Clinical Evaluation of a GP5+/6+-Based Luminex Assay Having Full High-Risk Human Papillomavirus Genotyping Capability and an Internal Control

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The LMNX genotyping kit HPV GP (LMNX) is based on the clinically validated GP5+/6+ PCR, with a genotyping readout as an alternative for the more established enzyme immunoassay (EIA) detection of 14 targeted high-risk human papillomavirus (HPV) types. LMNX is additionally provided with an internal control probe. Here, we present an analysis of the clinical performance of the LMNX using a sample panel and infrastructure provided by the international VALGENT (Validation of Genotyping Tests) project. This panel consisted of cervical specimens from approximately 1,000 women attending routine screening, "enriched" with 300 women with abnormal cytology. Cases were defined as women classified with cervical intraepithelial neoplasia (CIN) grade 2+ (CIN2+) ($n = 102$) or CIN3+ ($n = 55$) within the previous 18 months. Controls were women who had normal cytology results over two subsequent screening rounds at a 3-year interval ($n = 746$). The GP5+/6+-PCR EIA (EIA) was used as a comparator assay and showed sensitivities of 94.1% and 98.2% for CIN2+ and CIN3+, respectively, with a clinical specificity of 92.4% among women aged ≥30 years. The LMNX demonstrated clinical sensitivities of 96.1% for CIN2+ and of 98.2% for CIN3+ and a clinical specificity of 92.6% for women aged ≥30 years. The LMNX and EIA were in high agreement (Cohen's kappa = 0.969) for the detection of 14 hrHPVs in aggregate, and no significant difference was observed (McNemar's $P = 0.629$). The LMNX internal control detected 0.6% inadequate specimens. Based on our study results, we consider the LMNX, similarly to the EIA, useful for HPV-based cervical cancer screening.

Human papillomavirus (HPV) tests that have been validated for use in a clinical setting usually target 13 or 14 genotypes. These HPVs have been classified by the International Agency for Research on Cancer (IARC) as carcinogenic, i.e., HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, and HPV59 (class 1), probably carcinogenic, i.e., HPV68 (class 2A), or possibly carcinogenic, i.e., HPV66 (class 2B) (1). The Hybrid Capture 2 (HC2; Qiagen, Hilden, Germany) assay (2–4) and GP5+/6+ PCR-based enzyme immunoassay (EIA) kit HPV GP HR (EIA; Diassay, Rijswijk, the Netherlands) (5–9) were the first HPV tests to be clinically validated for primary screening on the basis of longitudinal results from large screening studies (10). Testing for high-risk HPV (hrHPV) nucleic acids is also useful for triage of women with equivocal or mildly abnormal cytology for colposcopy and as a test of cure of treatment (10).

Several novel HPV tests have been fully or partially validated by showing noninferiority to HC2 or EIA and high reproducibility (11), as has been extensively reviewed by Arbyn et al. (10) and reported in additional studies (12–14). These tests usually detect around 14 hrHPVs in aggregate, but some provide concurrent (partial) genotype-specific information. Concurrent genotyping for HPV16 and HPV18 could be beneficial for the triage of hrHPV-positive women (15), as these HPV types have a higher risk of causing cervical cancer than the other hrHPVs (16–19). The value of genotyping of hrHPVs other than HPV16 and HPV18 (20, 21) is currently unknown, although genotyping can resolve type-specific persistence issues more accurately than repeated measurements with a consensus test, which may assist in risk stratification for women in screening populations and diagnostic settings (22).

The LMNX Genotyping Kit GP HR (LMNX; Diassay BV,

Rijswijk, the Netherlands; previous version marketed as the *digene* HPV Genotyping LQ Test by Qiagen, Hilden, Germany) (20, 23–27), based on the clinically validated GP5+/6+ PCR assay (5–9), offers an alternative readout method for the EIA. LMNX provides high-throughput and full genotyping of the 14 high-risk (hr)HPV types described earlier and has recently been modified to incorporate an internal control for a human DNA target to minimize the chance of technical false-negative test results.

In the current study, the GP5+/6+-based PCR EIA system was firstly used to analyze a study panel of 1,300 cervical liquid cytology samples. The study forms part of an international collaboration for the clinical validation of HPV tests, which offer limited to extensive genotyping: Clinical Validation of HPV Genotyping Tests (VALGENT) (28). Here, we aimed to (i) compare the detection of 14 pooled hrHPVs by LMNX to detection by EIA for women with high-grade (HG) cervical intraepithelial neoplasia (CIN) and (ii) to evaluate the performance of the internal control recently included in LMNX, which allows verifying the quality of the specimen.

MATERIALS AND METHODS

Clinical specimens. The second study panel prepared by the VALGENT consortium (VALGENT-2) consisted of stored cervical specimens collected from 1,000 consecutive women attending the Scottish cervical cancer screening program between August and September 2012. In Scotland, women aged 20 to 60 years old are screened every 3 years by liquid-based cytology using the classification system of the British Society for Cervical Cytology (BSCC) (29; http://www.cancerscreening.nhs.uk /cervical/publications/nhscsp01.html). This screening population was "enriched" with specimens from 300 women that were selected for abnormal cytology, i.e., borderline nuclear change ($n = 100$), mild dyskaryosis ($n = 100$), moderate dyskaryosis ($n = 57$), and severe dyskaryosis ($n = 43$), in agreement with the VALGENT protocol (28). All cervical specimens had been collected by trained health care workers based in health care settings and were stored in PreservCyt ThinPrep medium (Hologic, Marlborough, MA), in agreement with European guidelines (30). Aliquots were prepared from the residual specimen and distributed to the laboratories participating in VALGENT by the Scottish HPV Reference Laboratory, Royal Infirmary of Edinburgh.

Ethical approval. Favorable ethical opinion for the study was provided by the West of Scotland Research Ethics Office, reference 11/WS/ 0038. Sample aliquots were labeled with a nonidentifiable study number only prior to dissemination to participating laboratories.

Clinical outcomes. Women with abnormal cytology were managed in accordance with guidelines of the United Kingdom National Health Service Cervical Screening Programme (NHSCSP) standard of care (http://www.cancerscreening.nhs.uk/cervical/publications/nhscsp01 .html), which are in agreement with European recommendations (31). Biopsy specimens were taken as routinely indicated and histologically graded according to the cervical intraepithelial neoplasia (CIN) classification system (32).

Women with high-grade cervical disease (case group) were defined as those diagnosed with histologically confirmed CIN2, CIN3, or invasive squamous cell carcinoma (CIN2+) within 18 months after collection of the cervical specimen. Diagnosis of CIN3 or invasive squamous cell carcinoma (CIN3+) was used as an additional disease outcome. Women without high-grade disease (control group) were those who had a normal cytology result in the current cervical specimen as well as in the specimen collected during the previous screening round approximately 3 years before. Among women without high-grade disease, those aged ≥30 years constituted the primary cohort for calculation of clinical specificity of the evaluated HPV tests in accordance with international guidelines (11).

Sample processing and HPV testing. Nucleic acids were isolated from 500 μl of the received aliquot on a MagNA Pure 96 instrument (Roche Diagnostics, Almere, the Netherlands) using a MagNA Pure 96 DNA and Viral NA Large Volume kit (Roche) and collected in 100 μl elution buffer in accordance with the manufacturer's instructions.

The GP5+/6+ EIA kit (EIA kit HPV GP HR; Diassay, Rijswijk, the Netherlands) was used according to the kit insert instructions. Briefly, 10 μl of extracted DNA was amplified by the GP5+/6+ broad-spectrum primer set. A probe cocktail specific for 14 hrHPV genotypes (i.e., HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, and HPV68) hybridized to 5 μl of GP5+/6+ amplimers in an enzyme immunoassay (EIA). This assay does not identify HPV genotypes individually.

A second aliquot of GP5+/6+ amplification product (4 μl) was genotyped by the use of the commercially available LMNX Genotyping kit HPV GP HR (LMNX) (Diassay; previous version marketed as the *digene* HPV Genotyping LQ Test by Qiagen, Hilden, Germany) according to the manufacturer's instructions (20, 23–27).

In short, GP5+/6+ amplification products were hybridized to HPV type-specific probes attached to color-coded beads, targeting the same 14 hrHPVs as the EIA. LMNX targets four additional possibly hrHPV types (i.e., HPV26, HPV53, HPV73, and HPV82), but these were not included in the assessment of the clinical performance. Readout was performed on

a Luminex 100 IS system (Luminex Corporation, Austin, TX) according to the instrument settings specified in the kit manual. Two general thresholds for the type-specific probes were evaluated in a previous study in relation to the established reverse line blot assay, i.e., median fluorescent intensities (MFI) of 30 and 100 (23). Based on a training panel (data not presented), a general cutoff MFI of 50 (recommended in the kit manual) provided optimal agreement with the EIA for hrHPV detection and was therefore used in this study.

The use of one Luminex 100 IS system (Luminex) combined with two MagNA Pure 96 instruments (Roche) for extraction and four GeneAmp PCR System 9700 modules (Applied Biosystems) for PCR allows high-throughput processing of approximately 3,000 specimens per week.

Internal control development. An internal control probe targeting a 313-bp byproduct coamplified by the GP5+/6+ primers, similar to that described in reference 33, has recently been incorporated into the LMNX assay. This coamplified fragment was originally visualized on an agarose gel, excised, purified, and determined by sequence analysis to be a human DNA fragment located on chromosome 14 (data not presented). The benefit of the use of this internal control strategy is that no modifications had to be made to the composition and test conditions of the GP5+/6+ PCR. Addition of separate primers for human DNA amplification (e.g., beta-globin) could have had an impact on HPV amplification due to competition between primer sets (34). The internal control probe was added to the mix of HPV-specific probes of the LMNX set at a threshold MFI of 50.

Statistics. Frequencies, distributions, and percentages with 95% binomial confidence intervals were computed. The two-tailed McNemar's test was used for comparison of EIA and LMNX hrHPV positivity rates, and the level of agreement was determined using Cohen's kappa statistics. The clinical sensitivity and specificity, calculated using the definitions of disease outcomes, of LMNX were compared to those of the EIA using a noninferiority score test (11). We applied a sensitivity threshold of ≥0.90 and a specificity threshold of ≥0.98 relative to those of GP5+/6+ EIA, under the null hypothesis of inferiority (11). The level of statistical significance was set at 0.05. Analyses were performed using SPSS version 19.0.

RESULTS

Study population characteristics. In total, 1,298 of the 1,300 cervical samples of the VALGENT-2 panel had sufficient material to be included in this study, since 2 specimens (from the screening population) were not included in the shipment. The average age of women in the overall population was 38 years (range, 18 to 68), with a significant proportion (32.3%) below the age of 30 years. In the screening population ($n = 998$), cytology scorings were negative (89.8%), borderline nuclear change (5.4%), mild dyskaryosis (3.8%), moderate dyskaryosis (0.5%), and severe dyskaryosis (0.5%). The colposcopic or histologic diagnoses for women referred for diagnostic follow-up within 18 months, originating from the screening or enriched population, were normal ($n = 63$), koilocytosis ($n = 11$), CIN grade 1 (CIN1) ($n = 25$), CIN2 ($n = 47$), CIN3 ($n = 52$), and invasive cervical squamous cell carcinoma (ICC) ($n = 3$) (Table 1). A total of 102 women had CIN2+, and 55 among them had CIN3+, whereas 746 subjects had two subsequent normal cytology results.

Characterization of the VALGENT study panel by GP5+/ 6+-PCR EIA. The study panel contained 387 (29.8%) women who were hrHPV positive by the GP5+/6+-PCR EIA, of whom 169 were from the screening cohort ($n = 998$) and 218 were from the enriched group ($n = 300$). The clinical sensitivities for CIN2+ and CIN3+ of the GP5+/6+-PCR EIA system were 94.1% (95% confidence interval [CI], 87.1% to 97.6%) and 98.2% (95% CI, 89.0% to 99.9%), respectively (Table 2). The clinical specificity for <CIN1 was 92.4% (95% CI, 90.0% to 94.3%) for women aged

TABLE 1 Characteristics of the two study populations, i.e., 998 women consecutively attending a cervical cancer screening program (screening population) and 300 women referred for abnormal cytology (enriched population)

Characteristic	Screening population		Enriched population		Overall population	
	n	%	n	%	n	%
Age						
Mean age in yrs (minimum–maximum)	40 (20–68)		31 (19–62)		38 (19–68)	
≥30 yrs	746	74.7	133	44.3	879	67.7
<30 yrs	252	25.3	167	55.7	419	32.3
Clinical outcome (worst diagnosis within 18 mos)						
Normal cytology, no histology	892	89.4	0	0.0	892	68.7
Normal cytology in current and previous screening round	746	74.7	0	0.0	746	57.5
Abnormal cytology, no histology	66	6.6	139	46.3	205	15.8
Normal colposcopic impression or normal histology	23	2.3	40	13.3	63	4.9
Koilocytosis	3	0.3	8	2.7	11	0.8
CIN1	1	0.1	24	8.0	25	1.9
CIN2	5	0.5	42	14.0	47	3.6
CIN3	8	0.8	44	14.7	52	4.0
ICC	0	0.0	3	1.0	3	0.2
Total	998	100	300	100	1,298	100

≥30 years ($n = 633$). For women of all ages, the clinical specificity was 90.3% (95% CI, 87.9% to 92.3%) ($n = 746$).

Comparison of HPV detection by LMNX and EIA. The LMNX was positive for at least 1 of 14 hrHPVs in 390 (30.0%) of the 1,298 samples tested in total, which was not significantly different from the overall positivity rate by the EIA (McNemar's $P = 0.629$). Both assays were in high agreement for hrHPV detection (kappa = 0.969) (Table 2). The hrHPV positivity rates by LMNX were 17.1% in the screening cohort ($n = 998$) and 73.0% in the enriched group ($n = 300$).

HPV results by EIA and LMNX were discordant in only 17 of 1,298 (1.3%) specimens tested in total. Seven women were positive by EIA but negative by LMNX. None of these women were diagnosed with histologically confirmed high-grade cervical disease. For these seven women, cytology results were negative ($n = 4$), borderline nuclear changes ($n = 1$), mild dyskaryosis ($n = 1$), and moderate dyskaryosis but with koilocytosis diagnosed in follow-up histology ($n = 1$). In contrast, LMNX detected hrHPV in 10 women who were negative by EIA. Among these 10 women, 5 had normal cytology, 3 had borderline nuclear changes, and 2 had

TABLE 2 Comparison of LMNX to EIA for hrHPV detection among women in the overall study population stratified for CIN2+ and CIN3+ outcome and for <CIN1 among women aged ≥30 years[a]

Study population	EIA result	No. of women with LMNX result[b]			Clinical performance characteristics [% (95% CI)]		Noninferiority score[c]
		Positive	Negative	Total	EIA	LMNX	
Overall population	Positive	380	7	387			
($n = 1,298$)	Negative	10	901	911			
	Total	390	908	1,298			
CIN2+ ($n = 102$)	Positive	96	0	96	Sensitivity (CIN2+):	Sensitivity (CIN2+):	$P < 0.001$
	Negative	2	4	6	94.1 (87.1–97.6)	96.1 (89.7–98.7)	
	Total	98	4	102			
CIN3+ ($n = 55$)	Positive	54	0	54	Sensitivity (CIN3+):	Sensitivity (CIN3+):	$P = 0.007$
	Negative	0	1	1	98.2 (89.0–99.9)	98.2 (89.0–99.9)	
	Total	54	1	55			
<CIN1 (≥30 yr)	Positive	46	2	48	Specificity:	Specificity:	$P < 0.007$
($n = 633$)	Negative	1	584	585	92.4 (90.0–94.3)	92.6 (90.2–94.4)	
	Total	47	586	633			

[a] The clinical sensitivity (for CIN2+ and CIN3+) and specificity of the LMNX were compared with those of the EIA using a noninferiority score test. The clinical sensitivity was calculated among women diagnosed with CIN2+ (CIN2, CIN3, or invasive squamous carcinoma) and CIN3+ (CIN3 or invasive squamous carcinoma) within 18 months of follow-up, and clinical specificity was determined among women aged ≥30 years with <CIN1 (normal cytology during the current as well as the previous screening round 3 years earlier).
[b] A positive LMNX result represents detection of one or more of the following 14 HPV genotypes: HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, and HPV59 (12 HPVs classified by the IARC as carcinogenic; class 1) and, additionally, HPV68 (class 2A) and HPV66 (class 2B).
[c] A sensitivity threshold of at least 90% and a specificity threshold of at least 98% relative to that of the EIA was applied in a noninferiority score test. The null hypothesis of inferiority of the LMNX was rejected if $P < 0.05$.

TABLE 3 hrHPV positivity rate by EIA and LMNX in relation to clinical outcome, i.e., among women who had histologically confirmed high-grade disease (CIN2+ or CIN3+) in the follow-up and women who had normal cytology in the current and the previous screening round (<CIN1)[a]

Study population and age group	CIN2+				CIN3+				<CIN1			
	EIA positive		LMNX positive[b]		EIA positive		LMNX positive[b]		EIA positive		LMNX positive[b]	
	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%	n/N
Screening population[c]	100	13/13	100	13/13	100	8/8	100	8/8	9.7	72/746	9.5	69/746
≥30 yrs	100	5/5	100	5/5	100	2/2	100	2/2	7.6	48/633	7.4	47/633
<30 yrs	100	8/8	100	8/8	100	6/6	100	6/6	21.2	24/113	19.5	22/113
Enriched population[d]	93.3	83/89	95.5	85/89	97.9	46/47	97.9	46/47	n/a	n/a	n/a	n/a
≥30 yrs	92.3	36/39	94.9	37/39	95.5	21/22	95.5	21/22	n/a	n/a	n/a	n/a
<30 yrs	94.0	47/50	96.0	48/50	100	25/25	100	25/25	n/a	n/a	n/a	n/a
Overall population	94.1	96/102	96.1	98/102	98.2	54/55	98.2	54/55	9.7	72/746	9.2	69/746
≥30 yrs	93.2	41/44	95.5	42/44	95.8	23/24	95.8	23/24	7.6	48/633	7.4	47/633
<30 yrs	94.8	55/58	96.6	56/58	100	31/31	100	31/31	21.2	24/113	19.5	22/113

[a] Positivity rates are stratified by population (screening, enriched, or overall) and subsequently by age group (women 30 years and older or women younger than 30 years old). <CIN1, women who had normal cytology during the current as well as the previous screening round 3 years earlier; CIN2+, women diagnosed with CIN2, CIN3, or invasive squamous carcinoma within 18 months of follow-up; CIN3+, women diagnosed with CIN3 or invasive squamous carcinoma within 18 months of follow-up; n/N, number of women with the indicated disease characteristics and test result/total number of women; n/a, not applicable.
[b] A positive LMNX result represents detection of one or more of the following 14 HPV genotypes: HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, and HPV59 (12 HPVs classified by IARC as carcinogenic; class 1) and, additionally, HPV68 (class 2A) and HPV66 (class 2B).
[c] Screening population: a study population of women consecutively attending a cervical cancer screening program.
[d] Enriched population: a study population of women referred for abnormal cytology.

mild/moderate dyskaryosis. The latter two women were diagnosed with CIN2 in follow-up histology. When specimens of these two women with underlying high-grade disease were retested by EIA and LMNX, one tested positive for hrHPV by both EIA and LMNX whereas one remained positive by LMNX only.

HPV genotyping by LMNX. Among the 390 hrHPV samples positive by LMNX, 68.5% contained a single HPV strain and 31.5% had multiple HPV strains. The proportions of multiple genotypes in HPV-positive smears differed according to clinical outcome, i.e., 34.6% for CIN1/koilocytosis, 29.5% for CIN2, 41.2% for CIN3, and 0% for ICC. The HPV genotyping results among the different groups are provided in Table S1 in the supplemental material.

The most prevalent types among the 390 HPV-positive women were HPV16 (28.5%), HPV31 (13.8%), and HPV66 (12.8%). In women diagnosed with CIN2 within 18 months (n = 44), HPV16 (50.0%), HPV18 (15.9%), and HPV31 (15.9%) were the most frequently detected types. Comparatively, in women with CIN3 (n = 51), the most prevalent types were HPV16 (52.9%), HPV31 (35.3%), and HPV33 (13.7%). Three women were diagnosed with ICC; all three had a single HPV16-positive smear. Table 2 details the 10 samples that were LMNX positive but EIA negative. These samples contained single infections by HPV33, HPV39, HPV45, HPV51 (n = 3), HPV52, HPV59 (n = 2), and HPV68.

Clinical performance LMNX and EIA. LMNX was compared to EIA for the detection of women diagnosed with high-grade disease within 18 months, i.e., CIN2+ and CIN3+ (Table 2). LMNX detected 98/102 (96.1%; 95% CI, 89.7 to 98.7%) women with CIN2+, and EIA was positive in 96/102 (94.1%; 95% CI, 87.1 to 97.6%) women with CIN2+. In this series, the clinical sensitivity of LMNX for CIN2+ was noninferior to that of EIA (noninferiority score test P < 0.001). The clinical sensitivity of LMNX for CIN3+ (54/55, 98.2%; 95% CI, 89.0 to 99.9%) was also noninferior to that of EIA (54/55, 98.2%; 95% CI, 89.0 to 99.9%) (noninferiority score test P < 0.001).

Despite high clinical sensitivity, EIA and LMNX were negative for six and four women with high-grade disease (CIN2+), respectively. Two women with CIN2 were HPV negative by EIA, but LMNX showed single infections with HPV52 and HPV59. In four women with high-grade disease, i.e., CIN2 (n = 3) and CIN3 (n = 1), EIA and LMNX were both negative for the presence of 14 hrHPVs. Three of these four women had single infections with types other than the 14 hrHPVs that were targeted by both assays, i.e., HPV53 (CIN3; by LMNX), HPV82 (CIN2; by LMNX), and HPV70 (CIN2; by sequence analysis). All these identified types have been classified as possibly carcinogenic by IARC (class 2B) (1).

The clinical specificity of LMNX for CIN2+ among women aged ≥30 years (586/633, 92.6%; 95% CI, 90.2% to 94.4%) was also noninferior to that of EIA (585/633, 92.4%; 95% CI, 90.0% to 94.3%) (noninferiority score test P < 0.001) (Table 2). The clinical specificities of LMNX and EIA among women of all ages (n = 746) were 90.8% (95% CI, 88.4% to 92.7%) and 90.3% (95% CI, 87.9% to 92.3%), respectively (noninferiority score test P < 0.001).

EIA and LMNX also showed similar clinical performance characteristics independently of study population (screening population versus enriched population) and subsequently of age category (younger than 30 years versus 30 years of age and older), as shown in Table 3. The hrHPV positivity in women from the screening population without underlying disease was dependent on age, irrespective of the HPV test used. hrHPV was more prevalent among women younger than 30 years old than among those 30 years of age and older, i.e., 19.5% versus 7.4% (P < 0.001) by LMNX and 21.2% versus 7.6% (P < 0.001) by EIA.

Assessment of specimen quality by internal control. The LMNX internal control probe was positive for human DNA in 968 (97.0%) of the screening specimens (n = 998). Among these specimens of sufficient quality, 158 (15.8%) were positive and 810 (81.2%) were negative for one or more of the 18 HPVs targeted by

LMNX. The internal control was negative in 30 samples, of which 24 (2.4%) were positive for HPV and were therefore considered of adequate quality. Six specimens (0.6%) were negative for both HPV and the internal control by LMNX and were therefore scored as inadequate. However, additional HPV testing by sequence analysis demonstrated the presence of HPVs not targeted by LMNX in three of these six samples, i.e., HPV11, HPV59 (a variant not targeted by the LMNX probe), and HPV90.

DISCUSSION

The clinically validated GP5+/6+-PCR EIA system was used to analyze this large study panel, which was composed as part of an international collaboration for the clinical validation of HPV genotyping tests (VALGENT) (28). The observed high clinical sensitivity of the EIA (94.1%) is in line with a meta-analysis of previous validation studies (94.5%; range, 94% to 100%) (10) and provides further support for the use of the EIA as the reference test in the VALGENT study protocols (28).

The LMNX test, which uses the same GP5+/6+ PCR primers and conditions as the EIA, showed a clinical performance that was noninferior to the EIA for the respective 14 hrHPV genotypes.

The clinical specificity of the EIA (among women aged ≥30 years; 92.4%) was slightly lower than that previously reported (94.8%; range, 86% to 96%) (10). The EIA positivity was higher among cytologically normal women below 30 years of age (21.2%) than among those aged 30 years and older (7.6%). A relatively low clinical specificity of HPV-based screening among young women has been observed previously (35). Therefore, guidelines for clinical validation of HPV tests have been defined for a screening population of women 30 years of age and older only (11).

Internal amplification controls aim to identify unsatisfactory specimens which may be the cause of false HPV-negative test results. The LMNX was upgraded with an internal control probe for a fragment of human DNA intrinsically coamplified by GP5+/6+ primers (33). In the screening population ($n = 998$), 30 specimens (3.0%) had a negative result for the internal control. Of these, 24 (2.4%) were positive for HPV. The negative result for the internal control probe was most likely related to PCR competition with HPVs present in the sample with a high load, since HPV DNA is preferentially amplified compared to human DNA by the GP5+/6+ primers. Specimens with these results can therefore be considered of adequate quality for hrHPV testing in a clinical setting. The remaining six samples (0.6%) were negative for both HPV and human DNA and were interpreted as invalid. These invalid results can be caused by inadequate specimen collection or by untargeted HPVs (confirmed by sequence analysis) present in high viral loads, which are preferentially amplified, resulting in an absent internal control signal (PCR competition). However, it should also be noted that a positive result for the internal control (i.e., the presence of human cells) does not provide full reassurance of correct sampling of indicator cells, i.e., of epithelial cells at the squamocolumnar junction. Also, there is currently no defined adequacy threshold for the quantity of human DNA that should be detected by a HPV test with an internal control.

The LMNX has the option of full genotyping of hrHPV types (23), including HPV16 and HPV18, which might be relevant for triage (15). The value of full genotyping is still unclear, but it might help in identifying type-specific persistent HPV infections. A persistent hrHPV infection is a known risk factor for the development of cervical (pre)cancer (22), and genotyping could therefore be of use in follow-up testing of screen-positive women. In addition, persistence of a hrHPV infection in women treated for high-grade cervical lesions could be indicative of residual or recurrent disease (36).

This study had several strengths. The composition and size of the VALGENT-2 study population in terms of cases (mainly from an enriched population) and controls (from a screening population) facilitated an accurate estimation of clinical performance of HPV tests, using a limited number of samples. Without the enrichment, a screening population of 10,000 to over 20,000 women would be needed to identify about 100 CIN2+ cases. Furthermore, a well-validated HPV test, i.e., the EIA, was used as a reference assay, to which all (genotyping) assays performed on the VALGENT-2 panel will ultimately be compared. Also, clinical outcome data are well registered in the Scottish screening program, which will allow a longitudinal evaluation of HPV tests over time. Finally, an advantage is that all HPV assays are performed on the original cervical specimen in VALGENT-2. This facilitates evaluations and comparisons of the complete test system (e.g., specimen processing, amplification, and readout), since all aspects of the diagnostic chain determine the final test result.

However, our study also had some limitations. International guidelines for clinical validation of hrHPV tests stipulate that samples should be derived from women in a screening cohort tested by a clinically validated hrHPV test, either combined with or not combined with cytology (11). The current study utilized a screening cohort based on cytology alone. Since the identification of CIN2+ cases in VALGENT-2 was driven by cytology, which is known be a clinically less sensitive technology, an advantage might be given to an HPV test with a relatively low sensitivity. The VALGENT-2 cohort will be followed up to identify CIN2+ cases that may develop over time in order to identify missed cases. In addition, an enriched population of 300 women with abnormal cytology was included to find a sufficient number of CIN2+ cases while avoiding the necessity of testing the whole population from which these 300 women were selected. Although this allows clinical validation according to international guidelines (11), the positive predictive value (PPV) of the evaluated tests is not relevant without appropriate statistical weighting. Furthermore, the specificity of hrHPV tests should be determined in a screening cohort of women at least 30 years of age. In our study, 25.3% of the screening population was below the age of 30. Nevertheless, the LMNX assay is based on the same primers and PCR procedure as EIA; therefore, it is highly unlikely that this assay would deviate from the EIA on another sample set. Finally, in addition to the fulfilled criteria for diagnostic accuracy by LMNX, the international guidelines (11) also require investigation of intralaboratory reproducibility in time and interlaboratory agreement. These were not investigated in the current study, since LMNX previously showed an intralaboratory reproducibility of at least 95% on a series of artificial and clinical samples that were assayed at different time points, by different technicians, and using different kit lots (described in the kit manual). High interlaboratory agreement (kappa = 0.987) was observed in a previous study (23). In summary, two HPV tests based on the GP5+/6+ PCR, i.e., EIA and LMNX, demonstrated equivalent clinical performance characteristics. The EIA and LMNX have the advantage that they are both based on the extensively clinically validated GP5+/6+ PCR. In addition, the LMNX has high-throughput and full genotyping capability, and test performance and sample quality can be veri-

fied through the use of the internal control. Based on our data, both the EIA and LMNX are suitable options for HPV-based cervical cancer screening.

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