9 (9.1-2286.4)	2/3	136.1 (13.1-1416.9)
	Multiple (vs single)	2/3	1.3 (0.1-28.7)	7/6	2.4 (0.3-23.2)
40	Single (vs negative)	1/4	73.9 (6.0-917.7)	1/3	31.7 (2.9-351.2)
	Multiple (vs single)	2/3	2.2 (0.1-50.5)	4/7	2.8 (0.2-41.2)
51	Single (vs negative)	2/18	15.8 (3.0-84.3)	0/22	—
	Multiple (vs single)	12/25	4.6 (0.9-23.4)	25/50	—
52	Single (vs negative)	4/6	121.6 (27.4-540.6)	3/9	32.0 (7.0-146.0)
	Multiple (vs single)	11/14	1.2 (0.3-5.5)	15/20	3.2 (0.6-15.8)
54	Single (vs negative)	1/10	13.9 (1.4-136.4)	1/14	5.2 (0.6-46.3)
	Multiple (vs single)	2/13	1.7 (0.1-27.8)	9/16	14.2 (1.4-140.6)
56	Single (vs negative)	5/6	150.7 (35.0-649.3)	2/4	76.3 (10.6-550.1)
	Multiple (vs single)	2/8	0.4 (0.1-3.5)	16/9	3.5 (0.5-23.2)
58	Single (vs negative)	3/14	32.3 (7.4-141.0)	1/16	6.1 (0.7-52.6)
	Multiple (vs single)	11/16	9.9 (1.8-55.0)	14/26	12.6 (1.3-120.2)
68	Single (vs negative)	1/8	20.0 (2.1-189.4)	2/9	27.5 (4.7-159.4)
	Multiple (vs single)	4/15	1.9 (0.2-21.7)	11/22	1.5 (0.2-9.5)
73	Single (vs negative)	1/9	18.0 (1.9-171.7)	2/8	26.4 (4.5-156.4)
	Multiple (vs single)	3/8	4.3 (0.3-59.9)	6/25	1.1 (0.2-7.9)

*Reference category.

Table 5. Age- and race-adjusted ORs and 95% CIs for the association between the concurrent number of HPV types detected at a given visit and cervical lesions observed at specified follow-up returns

Lesion outcome	Association measured	No. HPV types	OR (95% CI)*		
			All HPVs	All excluding HPV-16†	LR only‡
Any-grade SIL	At the same visit	0	1 (reference)	1 (reference)	1 (reference)
		1 type	25.7 (17.9-36.8)	25.1 (16.7-37.7)	13.0 (6.0-28.3)
		2-3 types	53.4 (34.5-82.7)	51.8 (29.8-90.2)	79.3 (9.1-687.7)
		4-6 types	84.1 (27.3-258.9)	41.0 (3.8-445.6)	x
		P_{trend}§	<0.001	0.004	ND
	6 mo later	0	1 (reference)	1 (reference)	1 (reference)
		1 type	15.4 (8.7-27.1)	11.7 (6.0-22.8)	2.7 (0.3-29.9)
		2-3 types	25.9 (12.0-56.3)	18.0 (5.3-61.1)	—
		4-6 types	247.3 (47.9-1276.4)	—	—
		P_{trend}	0.009	ND	ND
	12 mo later	0	1 (reference)	1 (reference)	1 (reference)
		1 type	7.6 (4.9-11.9)	7.5 (4.3-13.0)	9.9 (2.5-39.0)
		2-3 types	13.2 (6.9-25.4)	9.6 (3.1-29.3)	—
		4-6 types	60.3 (12.0-302.7)	—	—
		P_{trend}	*0.033*	*ND*	*ND*
	24 mo later	0	1 (reference)	1 (reference)	—
		1 type	4.2 (2.6-7.0)	3.8 (2.0-7.4)	—
		2-3 types	6.2 (2.7-14.1)	1.8 (0.2-15.9)	—
		4-6 types	—	—	—
		P_{trend}	*ND*	*ND*	ND
HSIL only	At the same visit	0	1 (reference)	1 (reference)	—
		1 type	19.8 (10.5-37.2)	16.6 (7.7-35.8)	—
		2-3 types	20.6 (8.2-51.7)	5.3 (0.5-52.4)	—
		4-6 types	122.0 (23.8-625.5)	—	—
		P_{trend}	0.507	ND	ND
	6 mo later	0	1 (reference)	1 (reference)	—
		1 type	35.8 (7.8-164.7)	43.9 (4.8-399.6)	—
		2-3 types	82.5 (14.6-467.8)	—	—
		4-6 types	1330.8 (98.9-17911.6)	—	—
		P_{trend}	0.011	ND	ND
	12 mo later	0	1 (reference)	1 (reference)	—
		1 type	8.5 (3.4-20.9)	6.1 (1.8-20.6)	—
		2-3 types	6.5 (1.2-36.2)	10.5 (1.1-98.5)	—
		4-6 types	185.2 (26.31304.5)	—	—
		P_{trend}	0.011	ND	ND
	24 mo later	0	1 (reference)	1 (reference)	—
		1 type	5.7 (1.9-17.4)	—	—
		2-3 types	11.8 (2.7-50.7)	3.3 (0.5-21.1)	—
		4-6 types	—	—	—
		P_{trend}	ND	ND	ND

NOTE: Analysis by generalized estimating equations.
Abbreviation: ND, not determined.
*Not estimated for combinations with no subjects in the stated exposure category or with no observed lesions.
†All women positive at least once for HPV-16 during the entire follow-up were removed from the analysis.
‡All women positive at least once for any HR-HPVs during the entire follow-up were removed.
§*P*s for linear trend in the relation between number of types and risk among HPV-positive women only.

the coinfections that are detected (11, 18). Consensus primer PCR assays, such as the MY09/11 protocol used in the Ludwig-McGill study, are not optimized for amplifying multiple types in the same mixture, and some HPV types are amplified less effectively than others. Underestimation of the number of types for those who developed cervical lesions could underestimate the effect of multiple types. In addition, some of the combinations restricted to certain infection categories lacked sufficient precision to be informative.

Arguably, the most important limitation of our study may be the reliance on cytologic ascertainment of cervical lesions without corresponding biopsy results. Admittedly, cytologic grade is a ''softer,'' surrogate expression of the histologic structure of the cervical epithelium at risk and may be more prone to be ''confounded'' by the cytopathic effects stemming from productive viral infections. On the other hand, for clinical prediction, management algorithms for lesion triage are triggered by cytology results, which constitute a more readily ''observable'' state of the natural history of cervical neoplasia than histologic assessment. Although the cytology assessments were carefully conducted in a reference laboratory following strict quality control and without knowledge of exposure status, cytologic misclassification may have resulted in an attenuation of risk estimates. We opted for an intensive, expert cytologic follow-up every 4 to 6 months of all smears collected in the study to avoid performing unnecessary biopsies, which would have interfered with the natural history of lesions. Use of intensive cytologic testing over time also permitted us to evaluate repeated lesion episodes.

Coinfection with multiple HPV types is a common finding of many molecular epidemiologic studies. Some HPV types might interact or act synergistically to induce lesion development or progression. The risk of high-grade lesions and invasive cervical cancer seems to be considerably increased among women with multiple-type infections compared with those harboring a single HPV type (19). Morrison et al. (16) and Herrero et al. (20) have shown that the risk of LSIL strongly increases when HPV-16 and other types are not present alone. Moreover, Fife et al. (11) suggested that HPV types 51, 52, 56, and 58 are among the types that might cooperate with HPV-16 to produce dysplasia or cancer. In the present study, we found that HPV types other than HPV-16 might also interact and add to baseline risks observed with single-type infections. Our analyses controlling for individual HPV types provided some

evidence that risk in the context of HPV types 16 and 58 may be particularly modulated by coinfections with other types. Even when we excluded women with HPV-16 from the analysis of HPV-58 (data not shown), we observed that coinfections in the context of the latter provide a substantial increment in risk. HPV types that belong to the same species share biological properties that may translate into similar courses of lesion induction and progression (3). It is possible that HPV types belonging to the A9 species (HPV-16, HPV-58, and related) or other types, such as HPV-51, may exert oncogenic effects more rapidly and more extensively in cervical epithelia infected with other HPV types.

In conclusion, it is well known now that HPV-16 and persistent infections with the same HR-HPV type are more likely to progress toward cervical neoplasia, but the present study provides evidence for the notion that infections with multiple types might contribute additional prognostic value. This finding has implications in terms of management of cervical lesions and clinical prediction of the outcome of HPV infections. Current standards for cervical cancer screening in North America and in Europe are based on aggregate detection of the main HPV types presumed to have oncogenic potential, but strong evidence has emerged in recent years in favor of HPV typing as a better prognostic (34, 35). Corroboration of our findings by future studies may assist in the identification of particularly high-risk combinations of types present in coinfections and will likely provide additional impetus for the incorporation of HPV typing in clinical practice. Likewise, as the new era of HPV vaccination begins, improved understanding of the epidemiology of HPV coinfections will also help in planning HPV testing for the surveillance of immunized individuals.

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