Human Papillomavirus Oncogenic mRNA Testing for Cervical Cancer Screening

Baseline and Longitudinal Results From the CLEAR Study

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ABSTRACT

Objectives: *This study determined the longitudinal clinical performance of a high-risk human papillomavirus (HR-HPV) E6/E7 RNA assay (Aptima HPV [AHPV]; Hologic, San Diego, CA) compared with an HR-HPV DNA assay (Hybrid Capture 2 [HC2]; Qiagen, Gaithersburg, MD) as an adjunctive method for cervical cancer screening.*

Methods: *Women 30 years or older with a negative result for intraepithelial lesions or malignancy cytology (n = 10,860) positive by AHPV and/or HC2 assays and randomly selected women negative by both assays were referred to colposcopy at baseline. Women without baseline cervical intraepithelial neoplasia (CIN) grade 2 or higher (CIN2+) continued into the 3-year follow-up.*

Results: *The specificity of AHPV for CIN2 or lower was significantly greater at 96.3% compared with HC2 specificity of 94.8% (*P < .001*). Estimated sensitivities and risks for detection of CIN2+ were similar between the two assays. After 3 years of follow-up, women negative by either human papillomavirus test had a very low risk of CIN2+ (<0.3%) compared with CIN2+ risk in women with positive AHPV results (6.3%) or positive HC2 results (5.1%).*

Conclusions: *These results support the use of AHPV as a safe and effective adjunctive cervical cancer screening method.*

Cervical cancer is one of the most frequent cancers in women worldwide, accounting for approximately 530,000 new cases and 275,000 deaths annually.[1] Countries with well-organized screening programs using conventional Papanicolaou (Pap) stain cytology have experienced substantially reduced mortality from the disease in the past 5 decades.[2-4] Despite this advance, the relatively low sensitivity and reproducibility of both conventional Pap smear and liquid-based cytology screening methods have prompted investigation into identifying adjunctive methods with Pap cytology for improving detection of cervical neoplasia.[5-9]

Infection with 14 high-risk human papillomavirus (HR-HPV) genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) is associated with almost all cases of cervical precancer, defined as cervical intraepithelial neoplasia (CIN) grade 2 (CIN2), grade 3 (CIN3), and cancer.[10] Addition of HR-HPV nucleic acid testing to a cervical cytology screening regimen offers higher sensitivity and negative predictive value (NPV) for detection of cervical precancer and cancer compared with cytology alone, especially in older women.[11-15] For this reason, HR-HPV nucleic acid testing is recommended as an adjunctive test to cytology to assess the presence of HR-HPV types in women 30 years of age or older.[16] In this context, HR-HPV testing guides patient management by identifying women at elevated risk for CIN2 or higher (CIN2+) but, importantly, also reassures women who are negative for HR-HPV of their extremely low cancer risk.[17-19]

First-generation HR-HPV molecular tests used for adjunctive cervical cancer screening function by detecting viral genomic DNA in cellular samples from the uterine cervix. However, because the presence of HR-HPV in the female genital tract is common and often transient in nature,[20,21]

and most cervical HPV infections resolve without becoming cancerous,[22,23] HR-HPV DNA-based test methods yield only moderate specificity for detection of high-grade cervical disease.[12,24] This leads to unnecessary follow-up and referral of patients to colposcopy, increasing the physical and emotional burdens on patients and elevating health care costs.

A test approved by the US Food and Drug Administration (FDA) for detection of HR-HPV E6/E7 messenger RNA (mRNA) (Aptima HPV [AHPV]; Hologic, San Diego, CA) has shown higher specificity with similar sensitivity for detection of CIN2+ compared with HPV DNA-based tests in patients referred for colposcopy due to an abnormal Pap smear result as well as in a screening setting.[25-30] Expression of mRNA from viral E6 and E7 oncogenes is highly associated with the development of CIN,[31,32] and extensive investigation into the role of E6 and E7 oncoproteins in the human papillomavirus (HPV) life cycle has revealed that the expression of the corresponding oncogenes is necessary and sufficient for cell immortalization, neoplastic transformation, and the development of invasive cancer.[33-35]

To confirm and extend the previous evidence on the clinical utility of HR-HPV oncogenic mRNA testing in a US population-based setting, the clinical performance of AHPV was evaluated as an adjunctive method for cervical cancer screening in women aged 30 years or older with negative for intraepithelial lesions or malignancy (NILM) cytology results from routine Pap testing in a pivotal, prospective, multicenter US clinical study including 3 years of follow-up (the Clinical Evaluation of Aptima mRNA [CLEAR] study). We report herein the results from this study.

Materials and Methods

Study Design, Conduct, and Participants

The CLEAR study consisted of two parts: the ASC-US (Atypical Squamous Cells of Unknown Significance) Study[30] and the Adjunct Study described here **Figure 1**. Women 30 years and older undergoing routine Pap testing who had a NILM cytology result were eligible to participate in the Adjunct Study and were recruited from 19 US family planning and obstetric/gynecologic clinics (private and academic), family practice medical groups, and clinical research centers encompassing a wide geographic area representative of the US population. Informed consent was obtained prior to enrollment of participants. The study protocol was approved by institutional review boards at the participating centers, and the study was conducted in accordance with applicable regulatory requirements and good clinical practices.

Women were excluded from the study if they were pregnant, were vaccinated against HPV, had a history of cervical disease (cancer or precancerous) or an abnormal Pap test result in the previous 12 months, or had a history of illness that could interfere with the study or create an unacceptable risk to the participant. Demographic information and relevant medical information (cervical cancer history, prior HPV diagnosis, and any abnormal cytology history) were collected from each participant. The study employed a baseline evaluation and a 3-year follow-up period with annual cytology visits for longitudinal disease ascertainment. Participants completed and exited the study once they had a CIN2+ diagnosis.

Cytology (Referral Pap)

At the baseline evaluation and each annual visit thereafter, a cervical specimen was collected with a broom-like device (Papette; Wallach Surgical Devices, Orange, CT) or an endocervical brush and spatula (Cytobrush Plus GT and Pap Perfect Plastic Spatula; Medscand, Trumbull, CT) and placed into a ThinPrep Pap Test (Hologic) vial containing PreservCyt Solution ("referral Pap" specimen). Pap specimens were processed locally using the ThinPrep 2000 System (Hologic) and evaluated for cytologic abnormalities. Cytology results were classified using the 2001 Bethesda System for reporting cervical cytology.[36]

HPV Testing

Baseline PreservCyt specimens (1-mL aliquot) were tested with the AHPV (Hologic) on both the automated Tigris DTS System and Panther System. Results from the two systems were similar; Panther System results are presented here. AHPV is a target amplification assay that uses transcription-mediated amplification to detect the E6/E7 oncogene mRNA of 14 HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). Three clinical laboratories each tested approximately one-third of all samples with AHPV. Most of the PreservCyt specimens were also tested at one laboratory for HR-HPV DNA using the Hybrid Capture 2 assay (HC2; Qiagen, Gaithersburg, MD), an FDA-approved test that detects HR-HPV DNA of 13 of the 14 HR-HPV types detected by the AHPV and is known to cross-react with the 14th type.[37] Testing and results interpretation of both HR-HPV tests were done according to the manufacturer's instructions.[38,39] Technicians performing HC2 and AHPV assays were masked to the other HPV test results and the participants' clinical status and colposcopic/histology results.

Disease Ascertainment

At baseline, women who tested positive in either the AHPV or the HC2 assay (HPV-positive women) and, to adjust for verification bias, approximately 6% of women who tested negative in both HPV assays (HPV-negative

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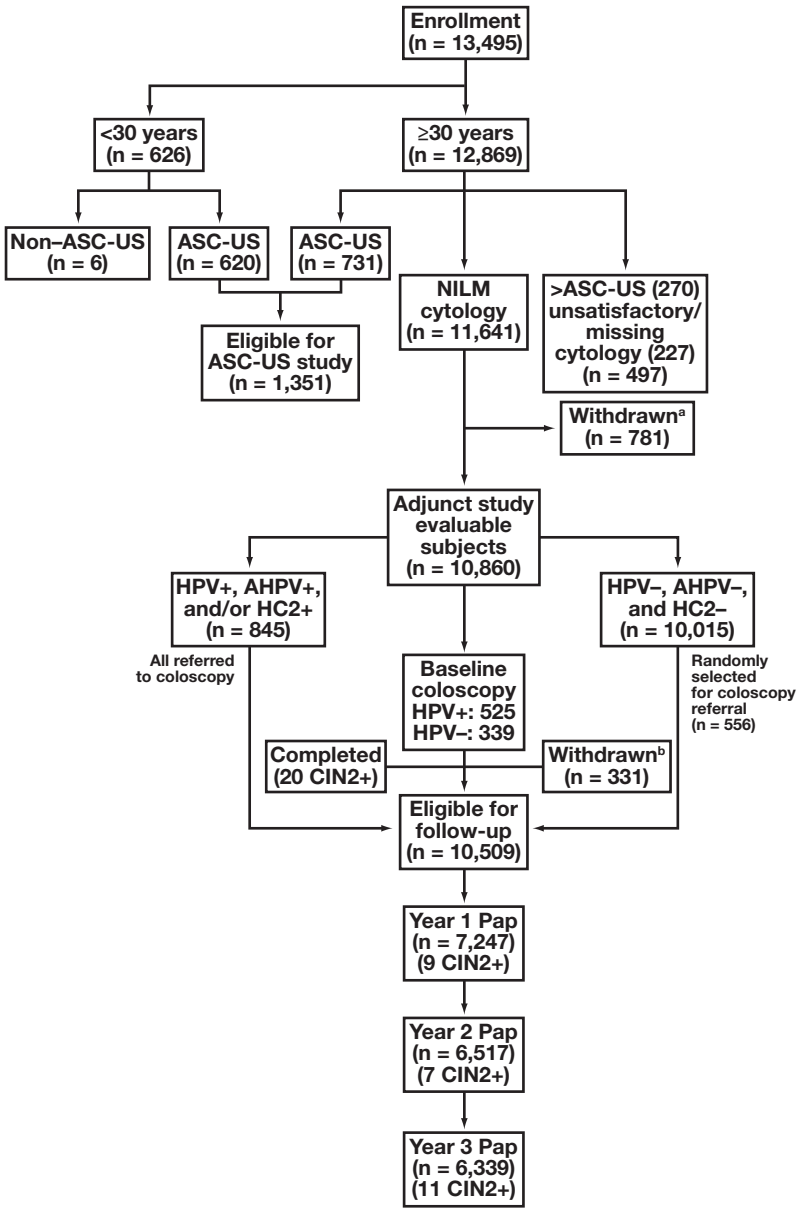

Figure 1 Clinical evaluation of Aptima mRNA study participant disposition. [a]Reasons for withdrawal: did not meet eligibility criteria (70); Pap volume insufficient for AHPV testing (117); specimen expired or unsuitable for testing (190); specimen lost (58); noncompliant site (320); other reasons (26). [b]Reasons for withdrawal: collection site did not participate in follow-up (243); subject terminated participation (37); participant had hysterectomy (22); participant not eligible (17); participant treated prior to CIN2+ diagnosis (8); other reasons (4). AHPV, Aptima HPV (Hologic, San Diego, CA); ASC-US, atypical squamous cells of unknown significance; CIN2+, cervical intraepithelial neoplasia grade 2 or higher; HC2, Hybrid Capture 2 (Qiagen, Gaithersburg, MD); HPV, human papillomavirus; NILM, negative for intraepithelial lesions or malignancy; Pap, Papanicolaou test.

women) were randomly selected and referred to colposcopy. Most colposcopy visits (>60%) were completed within 16 weeks from the baseline visit (median, 14 weeks; interquartile range, 8 weeks).

Colposcopists were masked to HPV results and collected cervical punch biopsy specimens from each visible lesion ("directed" biopsy) and an endocervical curettage (ECC) biopsy specimen. The biopsy specimens were processed according to the normal site procedures to produce H&E-stained slides. After local pathologist review, slides were reviewed by two central panel pathologists (T.C.W. and M.H.S.) and classified using the three-tiered CIN terminology.[40] Slides with discordant central panel diagnoses were reviewed by a third central pathologist to reach a consensus diagnosis (two out of three agreement). If agreement was not achieved, the three central panel pathologists reviewed

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the slides in conference to reach consensus. A participant's cervical disease status represents the highest grade consensus histology result from the colposcopy biopsy specimen. Review pathologists were masked to all other pathologists' diagnoses, the participants' clinical status, enrollment status (ASC-US Study or Adjunct Study), and HPV test results.

During follow-up, women with ASC-US or more severe cytology results were referred to colposcopy. Colposcopists collected the same types of biopsy specimens, which were processed and submitted to the same central pathology review as done at baseline to obtain the consensus histology result for that visit. Women with ASC-US or more severe cytology results who did not have a colposcopy were considered to have indeterminate disease status. Participants with NILM cytology at a follow-up visit were not referred to colposcopy and were considered to have a normal cervix.

The final disease status after 3-year follow-up was determined for each participant who completed the study. To complete the study, a woman must have either (1) a consensus result of CIN2 or worse or (2) at least one cytology visit during the first or second year of follow-up and one cytology visit during the third year of follow-up, including colposcopies for those with ASC-US or more severe cytology. Final disease status for women meeting the second criterion was based on their final consensus histology result or they were considered to have a normal cervix if they had NILM cytology at the last visit. Women with CIN2 or worse did not have further follow-up in the study.

Participants with an ASC-US or more severe cytology during follow-up who did not have a colposcopy or who attended the colposcopy visit but biopsy specimens were not collected, were lost, or the slides were inadequate to determine disease status were classified as indeterminate for cervical disease status.

Statistical Analysis

Test performance was evaluated with participants having a consensus histology result of CIN2+ (CIN2, CIN3, carcinoma in situ, or invasive cancer) classified as positive for cervical disease. A diagnosis of CIN1 or normal disease status classified participants as negative for cervical disease. In addition, test performance was evaluated using a more definitive disease end point where a consensus histology result of CIN3+ (CIN3, carcinoma in situ, or invasive cancer) classified participants as positive for cervical disease, and CIN2, CIN1, or normal classified participants as negative for cervical disease.

For the baseline risk analysis, a disproportionately smaller subset (3.4%) of HPV-negative vs HPV-positive women had disease status determined from the baseline colposcopy visit, resulting in verification bias. To adjust for this bias, a multiple imputation method[41] was used to impute missing disease status based on the observed consensus histology results and AHPV and HC2 assay results from women who had a baseline colposcopy. Verification bias-adjusted risk estimates and 95% confidence intervals (CIs) were generated using these imputed results.

The follow-up risk analysis included disease identified at baseline and during follow-up. Because all women did not have a colposcopy at baseline, individual by-year risk estimates may reflect disease that was either present but not detected at baseline or incident or progressive disease. Cumulative risks with 95% CIs were generated using the life table method, with participants not completing the study censored after the follow-up year last attended.

Sensitivity and specificity estimates with 95% CIs were generated including women who completed the study. The McNemar exact test of discordant matched pairs was performed to compare the assays, including only participants with results for both assays. All statistical tests and 95% CIs were two-tailed and performed at the 5% significance level, using SAS version 9.1 or higher (SAS Institute, Cary, NC).

Table 1
Demographics of Evaluable Participants (n = 10,860)

Characteristic	Value
Age, y	
Mean	44.2
Median	43
Minimum-maximum	30-89
IQR	15
Age groups, No. (%), y	
30 to <40	4,192 (38.6)
≥40	6,668 (61.4)
Race/ethnicity, No. (%)	
Non-Hispanic white	4,774 (44.0)
Hispanic white	1,814 (16.7)
Black	1,354 (12.5)
Asian	622 (5.7)
Other[a]	488 (4.5)
Unknown	1,808 (16.6)

IQR, interquartile range.
[a] Other includes American Indian, Alaska Native, Native Hawaiian, Pacific Islander, and multiple races.

Results

Participant Disposition and Demographic Information

A total of 13,495 women were included in this clinical study (Figure 1). Of the 12,869 women 30 years or older, 227 had an unsatisfactory or missing cytology result and 1,001 had an abnormal Pap result: ASC-US (5.7%), low-grade squamous intraepithelial lesion (1.5%), high-grade squamous intraepithelial lesion (0.2%), ASC-H (0.1%), atypical granular cells, atypical granular cells favor neoplastic, or

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Table 2
Disease Status at Baseline and After 3 Years of Follow-up and Corresponding AHPV and HC2 Test Results at Baseline

	Participants at Baseline, No.	AHPV+, No.			AHPV–, No.		
		HC2+	HC2–	HC2 Missing[a]	HC2+	HC2–	HC2 Missing[a]
Disease status at baseline[b]							
Total No.	10,860	383	97	32	282	9,467	599
Verified							
Normal	769	211	19	12	170	353	4
CIN1	29	12	0	1	7	9	0
CIN2	9	4	0	0	2	2	1
CIN3	8	7	0	0	1	0	0
AIS	3	2	1	0	0	0	0
CIN2+	20	13	1	0	3	2	1
CIN3+	11	9	1	0	1	0	0
Unverified	10,042	147	77	19	102	9,103	594
Disease status after 3-y follow-up[c]							
Total No.	10,843[d]	383	97	32	281	9,452	599
Normal	6,098	161	48	10	123	5,440	316
CIN1	56	10	0	0	6	36	4
CIN2	24	7	0	1	3	12	1
CIN3	20	14	0	1	2	3	0
AIS	3	2	1	0	0	0	0
CIN2+	47	23	1	2	5	15	1
CIN3+	23	16	1	1	2	3	0
Missing	4,378	167	44	17	130	3,756	264
Indeterminate	264	21	4	3	17	205	14

AHPV, Aptima HPV (Hologic, San Diego, CA); AIS, adenocarcinoma in situ; CIN1, cervical intraepithelial neoplasia grade 1; CIN2, cervical intraepithelial neoplasia grade 2; CIN2+, CIN2 or higher; CIN3, cervical intraepithelial neoplasia grade 3; CIN3+, CIN3 or higher; HC2, Hybrid Capture 2 (Qiagen, Gaithersburg, MD).
[a] In total, 631 women with AHPV assay results did not have HC2 test results primarily due to insufficient volume of the cytology specimen.
[b] Verified disease status was determined for women who attended colposcopy at baseline and had a consensus histology result. Women without a consensus histology result have an unverified disease status.
[c] Disease status after 3-year follow-up is based on completing 3-year follow-up with cytology performed at least once and colposcopy attendance for ASC-US or higher results during the first 2 years and during the third year.
[d] Seventeen women were determined ineligible after completion of baseline; results are excluded from follow-up analyses.

"other" (0.2% combined prevalence). The remaining 11,641 women 30 years or older with a NILM cytology at baseline were enrolled in the Adjunct Study and tested with the AHPV and HC2 tests. In total, 10,860 women were available for the baseline analysis, including 864 women (525 HPV+ and 339 HPV–) with a baseline colposcopy (781 women withdrew; see Figure 1 for reasons). Approximately 50% of the women referred to colposcopy had an ECC biopsy only, and approximately 50% had ECC plus one or more directed biopsies, resulting in the identification of 20 cases of CIN2+.

After the baseline evaluation, 10,509 women were eligible for follow-up (331 women withdrew for various reasons; see Figure 1). During follow-up, 7,247 women returned for an annual cytology visit during year 1, 6,517 returned during year 2, and 6,339 returned during year 3, with 6,201 women completing the study. Of the women who completed the study, 4,452 returned during all 3 years; the remaining returned only once during the first 2 years and in year 3 or had CIN2+ and exited the study prior to year 3. In each follow-up year, 4% to 6% of the women had ASC-US or greater cytology.

Demographics are presented in **Table 1**. The median age was 43 years, with 61.4% age 40 years or older; 44.0% were non-Hispanic white, 16.7% were Hispanic white, 12.5% were black, 5.7% were Asian, and 21.1% were categorized as "other" race or unknown.

HPV and Disease Prevalence

Cervical disease and HPV status are shown in **Table 2** for the baseline evaluation and cumulatively after 3 years of follow-up. Of the 10,860 evaluable participants with NILM cytology at baseline, 512 were positive for AHPV, yielding a prevalence of 4.7% for HR-HPV E6/E7 oncogenic mRNA, whereas prevalence of HR-HPV DNA was 6.5% among 10,229 women with HC2 results. A total of 845 HPV RNA-positive or DNA-positive women and 556 randomly selected HPV-negative women were referred to colposcopy at baseline (Figure 1).

At baseline, the percentage of colposcopy attendance was similar between HPV-positive (62%, n = 526) and randomly selected HPV-negative (61%, n = 339) women with 29 cases of CIN1, nine cases of CIN2, eight cases of CIN3, and three cases of adenocarcinoma in situ (AIS) identified (Table 2). Four of the CIN2 cases and two of the AIS cases were identified based on an ECC biopsy specimen only.

In total, 6,271 women completed the 3-year follow-up with a known disease status (Table 2). Of these, 6,098

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Table 3
Absolute and Relative Risk of CIN2+ and CIN3+ Disease at Baseline (Verification Bias Adjusted)

	AHPV Assay			HC2 Test		
Disease Status/Assay Result	Absolute Risk (95% CI), %	Relative Risk (95% CI)	Prevalence, %	Absolute Risk (95% CI), %	Relative Risk (95% CI)	Prevalence, %
CIN2+						
Positive	4.5 (2.7-7.4)	7.5 (2.1-26.3)	0.9	3.7 (2.3-6.1)	7.3 (1.6-33.5)	0.9
Negative	0.6 (0.2-1.9)			0.5 (0.1-2.1)		
CIN3+						
Positive	3.0 (1.6-5.5)	24.9 (2.0-307.0)	0.4	2.3 (1.3-4.1)	21.0 (1.0-423.8)	0.4
Negative	0.1 (0.0-1.7)			0.1 (0.0-2.4)		

AHPV, Aptima HPV (Hologic, San Diego, CA); CI, confidence interval; CIN2+, cervical intraepithelial neoplasia grade 2 or higher; CIN3+, cervical intraepithelial neoplasia grade 3 or higher; HC2, Hybrid Capture 2 (Qiagen, Gaithersburg, MD).

Table 4
Cumulative Absolute and Relative Risk of CIN2+ and CIN3+ Disease by Age Group After 3-Year Follow-up (Life Table Analysis)

		AHPV Assay			HC2 Test		
Disease Status/Age Group, y	Assay Result	Absolute Risk (95% CI), %	Relative Risk (95% CI)	Prevalence, %	Absolute Risk (95% CI), %	Relative Risk (95% CI)	Prevalence, %
CIN2+							
Overall	Positive	6.32 (4.29-9.27)	23.94 (13.59-42.18)	0.55	5.12 (3.53-7.41)	22.39 (12.19-41.12)	0.55
	Negative	0.26 (0.17-0.41)			0.23 (0.14-0.38)		
30-39	Positive	7.76 (4.81-12.40)	31.11 (13.04-74.21)	0.76	6.46 (3.99-10.39)	27.36 (10.88-68.80)	0.79
	Negative	0.25 (0.12-0.53)			0.24 (0.10-0.54)		
≥40	Positive	4.51 (2.34-8.63)	16.57 (7.26-37.82)	0.42	3.77 (2.10-6.71)	16.85 (7.21-39.35)	0.40
	Negative	0.27 (0.16-0.46)			0.22 (0.12-0.42)		
CIN3+							
Overall	Positive	4.42 (2.76-7.03)	67.87 (25.32-181.88)	0.27	3.43 (2.14-5.48)	59.14 (20.09-74.12)	0.28
	Negative	0.07 (0.03-0.16)			0.06 (0.02-0.16)		
30-39	Positive	5.74 (3.22-10.11)	102.84 (23.17-456.51)	0.44	4.78 (2.67-8.48)	171.50 (22.39-1,313.63)	0.45
	Negative	0.06 (0.01-0.22)			0.03 (0.00-0.20)		
≥40	Positive	2.81 (1.27-6.16)	41.80 (10.53-166.00)	0.16	2.05 (0.93-4.52)	28.46 (7.15-113.20)	0.17
	Negative	0.07 (0.02-0.21)			0.07 (0.02-0.22)		

AHPV, Aptima HPV (Hologic, San Diego, CA); CI, confidence interval; CIN2+, cervical intraepithelial neoplasia grade 2 or higher; CIN3+, cervical intraepithelial neoplasia grade 3 or higher; HC2, Hybrid Capture 2 (Qiagen, Gaithersburg, MD).

(97.2%) women had normal (negative) disease status, and 56 (0.9%) had low-grade lesions (CIN1). In addition to the 20 women with CIN2+ identified at baseline, 15 (0.2%) women had CIN2 and 12 (0.2%) women had CIN3 identified during follow-up, with two cases identified from an ECC biopsy specimen only.

Of the 27 women with CIN2+ identified during follow-up, two had CIN1 at baseline, with CIN3 identified during year 1. Ten women had no disease found at baseline, with five cases of CIN2+ identified during year 1, one case of CIN2+ identified during year 2, and four cases of CIN2+ identified during year 3. The remaining 15 women with CIN2+ identified during follow-up did not have a baseline colposcopy; among them, two cases of CIN2+ were identified during year 1, six cases of CIN2+ during year 2, and seven cases of CIN2+ during year 3.

AHPV Assay Performance

Baseline risk and prevalence estimates adjusted for verification bias are provided in **Table 3**. The prevalence of CIN2+ was 0.9% in the overall population. CIN2+ occurred in 4.5% (95% CI, 2.7%-7.4%) of women with positive AHPV results and in 0.6% (95% CI, 0.2%-1.9%) of women with negative AHPV results, yielding a relative risk of 7.5 (95% CI, 2.1-26.3). This indicates that women with a positive AHPV result are at significantly greater risk of CIN2+ than women with a negative AHPV result. The CIN2+ relative risk obtained for the HC2 test at baseline was similar (7.3; 95% CI, 1.6-33.5). For CIN3+ diagnosis, the overall prevalence was 0.4%. The AHPV relative risk was 24.9 (95% CI, 2.0-307.0), again with a similar relative risk for HC2 (21.0; 95% CI, 1.0-423.8).

Cumulative absolute and relative risks for AHPV and HC2 over the 3-year follow-up period for HPV-positive and HPV-negative women are shown in **Table 4**. Women with an HPV-negative result with either test had very low cervical disease risk after 3 years of follow-up (<0.3%). Comparatively, 5% to 6% of women with an HPV-positive result had CIN2+ and 3% to 4% had CIN3+, with overall cumulative absolute and relative risks slightly higher for

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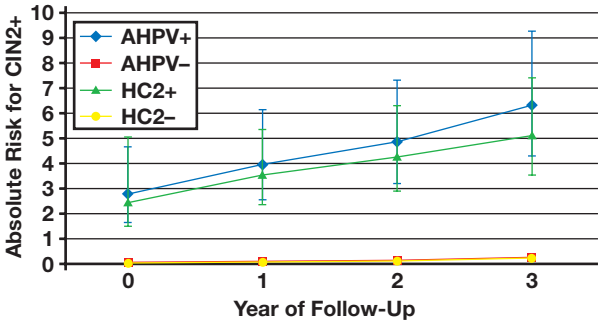

Figure 2 Cumulative absolute risk of cervical intraepithelial neoplasia grade 2 or higher (CIN2+) by year. AHPV, Aptima HPV (Hologic, San Diego, CA); HC2, Hybrid Capture 2 (Qiagen, Gaithersburg, MD).

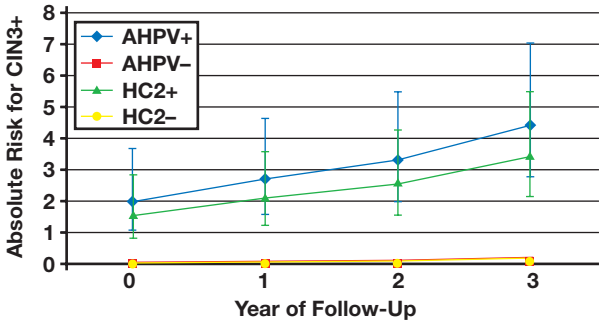

Figure 3 Cumulative absolute risk of cervical intraepithelial neoplasia grade 3 or higher (CIN3+) by year. AHPV, Aptima HPV (Hologic, San Diego, CA); HC2, Hybrid Capture 2 (Qiagen, Gaithersburg, MD).

the AHPV assay than for HC2. Younger women aged 30 to 39 years who were HPV positive had twice the prevalence of disease but a similar increase in relative risk of cervical disease compared with HPV-positive women 40 years and older (Table 4). Risk of cervical disease in HPV-negative women did not vary by age group.

Figure 2 and **Figure 3** show the cumulative absolute risk of CIN2+ and CIN3+, respectively, by year according to AHPV or HC2 positivity status at baseline. Both assays show a similar trend, with consistent slightly higher risk for the AHPV assay each year.

After 3 years of follow-up, the specificity of AHPV for CIN2 or lower was 96.3% (95% CI, 95.8%-96.7%), significantly greater ($P < .001$) compared with HC2 specificity of 94.8% (95% CI, 94.3%-95.4%) **Table 5**. AHPV specificity for CIN3 or lower (96.2%; 95% CI, 95.5%-96.5%) was also significantly greater ($P < .001$) than HC2 specificity (94.7%; 95% CI, 94.1%-95.2%). Estimated sensitivities for detection of CIN2+ and CIN3+ were similar between the two assays ($P = .219$ and $P = 1.0$, respectively). For detection of CIN2+, AHPV sensitivity was 55.3% (95% CI, 41.2%-68.6%), and HC2 sensitivity was 63.6% (95% CI, 48.9%-76.2%). For CIN3+ detection, AHPV sensitivity was 78.3% (95% CI, 58.1%-90.3%), and HC2 sensitivity was 81.8% (95% CI, 61.5%-92.7%) (Table 5).

Discussion

This study presents the results of a 3-year longitudinal evaluation of the AHPV assay as an adjunctive method for screening women 30 years and older who have NILM Pap cytology results. Consistent with previously published data,[28,29] these results demonstrate that HR-HPV oncogenic E6/E7 mRNA testing has a sensitivity similar to an HR-HPV DNA-based test for detection of CIN2+ and CIN3+ and slightly, but significantly, improved specificity compared with HR-HPV DNA testing for both end points. We found that use of AHPV as an adjunctive method for HPV-induced cervical disease screening provided disease detection capability similar to HC2 while reducing the false-positive rate (from 5.2% to 3.7%) relative to the HPV DNA-based test. Reduction of HPV detection in women without cervical disease minimizes the anxiety and burden associated with spurious positive HPV molecular test results in women with NILM cytology, decreases health care costs, and reduces unnecessary follow-up procedures, thereby improving the safety of cervical cancer screening (unnecessary colposcopy is considered a significant "harm" in the recent American Cancer Society guidelines[16]).

Importantly, we show that women with a NILM cytology result who also had a positive AHPV result are approximately 24 times more likely to have CIN2+ disease after 3 years than women with a negative AHPV result. This risk increased to approximately 68-fold for detection of CIN3+ disease. Similar but slightly lower risk estimates were obtained with HC2, demonstrating comparable accuracy of the AHPV and HC2 for identifying participants with CIN2+ and CIN3+ in this respect.

After 3 years of follow-up, women in this study who were HPV negative at baseline using any test method had very low risk for CIN2+ (<0.3%), a result similar to previously published studies with HC2.[42,43] These findings reinforce evidence from previous studies showing that HR-HPV nucleic acid testing should be performed as an adjunctive test to routine Pap for cervical cancer screening of women 30 years or older to increase sensitivity of disease detection.[28] Correspondingly, compared with annual cytology-only screening, this study supports longer screening intervals for women negative for both abnormal cytology and HPV E6/E7 mRNA, due to the high NPV and low risk of disease afforded by this screening algorithm for 3 years following a test-negative

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Table 5
Clinical Sensitivity and Specificity for CIN2+ and CIN3+ Disease After 3-Year Follow-up[a]

	AHPV, No.			Sensitivity/Specificity, % [No./Total No.] (95% CI)[b]		Difference (95% CI)	*P* Value
HC2	Positive	Negative	Total	AHPV	HC2		
CIN2+				55.3 [26/47] (41.2 to 68.6)	63.6 [28/44] (48.9 to 76.2)	–9.1 (–21.9 to 3.8)	.219
Positive	23	5	28				
Negative	1	15	16				
Missing/equivocal	2	1	3				
Total	26	21	47				
<CIN2				96.3 [5,925/6,154] (95.8 to 96.7)	94.8 [5,524/5,824] (94.3 to 95.4)	1.4 (0.9 to 1.9)	<.001
Positive	171	129	300				
Negative	48	5,476	5,524				
Missing/equivocal	10	320	330				
Total	229	5,925	6,154				
CIN3+				78.3 [18/23] (58.1 to 90.3)	81.8 [18/22] (61.5 to 92.7)	–4.5 (–24.4 to 15.3)	1.000
Positive	16	2	18				
Negative	1	3	4				
Missing/equivocal	1	0	1				
Total	18	5	23				
<CIN3				96.2 [5,941/6,178] (95.5 to 96.5)	94.7 [5,536/5,846] (94.1 to 95.2)	1.4 (1.0 to 1.9)	<.001
Positive	178	132	310				
Negative	48	5,488	5,536				
Missing/equivocal	11	321	332				
Total	237	5,941	6,178				

AHPV, Aptima HPV (Hologic, San Diego, CA); CI, confidence interval; CIN2+, cervical intraepithelial neoplasia grade 2 or higher; <CIN2, cervical intraepithelial neoplasia grade 2 or lower; CIN3+, cervical intraepithelial neoplasia grade 3 or higher; <CIN3, cervical intraepithelial neoplasia grade 3 or lower; HC2, Hybrid Capture 2 (Qiagen, Gaithersburg, MD).
[a] Differences (95% CI) and *P* values (McNemar exact test) are calculated including only women with both AHPV and Digene HC2 assay results (excluding samples with missing or equivocal Digene HC2 results).
[b] Sensitivity is reported for CIN2+ and CIN3+; specificity is reported for <CIN2 and <CIN3.

baseline visit. Extension of cervical cancer screening intervals following negative HPV and cytology test results in women 30 years or older is a key recommendation of current US screening guidelines from both the American Cancer Society and the US Preventive Services Task Force.[16]

Conversely, since the positive predictive value of any HPV test in women with NILM cytology is low, additional AHPV testing to detect persistent HR-HPV infection during follow-up care in women with an initial AHPV-positive result is likely a better option than direct referral to colposcopy. Alternatively, genotyping with referral for HPV 16– or HPV 18–positive women can optimize referral and minimize loss to follow-up.[44]

Several design features were employed in the CLEAR study to achieve accurate determination of the performance characteristics for both AHPV and HC2 assays. First, all biopsy samples were subjected to adjudicated review by three independent expert pathologists. Second, molecular test performance was compared with a consensus histology diagnosis, the gold standard for determining cervical disease status. Third, AHPV performance was compared directly with HC2 performance, the most broadly used and characterized HPV DNA test. Fourth, performance characteristics of both assays obtained from baseline results were adjusted for verification bias by conducting colposcopy and biopsy in 3.4% of HPV-negative women. This process is recommended in low-prevalence populations to avoid overestimating assay sensitivity and underestimating assay specificity.[45-47] Finally, women were followed for 3 years with annual cytology testing and referral to colposcopy for abnormal results.

A limitation of this study was that a portion of HPV-negative women with normal cytology were subjected to colposcopy and biopsy at the baseline visit but not at the subsequent follow-up visits. Thus, the relative risk estimates reported here for disease in HPV-positive vs HPV-negative women evaluated during years 1, 2, and 3 of the follow-up period may be overstated. This potential bias is present in previously reported longitudinal cotesting studies[17,19,48] and is unavoidable, since implementation of invasive procedures on thousands of women with normal cytology and negative HPV test results presents a burden to study participants and is not supported by current US and European practice guidelines. However, as in previous longitudinal cotesting studies, women enrolled in CLEAR who exited at the final (third) year of follow-up had yielded negative cytology and/or negative HC2 and AHPV results from four consecutive examinations. Thus, their risk of harboring an occult CIN lesion is likely to be exceedingly small,[42,43] such that any potential error encountered here most likely constitutes a very small fraction of the overall magnitude of the risks reported.

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Another limitation of this study was that colposcopists were aware of the women's HPV test status during the first half of the baseline portion of the study, because during that period, only women who tested positive in the AHPV or HC2 were referred to colposcopy. When the colposcopists were unmasked, they may have been more diligent to find cervical disease with prior knowledge of current HPV infection status. However, after randomly selected HPV-negative women were referred to colposcopy, the colposcopists were masked to HPV status, and throughout the entire study, colposcopists were masked as to which HPV assay caused the referral. Thus, any potential "colposcopy bias" would be identical for both molecular tests.

In summary, these results demonstrate that the clinical performance of HR-HPV E6/E7 mRNA testing using the AHPV is consistent with current US cervical cancer screening guidelines for women with a NILM cytology result who are 30 years of age or older. There was a significantly greater risk of CIN2+ in AHPV-positive vs AHPV-negative participants, as well as a statistically and clinically significant improvement in specificity for detection of CIN2+ by the AHPV compared with HPV DNA testing with the HC2 assay. Thus, these data confirm the clinical utility of AHPV testing in an adjunct cervical cancer screening setting.

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The CLEAR study testing sites and participants are as follows: Laboratory testing sites: B. A. Body, Laboratory Corporation of America, Burlington, NC; B. Yen-Lieberman, Cleveland Clinic Foundation, Cleveland, OH; C. Ginocchio, North Shore Long Island Jewish Health System Laboratory, Lake Success, NY; and T. Davis, Eskenazi Health Services, Indianapolis, IN. Collection sites (principal investigators and institutions): M. Rabinowitz, Community Medical Research, Miami Beach, FL; A. T. Hanna, Diverse Research Solutions, Oxnard, CA; K. Ault, Emory University School of Medicine, Atlanta, GA; G. M. Grossman, HealthCare Partners, Los Angeles, CA; W. Campbell, HealthCare Partners, Torrance, CA; M. Stripling, NEA Baptist Clinic Women's Clinic, Jonesboro, AR; D. Ronk, Planned Parenthood of Eastern Arkansas and Oklahoma, Tulsa, OK; R. Muckerman, PPS Clinical Research, Chesterfield, MO; B. Naghi, Peninsula Research Associates, Rolling Hills Estates, CA; T. Easter, REMEK Research, Montclair, CA; E. Guzman, Guzman MD Research, Downey, CA; A. Wagner, Saginaw Valley Medical Research, Saginaw, MI; C. Saffer MD, West Coast OB/GYN, San Diego, CA; R. Healy, Adams Patterson OB-GYN, Memphis, TN; and W. Wilkerson, Insignia Clinical Research, Tampa, FL.

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