

Multiple Human Papillomavirus Infections with High Viral Loads Are Associated with Cervical Lesions but Do Not Differentiate Grades of Cervical Abnormalities

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Multiple human papillomavirus (HPV) genotypes often coexist within cervical epithelia and are frequently detected together in smears of different grades of cervical neoplasia. Describing the association between multiple infections and cervical disease is important in generating hypotheses regarding its pathogenesis. We analyzed the prevalence of multiple HPV infections and their attribution to cervical disease in a screening population of 999 consecutive BD SurePath liquid-based cervical cytology samples enriched with atypical squamous cells of undetermined significance (ASCUS) ($n = 100$), low-grade squamous intraepithelial lesions (LSIL) ($n = 100$), and high-grade squamous intraepithelial lesions (HSIL) ($n = 97$). HPV genotyping was performed only on cytology specimens using a broad-spectrum GP5⁺/6⁺-PCR/multiplex HPV genotyping (BSGP5⁺/6⁺-PCR/MPG) assay that detects and quantifies 51 HPV genotypes and 3 subtypes. Using a recently defined high viral load cutoff, the quantitative data were scored as high or low viral load. In the 36-month follow-up, 79 histologically confirmed cervical intraepithelial neoplasia grade 2 or greater (CIN2+) cases were identified. In the screening population, there was a trend of having more multiple infections at a younger age. Multiple HPV infections were common. Multiple HPV types were most prevalent in LSIL (75.9% of HPV positives), followed by HSIL (65.5%), ASCUS (64.6%), and negative for intraepithelial lesion or malignancy (NILM) (36.8%). On average, 3.2 and 2.5 HPV types were detected per LSIL and HSIL sample, respectively. Multiple HPV types with high viral loads were most prevalent in LSIL (62.6% of high viral load positives), followed by HSIL (51.9%), ASCUS (40.7%), and NILM (19.3%). Patients with multiple high viral loads showed a 4- to 6-fold-higher risk of having cervical precancerous cytological lesions than did patients with single high viral loads. Compared to NILM, multiple infections, especially with multiple high viral loads, were significantly associated with cytological precancerous lesions. However, the presence of multiple infections did not distinguish low-grade from high-grade cytological lesions.

Human papillomaviruses (HPV) are DNA viruses that infect cutaneous and mucosal epithelia. Until now, 120 HPV genotypes have been characterized based on sequence information (1), with evidence that even more exist (2). There are 51 mucosal HPV types that have been recognized, and these are further divided into three groups based on their epidemiological association with cervical cancer (CxCa): 14 high-risk HPV (Hr-HPV) types (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68), 6 possibly high-risk HPV (pHr-HPV) types (HPV26, -53, -67, -70, -73, and -82), and 31 low-risk HPV (Lr-HPV) types (HPV6, -7, -11, -13, -30, -32, -34, -40, -42, -43, -44, -54, -61, -62, -69, -71, -72, -74, -81, -83, -84, -85, -86, -87, -89, -90, -91, -97, -102, -106, and -114) (3, 4). Recently, HPV types HPV55 and HPV64 have been removed from the list of distinct HPV types, as they share 93% sequence homology to HPV44 and HPV34, respectively (5). By definition, these types are referred to as subtypes, as is also the case for HPV68a and HPV68b. Hr-HPV DNA has been detected in 99.7% of cervical cancer tissues (6), and persistent infection with an Hr-HPV type, particularly HPV16 or HPV18, is recognized as a necessary cause of CxCa (7). CxCa develops from common acute HPV infections through less-common persistent Hr-HPV infections and a series of stages histologically classified as cervical intraepithelial neoplasia 1 to 3 (CIN1 to CIN3), or cytologically defined as low- or high-grade squamous intraepithelial lesion (LSIL or HSIL) (8).

Identifying the individual HPV types in each grade of cervical neoplasia is important for the development of HPV vaccines and screening strategies (9). These data are required to estimate the efficacy of current vaccines and to determine which HPV types should be included in the next generation of vaccines. In addition, these data will also form the basis for selecting the HPV types to be included in future HPV screening assays. Moreover, it will be important to monitor possible changes in HPV type distributions in the vaccinated and HPV-screened population to better understand cross-protection and to identify putative new Hr-HPV types once HPV16 and HPV18 are eliminated. However, identifying the “driving” genotype for each grade of cervical neoplasia is difficult, because multiple HPV genotypes are frequently detected within the epithelium of the uterine cervix (10). In addition, multiple cervical lesions within an individual patient might be caused by different genotypes, and a precursor lesion, caused by a specific

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carcinogenic genotype, can be surrounded by transient HPV infections (11).

In the present analysis, we evaluated the distributions of multiple HPV DNA-positive infections and multiple high viral load infections by analyzing 51 mucosal HPV types and 3 subtypes by the broad-spectrum GP5⁺/6⁺-PCR/multiplex HPV genotyping (BSGP5⁺/6⁺-PCR/MPG) assay in liquid-based cervical samples comprising the disease continuum from HPV infection to invasive cancer. The specimens were collected as part of routine cytological screening practices (which, due to their widespread nature, are then assumed to offer sufficient representation for general population).

MATERIALS AND METHODS

Clinical specimens. The current study is part of the VALGENT project, which provides a comprehensive design to validate and compare general HPV tests (identifying Hr-HPV infections) and HPV genotyping assays (identifying some or all high-risk HPV types separately) (12–14). Starting 1 October 2006, 999 consecutive cytology samples were collected during routine gynecological health checks from women in Flanders, Belgium. All samples were collected with a Cervex-Brush Combi and prepared using BD SurePath technology for liquid-based cytology (15, 16). The study samples were supplemented by smears from patients with atypical squamous cells of undetermined significance (ASCUS) ($n = 100$), LSIL ($n = 100$), and HSIL ($n = 97$), as described recently (13, 14). Study-specific patient identification codes were assigned and transmitted in such a manner that patient confidentiality was preserved. According to Belgian law (as of 20 May 2004), no informed consent or any action of the patient was required for this retrospective study because the anonymity of the patients was guaranteed. During the 36-month follow-up, 79 histologically confirmed CIN2+ (CIN2, $n = 27$; CIN3, $n = 47$, or carcinoma, $n = 5$) were identified. Among the 297 women with abnormal cytology, 15 samples (5 ASCUS, 8 LSIL, and 2 HSIL) with 3 confirmed CIN3 cases were excluded from the analysis due to insufficient DNA material for PCR in Heidelberg (<5 μ l), resulting in a total of 1,281 specimens.

Cytology. Thin-layer slide preparations were made with the fully robotic AutoCyte PREP System7 (Tripath Imaging, Inc.) and were prepared as described previously (17). HPV-targeted cytology was read on all cytology samples with prior knowledge of quantitative PCR (qPCR) HPV DNA data (14). The cytological results were classified according to the 2001 Bethesda System (18). Women with cytological abnormalities were managed according to Belgian follow-up guidelines (19).

Clinical outcomes. We considered the presence of CIN2+, identified through the usual diagnostic workup of screen-test-positive women, as a threshold of clinical disease outcome. Screen-test-negative women were not submitted to diagnostic verification. We therefore considered women who had two or more consecutive cytological results of “no intraepithelial lesion or malignancy” (NILM) with a time interval of ≥ 1 year within the 36 months of follow-up to be free of CIN2+. This group was selected for computing clinical specificity. An assessment of accuracy was restricted to women with available clinical outcome information.

Isolation of DNA from cervical cells. DNA isolation from BD SurePath liquid-based cytology leftover was performed within 1 to 2 days after the collection of the samples, as described previously (16, 20). The isolated DNA was divided into 5 aliquots, and one aliquot was sent to Heidelberg for HPV genotyping using the BSGP5⁺/6⁺-PCR/MPG assay.

BSGP5⁺/6⁺-PCR/MPG assay. The BSGP5⁺/6⁺-PCR/MPG assay comprises the BSGP5⁺/6⁺-PCR, which homogeneously amplifies all 51 genital HPV types and 3 HPV subtypes generating biotinylated amplicons of ~150 bp from the L1 region (21) and a multiplex HPV genotyping (MPG) assay with bead-based Luminex xMAP suspension array technology. While the previous version of the assay identified 27 HPV types (Hr-HPV types HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68b, pHr-HPV types HPV26, -53, -67, -70, -73, -82, and Lr-HPV

types HPV6, -11, -30, -42, -43, -44, and -69) and the β -globin gene (21, 22), an additional 24 HPV types (Lr-HPV types HPV7, -13, -32, -34, -40, -54, -61, -62, -71, -72, -74, -81, -83, -84, -85, -86, -87, -89, -90, -91, -97, -102, -106, and -114) and 3 subtypes (Lr-HPV55, HPV64, and Hr-HPV68a) have been recently added to MPG (13).

One aliquot of isolated DNA was analyzed, blinded to cytological and histological results, by the BSGP5⁺/6⁺-PCR/MPG assay. The HPV genotyping and β -globin assessment by the BSGP5⁺/6⁺-PCR/MPG assay were performed as described previously (10, 14, 21, 22). HPV amplification was carried out using the Multiplex PCR kit (Qiagen, Hilden, Germany). The results were expressed in median fluorescence intensities (MFI) of ≥ 100 beads per sample. The cutoff value (5 net MFI) to define HPV DNA positivity was applied as described previously (21). Quantification of HPV signals was accomplished by computing for each positive reaction mixture the relative HPV MFI signal (%) by dividing the measured HPV MFI value by the maximum value detected of this HPV type using colony PCR products. Finally, the relative MFI (expressed as a %) was divided by the measured β -globin MFI value to form a nondescriptive viral load value (%HPVMFI/ β -globin MFI). High viral load was classified for all HPV types by a predefined high viral load cutoff (0.0007 units) that was recently described for Hr-HPV types (14). This high viral load cutoff was developed to distinguish smears with normal cytology from those with cervical abnormalities, and it correlated to 0.46 HPV copies per cell using real-time PCR.

Statistical analysis. Statistical analyses were performed with the SAS software, version 9.2 (SAS Institute). *P* values of < 0.05 were considered statistically significant. Logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CI), which describe the association between multiple infections and cervical abnormalities. In general, a woman infected with > 1 of the 51 HPV types was considered to have multiple infections. For the computation of OR, multiple infections were further stratified into multiple infections analyzed by 14 Hr-, 20 Hr- and pHr-, or 51 Hr-, pHr-, and Lr-HPV types. Cytologically defined LSIL and HSIL, as well as histologically defined CIN1, CIN2, and CIN3, were defined as cervical abnormalities.

RESULTS

Multiple HPV infections in the screening population stratified by age. The overall frequency of HPV DNA positivity and high viral load in the screening population was highest in the youngest age group (15 to 24 years) (HPV DNA positivity, 55.6%; high load, 31.3%) and gradually decreased with increasing age (Table 1). Two weaker peaks were observed in the age groups of 55 to 64 years (HPV DNA positivity) and 45 to 54 years (high load). In contrast, the relative frequency of multiple infections among HPV-positive women peaked in the age group of 25 to 34 years (HPV DNA positivity, 54.0%; high load, 40.7%).

The overall HPV prevalence, as well as the relative frequency of multiple infections, decreased with age when we analyzed only 14 Hr-HPV types. The overall frequencies of HPV DNA positivity and high viral load were highest in the youngest age group, reaching 40.4% and 20.23%, respectively. Irrespective of the number of HPV types analyzed, multiple infections (DNA positive and high viral load) were detected more frequently than single infections at a younger age (Table 1).

Multiple HPV infections stratified by cytology. Multiple infections were most prevalent in LSIL (75.9% of HPV-positive smears, with an average of 3.2 HPV types per HPV positive sample), followed by HSIL (65.5%, 2.5 HPV types), ASCUS (64.6%, 2.6 HPV types), and NILM (36.8%, 1.6 HPV types) (Table 2). In a single ASCUS sample, 17 concurrent HPV genotypes were detected. Restricting the analysis to high viral load infections, there was a reduction in the number of multiple infections; however, the

TABLE 1 Multiple infection DNA positivity and multiple high viral loads with all 54 HPV (sub)types or 14 Hr-HPV in the Belgian screening population stratified by age

HPV type	Age group (yr) ^a	HPV-negative (n)	DNA positivity					High load					Total samples (n)		
			Single infection		Multiple infection		Overall frequency (%) ^b	Multiple infections vs all DNA positives (%)	Single infection		Multiple infection			Overall frequency (%) ^b	Multiple infections vs all high loads (%)
			n	%	n	%			n	%	n	%			
54 HPV (sub)types	15–24	44	27	27.3	28	28.3	55.6	50.9	20	20.2	11	11.1	31.3	35.5	99
	25–34	133	46	19.7	54	23.2	42.9	54.0	35	15	24	10.3	25.3	40.7	233
	35–44	186	50	18.6	33	12.3	30.9	39.8	20	7.4	12	4.5	11.9	37.5	269
	45–54	133	42	20.9	26	12.9	33.8	38.2	18	9.0	7	3.5	12.4	28.0	201
	55–64	84	30	22.9	17	13.0	35.9	36.2	12	9.2	2	1.5	10.7	14.3	131
	≥65	46	12	18.5	7	10.8	29.2	36.8	7	10.8	1	1.5	12.3	12.5	65
	Total	626	207	20.7	165	16.5			112	11.2	57	5.7			998
14 Hr-HPV	15–24	59	24	24.2	16	16.2	40.4	40.0	16	16.2	4	4.0	20.2	20.0	99
	25–34	151	51	21.9	31	13.3	35.2	37.8	30	12.9	12	5.2	18.0	28.6	233
	35–44	214	37	13.8	18	6.7	20.4	32.7	14	5.2	4	1.0	6.7	22.2	269
	45–54	152	39	19.4	10	5.0	24.4	20.4	14	7.0	2	1.0	8.0	12.5	201
	55–64	102	21	16.0	8	6.1	22.1	27.6	5	3.8	1	0.8	4.6	16.7	131
	≥65	51	10	15.4	4	6.2	21.5	28.6	4	6.2	0	0.0	6.2	0.0	65
	Total	729	182	18.2	87	8.7			83	8.3	23	2.3			998

^a For one patient in the screening population, no age information was available.

^b Overall frequency is the number of HPV positives to the number of all samples, expressed as a percentage.

order remained the same, with greatest prevalence in LSIL (62.6% of HPV-positive smears, with an average of 2.7 HPV types), followed by HSIL (51.9%, 2.1 HPV types), ASCUS (40.7%, 1.9 HPV types), and NILM (19.3%, 1.2 HPV types). Multiple high viral load infections with up to 7 (HSIL), 6 (ASCUS), and 12 concurrent HPV types (LSIL) were detected. In contrast, in NILM, only triple high viral load infections were observed. Age-adjusted odds ratios showed a significant positive association between multiple infections and the presence of LSIL and HSIL compared to NILM, which was stronger for multiple high viral load infections than for multiple DNA positivity (Table 3). No differences were seen in the relative frequencies of multiple infections between HSIL and LSIL.

Multiple HPV infections in baseline cytology samples with histologically confirmed CIN at follow-up. Multiple HPV DNA-positive infections were most prevalent in baseline cytology samples with histologically confirmed CIN3 (70.5%), followed by CIN2 (70.4%), CIN1 (66.7%), and no CIN (CIN0) (52.0%) at follow-up (Table 4). In cytology samples with CIN2 and CIN3 at follow-up, up to 7 concurrent HPV genotypes were detected. In the 5 cytology samples with cervical cancer at follow-up, no multiple infections were found. Restricting the analysis to high viral load infections, multiple infections were most prevalent in cytology samples with CIN1 (60%), CIN3 (56.8%), CIN2 (50%), and CIN0 (35.1%) at follow-up (Table 4). A multiple high viral load infection with up to 7 HPV types was detected in one cytology sample with CIN3 at follow-up. Age-adjusted OR showed a significant positive association between multiple infections in cytology samples and the presence of CIN1 to CIN3 at follow-up compared to NILM at baseline (Table 3). This association was stronger for multiple high viral load infections than for multiple HPV DNA-positive infections, irrespective of the number of HPV types analyzed. When we analyzed the 14 Hr-HPV types, only multiple high viral load infections were significantly associated with

CIN2+ at follow-up compared to NILM at baseline. Patients with multiple high viral loads showed a 4- to 6-fold-higher risk of having cervical precancerous lesions than did patients with single high viral loads. No significant difference was seen in the frequency of multiple infections in the cytology samples of patients who developed cervical intraepithelial neoplasia grades 1 to 3 (CIN1 to CIN3) at follow-up.

DISCUSSION

To our knowledge, this is the first study to describe multiple high viral load infections and their association with cervical lesions by analyzing the whole spectrum of all known genital HPV types in a screening population and in a representative set of women with cervical abnormalities. For the first time, estimates are provided on the prevalence of multiple HPV infections, including HPV types that have never been assessed before. Using the BSGP5+/6+-PCR/MPG assay, a surprisingly high proportion of multiple infections were detected among HPV-positive women that were highest in LSIL, followed by HSIL. The high number of multiple infections observed in our study can be attributed to the use of a sensitive assay that is capable of simultaneously detecting 51 HPV types and 3 subtypes. In previous studies, 20 to 58% of HPV-positive women were reported to be infected with multiple HPV types (10, 21, 23–25). However, HPV typing in these studies covered fewer types. Consequently, technical and study design differences hamper a direct comparison of these results.

Multiple infections have been found more frequently among young women at the peak of their sexual activity or with an impaired immune response (26–28). Consistently, half of HPV-positive young women between 15 and 34 years of age showed multiple HPV infections. In our screening population, the median age of women with 2 or more high viral load HPV infections is 31 years. However, previous studies mostly failed to find an associa-

TABLE 2 Multiple DNA positivity and multiple high viral loads in the screening population and abnormal cytology analyzing 54 HPV (sub)types

x-fold HPV DNA-positive samples	Screening population		NILM		ASCUS		LSIL		HSIL											
	Positive	High load	Positive	High load	Positive	High load	Positive	High load	Positive	High load										
	n	%	n	%	n	%	n	%	n	%										
HPV negative	626	62.7	829	83.0	609	66.7	804	88.1	23	16.9	55	40.4	2	1.8	7	6.1	110	100.0	2	1.8
HPV positive	373	37.3	170	17.0	304	33.3	109	11.9	113	83.1	81	59.6	112	98.2	107	93.9	110	100.0	108	98.2
	207	20.7	112	11.2	192	21.0	88	9.6	40	29.4	48	35.3	27	23.7	40	35.1	38	34.5	52	47.3
1	88	8.8	35	3.5	70	7.7	16	1.8	27	19.9	14	10.3	26	22.8	22	19.3	31	28.2	30	27.3
2	39	3.9	11	1.1	22	2.4	5	0.5	26	19.1	9	6.6	18	15.8	17	14.9	19	17.3	8	7.3
3	15	1.5	4	0.4	10	1.1			5	3.7	5	3.7	21	18.4	11	9.6	6	5.5	7	6.4
4	10	1.0	4	0.4	4	0.4			7	5.1	2	1.5	6	5.3	7	6.1	7	6.4	6	5.5
5	6	0.6	3	0.3	4	0.4			4	2.9	3	2.2	4	3.5	4	3.5	4	3.6	4	3.6
6	4	0.4	1	0.1					1	0.7			5	4.4	3	2.6	5	4.5	1	0.9
7	2	0.2							1	0.7			3	2.6	2	1.8				
8	1	0.1							1	0.7			1	0.9	1.0	0.9				
9									1	0.7			1	0.9	1.0	0.9				
12																				
14																				
17	1	0.1						1	0.7				1	0.9	1.0	0.9				
Total	999		999		913		913		136		136		114		114		110		110	
Multiple infection/HPV-pos (%)	44.5		34.1		36.8		19.3		64.6		40.7		75.9		62.6		65.5		51.9	
Avg no. of HPV types per HPV-positive sample	1.9		1.6		1.6		1.2		2.6		1.9		3.2		2.7		2.5		2.1	
Median no. of HPV types per HPV-positive sample (interquartile range)	1 (1-2)		1 (1-2)		1 (1-2)		1 (1-1)		2 (1-3)		1 (1-2)		3 (2-4)		2 (1-4)		2 (1-3)		2 (1-2)	

^a Positive results are based on the standard cutoff, and high load is above the cutoff for high viral load. Atypical glandular cells (AGC) and atypical squamous cells—cannot exclude a high-grade squamous intraepithelial lesion (ASC-H) were excluded from analyses.

TABLE 3 Age-adjusted odds ratios for disease categories associated with multiple infection compared to single infections or multiple high viral loads compared to single high viral loads

Disease category ^a	Infection type and HPV status ^b	OR (95% CI)	
		Positivity	High load
HSIL vs NILM	Single infection ^c	1.0 (reference)	1.0 (reference)
	Multiple 14 Hr-HPV	1.78 (1.08–2.95) ^d	5.73 (1.88–17.5) ^d
	Multiple 20 (p)Hr-HPV	2.63 (1.62–4.25) ^d	5.21 (2.13–12.7) ^d
	Multiple 54 HPV	3.00 (1.89–4.76) ^d	4.29 (2.31–7.97) ^d
LSIL vs NILM	Single infection	1.0 (reference)	1.0 (reference)
	Multiple 14 Hr-HPV	2.35 (1.41–3.93) ^d	6.58 (2.14–20.2) ^d
	Multiple 20 (p)Hr-HPV	2.60 (1.59–4.26) ^d	5.82 (2.38–14.2) ^d
	Multiple 54 HPV	4.74 (2.87–7.83) ^d	6.39 (3.43–11.9) ^d
HSIL vs LSIL	Single infection	1.0 (reference)	1.0 (reference)
	Multiple 14 Hr-HPV	0.77 (0.44–1.35)	0.87 (0.48–1.58)
	Multiple 20 (p)Hr-HPV	1.03 (0.59–1.79)	0.90 (0.51–1.60)
	Multiple 54 HPV	0.66 (0.36–1.21)	0.69 (0.40–1.21)
CIN3 vs NILM	Single infection	1.0 (reference)	1.0 (reference)
	Multiple 14 Hr-HPV	1.56 (0.79–3.04)	4.32 (1.29–14.4) ^d
	Multiple 20 (p)Hr-HPV	2.12 (1.11–4.03) ^d	4.29 (1.60–11.5) ^d
	Multiple 54 HPV	2.64 (1.39–4.99) ^d	3.89 (1.84–8.21) ^d
CIN2+ vs NILM	Single infection	1.0 (reference)	1.0 (reference)
	Multiple 14 Hr-HPV	1.62 (0.91–2.87)	4.41 (1.40–13.9) ^d
	Multiple 20 (p)Hr-HPV	2.49 (1.43–4.32) ^d	4.64 (1.84–11.7) ^d
	Multiple 54 HPV	2.90 (1.69–4.98) ^d	4.06 (2.06–7.98) ^d
CIN2 vs NILM	Single infection	1.0 (reference)	1.0 (reference)
	Multiple 14 Hr-HPV	1.87 (0.80–4.37)	5.43 (1.41–20.8) ^d
	Multiple 20 (p)Hr-HPV	3.65 (1.53–8.68) ^d	6.26 (2.03–19.2) ^d
	Multiple 54 HPV	3.56 (1.49–8.53) ^d	4.39 (1.73–11.1) ^d
CIN1 vs NILM	Single infection	1.0 (reference)	1.0 (reference)
	Multiple 14 Hr-HPV	2.02 (0.87–4.70)	5.59 (1.36–23.0) ^d
	Multiple 20 (p)Hr-HPV	2.20 (0.97–4.99)	7.62 (2.37–24.5) ^d
	Multiple 54 HPV	3.24 (1.40–7.50) ^d	6.20 (2.39–16.0) ^d
CIN2+ vs CIN1	Single infection	1.0 (reference)	1.0 (reference)
	Multiple 14 Hr-HPV	0.79 (0.31–2.00)	0.98 (0.36–2.65)
	Multiple 20 (p)Hr-HPV	1.09 (0.43–2.76)	0.72 (0.28–1.83)
	Multiple 54 HPV	0.80 (0.30–2.14)	0.63 (0.24–1.65)
CIN3+ vs CIN1	Single infection	1.0 (reference)	1.0 (reference)
	Multiple 14 Hr-HPV	0.73 (0.27–2.00)	0.88 (0.30–2.56)
	Multiple 20 (p)Hr-HPV	0.89 (0.32–2.44)	0.62 (0.23–1.69)
	Multiple 54 HPV	0.71 (0.24–2.06)	0.61 (0.22–1.72)

^a HPV data were generated using baseline cytology samples only and histological results were obtained during the 36 months of follow-up time.

^b Number of HPV types analyzed: “14” indicates multiple infection with at least two of the following 14 high-risk HPV types: HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, -68; “20” adds the types HPV26, -53, -67, -70, -73, and -82 that are considered as possibly high-risk; “54” indicates 34 additional low-risk (sub)types.

^c Single infections detected by analyzing 14 Hr-, 20 (p)Hr-, or 54 HPV types.

^d Statistically significant.

tion between multiple HPV infections and disease. In the present study, we demonstrated a highly significant association between multiple HPV DNA-positive infections and cervical lesions or neoplasia.

In our study, when we analyzed high viral load infections, the percentage of multiple high viral load infections decreased compared to HPV DNA positivity alone; however, it remained surprisingly high. For example, >10% of women of age 15 to 34 years showed multiple high viral load infections. When we restricted the analysis to the 14 Hr-HPV types, 5% of the young women still

were positive for multiple high viral load infections. Compared to multiple HPV DNA-positive infections, we observed an even stronger association between multiple high viral load infections and the presence of cervical lesions. Assuming that high viral load assessment is a direct measure of biological activity, it might serve to better identify persistent coinfections in clinical samples rather than identify latent infections or deposited HPV DNA.

The value of diagnosing multiple high viral load infections in cervical cancer screening is limited, as only 50 to 60% of CIN2 and CIN3 samples and only a very small fraction of CxCa patients would test positive. In addition, multiple high viral load infections are not more associated with high-grade than with low-grade lesions, indicating that the presence of multiple infections is a poor characteristic for discriminating (in clinical practice) between lesions requiring further follow-up and/or treatment and those that have a low risk of cervical cancer. However, for the follow-up of HPV DNA-positive women with normal cytology, the diagnosis of multiple high viral load infections might be predictive of the future development of cervical lesions. A supporting finding was made in this study: among high viral load-positive women with normal cytology at baseline, the proportion of patients with multiple high viral load infections was higher in the group that developed abnormal cytology at follow-up than in the group that remained cytologically normal. However, due to small sample numbers, the observed difference was not significant by chi-square test (data not shown).

Several recent studies have shown convincingly that the presence of one HPV type does not increase the likelihood of acquiring a second, and that the natural competition between different HPV types might not occur in the uterine cervix (9, 29–33). In the present study, this is supported by the high variety of different HPV genotypes found with high viral loads in a single sample. We assume that these infections are biologically independent (34) and that multiple infections might be the result of an impaired immune system and/or frequent changes in sexual partners. While multiple high viral loads are significantly associated with cervical cancer precursors, it is very unlikely that multiple HPV types are contributing to the same lesion and should thus be considered true risk factors. However, being unable to efficiently erase or suppress HPV infections and, thus, being infected by several HPV types at different sites with high viral loads might in the end increase the chance of a malignant transformation in cervical cells. Currently, studies are ongoing to investigate which HPV type in cervical smears with multiple HPV infections is the driving HPV type in the corresponding cancer tissue.

A limitation of this study is that HPV genotyping was not performed on the histological specimens, but was performed only on cytological specimens for which histological follow-up data were available within the next 36 months. While for some cytological samples, a histologically confirmed CIN2+ was available immediately after the referral cytology at baseline, other CIN2+ were identified during follow-up over a maximum of 3 years. To reach statistically meaningful group sizes, all analyses were performed on cumulated histological diagnoses and were not stratified by duration of follow-up.

In conclusion, the results of the present investigation provide evidence for the notion that multiple high viral load infections are strongly associated with cervical lesions. Especially in an HPV vaccination era, an improved understanding of the natural history and dynamics of HPV infection and the pathogenic effect of mul-

TABLE 4 Multiple DNA positivity and multiple high viral loads in cytology samples with subsequent histological follow-up analyzing 54 HPV (sub)types

x-fold HPV DNA-positive samples	Histology ^a																			
	CIN0 ^b				CIN1				CIN2				CIN3				CxCa			
	Positive		High load		Positive		High load		Positive		High load		Positive		High load		Positive		High load	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
HPV negative	17	25.4	30	44.8	2	6.9	4	13.8			1	3.7								
HPV positive	50	74.6	37	55.2	27	93.1	25	86.2	27	100.0	26	96.3	44	100.0	44	100.0	5	100.0	5	100.0
1	24	35.8	24	35.8	9	31.0	10	34.5	8	29.6	13	48.1	13	29.5	19	43.2	5	100.0	5	100.0
2	12	17.9	6	9.0	5	17.2	7	24.1	9	33.3	5	18.5	16	36.4	16	36.4				
3	4	6.0	3	4.5	6	20.7	3	10.3	5	18.5	3	11.1	7	15.9	2	4.5				
4	4	6.0			2	6.9	3	10.3	4	14.8	5	18.5	1	2.3	2	4.5				
5			2	3.0	4	13.8	2	6.9	1	3.7			3	6.8	2	4.5				
6	3	4.5	2	3.0	1	3.4							1	2.3	2	4.5				
≥7	3	4.5											3	6.8	1	2.3				
Total	67		67		29		29		27		27		44		44		5		5	
Multiple infection/HPV-pos (%)	52.0		35.1		66.7		60.0		70.4		50.0		70.5		56.8		0		0	

^a Positive results are based on standard cutoff; high load indicates above the cutoff for high viral load. HPV data were generated using baseline cytology samples only. Histological results were obtained during the 36 months of follow-up time.

^b CIN0, normal histology.

multiple types is necessary in order to monitor the impact of vaccination on changes in the distribution of individual HPV types. The BSGP5⁺/6⁺-PCR/MPG assay would be an ideal method to address these questions in future studies, as it provides both qualitative and quantitative genotyping results for all 54 mucosal HPV types and subtypes.

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M.A. designed the VALGENT protocol. M.A., M.S., and M.P. initiated and coordinated the study. C.D. and I.B. provided extracted clinical specimens, and J.B. performed histological and cytological analyses. M.S. performed viral load experiments and wrote the initial draft of the manuscript. J.A. managed the HPV, cytology, and histology databases. M.S. performed statistical analyses. All authors contributed and agreed to the final manuscript.

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