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Effectiveness of novel, lower cost molecular human papillomavirus-based tests for cervical cancer screening in rural china

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This study examined the efficacy of the Onco*E6*™ Cervical Test, *care*HPV™ and visual inspection with acetic acid (VIA) in identifying women at risk for cervical cancer and their capability to detect incident cervical precancer and cancer at 1-year follow-up. In a population of 7,543 women living in rural China, women provided a self-collected and two clinician-collected specimens and underwent VIA. All screen positive women for any of the tests, a ~10% random sample of test-negative women that underwent colposcopy at baseline, and an additional ~10% random sample of test-negative women who did not undergo colposcopy at baseline ($n = 3,290$) were recruited. 2,904 women were rescreened 1 year later using the same tests, colposcopic referral criteria, and procedures. Sensitivities of baseline tests to detect 1-year cumulative cervical intraepithelial neoplasia Grade 3 or cancer (CIN3+) were 96.5% and 81.6% for *care*HPV™ on clinician-collected and self-collected specimens, respectively, and 54.4% for Onco*E6*™ test. The Onco*E6*™ test was very specific (99.1%) and had the greatest positive predictive value (PPV; 47.7%) for CIN3+. Baseline and 1-year follow-up cervical specimens testing HPV DNA positive was sensitive (88.0%) but poorly predictive (5.5–6.0%) of incident CIN2+, whereas testing repeat HPV16, 18 and 45 E6 positive identified only 24.0% of incident CIN2+ but had a predictive value of 33.3%. This study highlights the different utility of HPV DNA and E6 tests, the former as a screening and the latter as a diagnostic test, for detection of cervical precancer and cancer.

Well organized, comprehensive cytology-based screening programs have reduced the incidence of cervical cancer by 70% or more.[1] More than 80% of the 530,000 annual cervical cancer cases and 270,000 annual cervical cancer-related deaths occur in women living in low- and middle-income countries (LMICs)[2,3] due to their inability to establish or maintain a high-quality, high-coverage cytology-based screening programs.[1,4,5] As a consequence, and due to the common exposure to human papillomavirus (HPV) infection worldwide, cervical cancer remains a leading cause of cancer-related deaths and years of life lost in women living in developing countries, a situation markedly different from the developed world.[1,6]

New cervical cancer screening strategies have emerged including visual inspection after acetic acid (VIA) and molecular testing for high-risk human papillomavirus (hrHPV). Recent World Health Organization guidelines recommend hrHPV testing, if it can be afforded, or VIA instead of cytology for those countries that are unable to establish a high-coverage, effective cytology testing-based screening program.[7] In randomized clinical trials, testing for hrHPV DNA has shown to be more effective than cytology in reducing cervical cancer incidence in about 5 years[8] and more effective than cytology and VIA in reducing cervical cancer-related deaths in 8 years.[9] Importantly, hrHPV DNA testing is highly

Key words: E6, HPV, cervical intraepithelial neoplasia, cervical cancer screening

Abbreviations: CIN: cervical intraepithelial neoplasia; CICAMS: Cancer Institute and Hospital, Chinese Academy of Medical Sciences; HC2: Hybrid Capture 2; HPV: human papillomavirus; hrHPV: high-risk human papillomavirus; LMICs: low- and middle-income countries; Pap: Papanicolaou; PPV: positive predictive value; START-UP: Screening Technologies to Advance Rapid Testing for Cervical Cancer, Prevention—Utility and Program Planning; VIA: visual inspection with acetic acid

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What's new?

Low-cost technologies for the detection of high-risk human papillomavirus (hrHPV) types are of particular interest for use in cervical cancer screening in developing countries. Promising technologies include those that are capable of detecting the HPV E6 oncoprotein or hrHPV DNA. This evaluation of women in rural China shows that tests for E6 and HPV DNA differ in their detection performance yet are complementary in cervical cancer assessment. HPV DNA detection showed superior screening performance, because of its high sensitivity and negative predictive value. HPV E6 detection performed better in diagnosis, because of its specificity and positive predictive value.

sensitive, and therefore a negative test provides excellent reassurance against cervical cancer or precancer over the coming years, permitting screening intervals to be safely extended.[10–12]

For LMICs, cervical cancer screening strategies must be effective, affordable and sustainable, properties that cytology-based testing cannot satisfy. A new generation of hrHPV based tests has been developed to better meet these requirements for successful implementation of cervical cancer screening programs in LMICs. These new hrHPV tests are lower cost, faster and easier to use and, if validated, might offer an alternative method for cervical cancer screening of underserved women in LMICs.

Two lower cost molecular tests for hrHPV have been developed: (*i*) *care*HPV™ (QIAGEN, Gaithersburg, MD) detects the DNA for a pool of 14 hrHPV genotypes[13] and (*ii*) the Onco***E6***™ Cervical Test (Arbor Vita Corporation, Fremont, CA) detects hrHPV E6 oncoproteins from HPV16, 18 and 45 in the version used in this study.[14] Preliminary studies of *care*HPV™ have shown sensitivity and specificity that approach those of Hybrid Capture 2 (HC2), a US Food and Drug Administration-approved hrHPV DNA test upon which *care*HPV™ is based, and can be run by secondary school graduates without laboratory experience, using a training of trainer model.[13]

The Onco***E6***™ Cervical Test detects elevated levels of E6 oncoproteins, which are required for epithelial cell transformation to occur. Thus detecting E6 proteins represents an attractive, disease-specific biomarker of viral oncogenic activity in high hrHPV prevalence populations and as a triage of hrHPV DNA-positive women. The Onco***E6***™ Cervical Test does not require sophisticated equipment, and operator training is simple, thus favoring its adoption in low-resource settings. Previous findings of the Onco***E6***™ Cervical Test revealed a very high positive predictive value (PPV). These studies showed it was the most specific test in detecting the presence of cervical precancer and cancer lesions compared with other HPV DNA tests[15] but its sensitivity for primary screening was suboptimal.

Large-scale evaluations of both lower cost tests and strategies for utilizing them are currently lacking. To address this gap, we conducted a clinical trial in 7,500 women living in rural China as part of the Screening Technologies to Advance Rapid Testing for Cervical Cancer Prevention—Utility and Program Planning (START-UP) project. Here we report the ability of these tests to predict and detect newly diagnosed cervical precancer and cancer at the 1-year follow-up in a high-risk sub-cohort of the START-UP population.

Material and Methods

Population

Recruitment and sample size calculations, from October 2010 through June 2011, were previously described in detail.[15] The institutional review boards of PATH, Cancer Institute and Hospital, Chinese Academy of Medical Sciences (CICAMS) and US National Cancer Institute approved the study.

Enrollment visit

After an education session describing the study procedures and providing written informed consent, participants (*i*) completed a short risk-factor questionnaire administered by study personnel, (*ii*) self-collected a vaginal specimen in a private room, (*iii*) underwent a routine pelvic exam by female clinicians, at which time two cervical specimens were collected, the first specimen was a swab collected into a dry tube for the Onco***E6*** test and the second specimen was collected into *care*HPV™ Collection Medium (DCM; QIAGEN) for HPV DNA testing and (*iv*) were screened by visual inspection after applying 5% acetic acid (VIA) to the cervix and waiting for 1 min.

Clinical management

Women were referred to colposcopy at baseline if they either were (*i*) "screen positive" at enrollment, *i.e.*, positive by any one or more of the six tests performed (positive on HC2 and *care*HPV™ testing of self-collected and clinician-collected specimens, E6 and/or VIA) or (*ii*) selected as part of a 10% random sample of "screen-negative" women (negative on all six test results). All women referred to colposcopy had a rigorous evaluation that included using a 4-quadrant microbiopsy protocol as previously described.[15,16] As dictated by the institutional review boards, women who had no visible acetowhite lesions had their screening results revealed: those who had any positive screening test underwent the 4-quadrant microbiopsy protocol whereas those who were screen negative had no biopsies taken. Women with a CIN2+ underwent an excisional procedure such as loop

electrosurgical excision procedure or if cancer was found, cancer management.

One-year Follow-up

Women who were referred to colposcopy at baseline as described above or selected as part of a second 10% random sample of screen-negative women at baseline had a 1-year follow-up visit, and the same tests and colposcopic referral criteria were applied.

Laboratory testing

As previously described,[15,17] the self-collected and second clinician-collected specimen were tested for hrHPV DNA by HC2 and *care*HPV^{TM}; *care*HPV^{TM}-positive specimens were also tested by a research-use only pooled probe set that targets HPV16, 18 and 45 ("*care*HPV16, 18 and 45") using the *care*HPV^{TM} platform and protocol. A signal strength of 1.0 relative light units per positive control or greater was considered positive for HC2, *care*HPV^{TM} and *care*HPV^{TM}16/18/45. The Onco***E6***^{TM} Cervical Test is an immunochromatographic test using lateral flow format for the E6 oncoproteins of HPV Types 16 and/or 18 and/or 45 as previously described.[14,15,17]

Statistical analysis

As shown in Figure 1, of the 7,543 women recruited into the study; 7,539 women completed screening. After 1 year, 3,290 were invited for follow-up; of these 2,904 agreed to participate in the 1-year follow-up and 2,794 completed their follow-up. Additionally 2,625 completed follow-up and had a nonmissing baseline diagnosis of <CIN2. Combining baseline and 1-year follow-up diagnoses, excluding those women who had recurrent disease following baseline treatment for CIN2+, there were cumulatively 55 cases of CIN2, 100 cases of CIN3 and 14 cases of invasive cervical cancer diagnosed or 169 cases of CIN2+ and 114 cases of CIN3+. In the 1-year follow-up, 25 cases of CIN2+ and 12 cases of CIN3 were diagnosed. Only a single case of CIN2+ was diagnosed (at the 1-year follow-up) among women who were negative for all baseline screen results. As a consequence, we did not adjust for verification bias in our sensitivity and specificity calculations.

We calculated the percent positive of each screening test done at baseline for the worst cumulative 1-year diagnosis, under the assumption that there was virtually no disease to be diagnosed among women who were negative for all six screening results who were not followed for a year. Among those women who had a 1-year follow-up visit and a diagnosis of <CIN2 at baseline, we calculated the percent positive of each screening test done at the 1-year follow-up for the 1-year diagnosis.

We estimated the sensitivity, specificity and positive and negative predictive values for all screening tests performed at baseline for both 1-year cumulative worst diagnoses of CIN2+ and CIN3+. We also evaluated the sensitivity and specificity for CIN2+ and CIN3+ of the screening tests at 1-year follow-up among all women in the follow-up cohort.

We compared baseline HPV16, 18 and 45 DNA and E6 results, individually and pairwise (positive/positive, positive/negative, negative/positive and negative/negative) with baseline diagnoses and 1-year follow-up diagnoses. Finally, for all screening tests, we compared paired baseline and 1-year results with the 1-year follow-up diagnoses.

Results

Table 1 shows the comparisons of (*i*) baseline test results for the six screening tests and 1-year cumulative worst diagnoses and (*ii*) the 1-year test results for the six screening tests and the 1-year, newly diagnosed diagnoses. Similar relationships between the tests and the diagnoses were observed for both comparisons. HPV DNA detection in clinician-collected specimens was ~80% in CIN1 and >90% for CIN2, CIN3 and cancer. By comparison, HPV16, 18 and 45 E6 detection increased from ≤10% for CIN1 to ~50% in CIN3, and was positive in 78.6% of the cancers, all 14 of which were diagnosed at baseline. Baseline VIA was only sensitive (78.8%) for cancer; VIA at the 1-year follow-up identified only 2 of the 12 (16.7%) newly diagnosed CIN3, which was nonsignificantly less ($p = 0.1$) than 44.3% of the baseline CIN3 cases that VIA identified as previously reported.[15]

The performances of the screening tests for detection of 1-year cumulative CIN2+ and CIN3+ are shown in Table 2. All combinations of HPV DNA tests and specimens were very sensitive for CIN3+: 96.5% for *care*HPV^{TM} testing of clinician-collected specimens, 81.6% for *care*HPV^{TM} testing of self-collected specimens, 96.5% for HC2 testing of clinician-collected specimens and 89.5% HC2 testing of self-collected specimens. HPV DNA tests were the least specific for CIN3+, with specificities ranging from 83.2% (HC2 testing on self-collected specimens) to 86.9% (*care*HPV^{TM} testing on clinician-collected specimens). HPV16, 18 and 45 E6 detection was very specific for CIN3+ and as a consequence, had a PPV of 47.7%. VIA was similarly sensitive (45.6% *vs.* 54.4%, $p = 0.8$) and less specific (93.3% *vs.* 99.1%, $p < 0.001$) for CIN3+ than HPV16, 18 and 45 E6. Similar patterns were observed for 1-year cumulative CIN2+ and for 1-year test performance for newly diagnosed CIN2+ and CIN3+ (Supporting Information table), with the notable exception for 1-year test performance of VIA on newly diagnosed CIN3 as discussed above.

In Table 3, the relationship of baseline HPV16, 18 and 45 E6 and DNA detection among *care*HPV^{TM} positives (on the clinician-collected specimen) with the worst cumulative 1-year diagnoses for women who had <CIN2 at baseline is presented. Testing baseline E6 positive/DNA negative was rare and there were no concurrent or newly diagnosed cases of CIN2, CIN3 or cancer with those results. Whereas 8 of 25 (32.0%) of the baseline HPV DNA-positive CIN2 cases were also concurrently E6 positive, 49 of 72 (68.1%) of the baseline

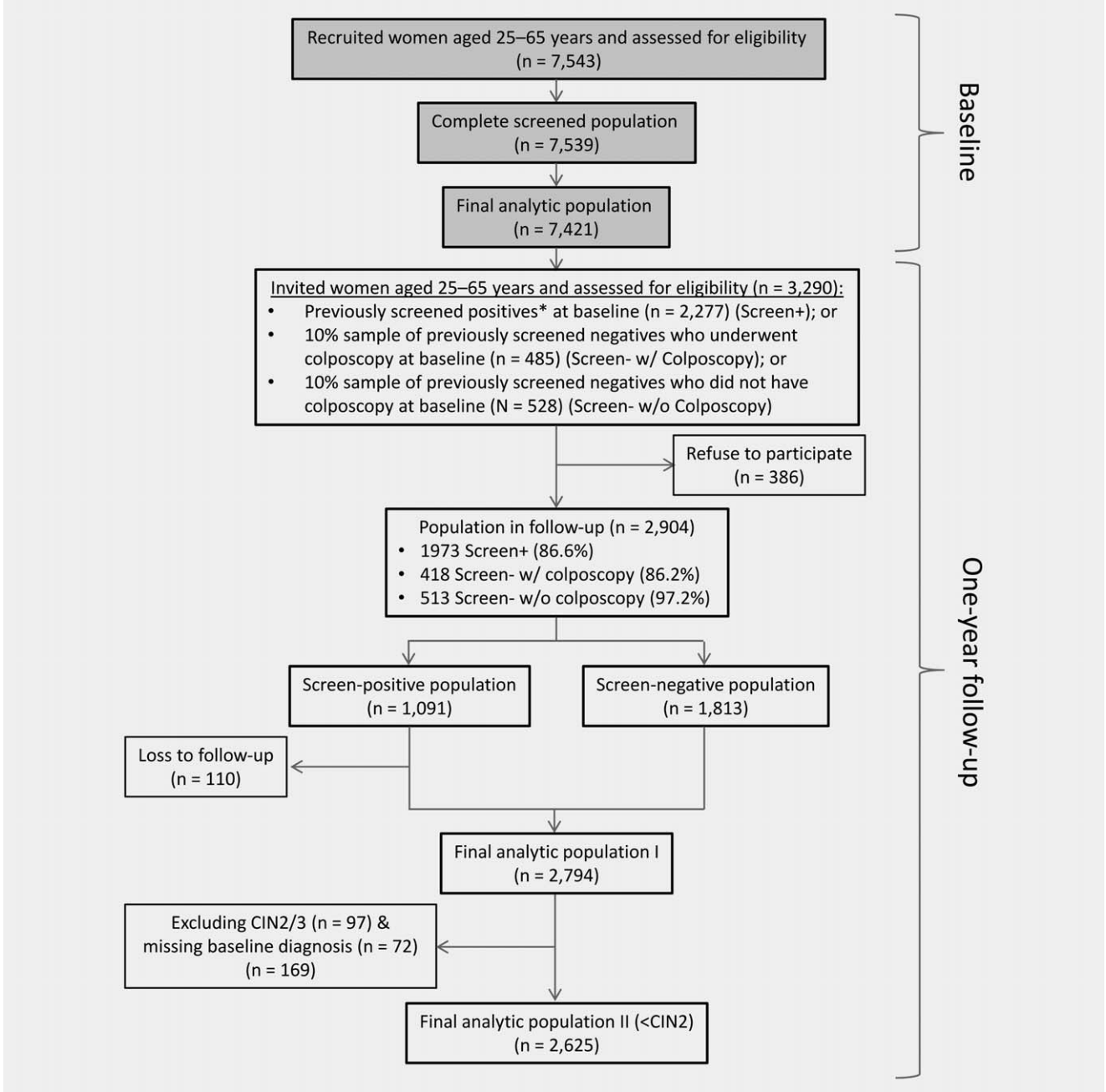

Figure 1. Consort diagram describing the enrollment and follow-up populations. Women diagnosed with cancer at enrollment were excluded from follow-up.

HPV DNA-positive CIN3 cases were also concurrently E6 positive ($p = 0.002$, Fisher's exact).

Table 4 examines the relationship of paired baseline and 1-year follow-up results for each of the six screening tests to 1-year newly diagnosed cases of CIN2+ among those in the high-risk cohort of women undergoing follow-up, had a diagnosis at baseline that was <CIN2, and completed follow-up ($n = 2{,}625$). In this cohort, 51.7% of HC2 positives and 47.8% of *care*HPV^{TM} positives on clinician-collected specimens, 49.4% of HC2 positives and 44.0% of *care*HPV^{TM} positives on self-collected specimens, 13.5% of VIA positives and 31.0% of HPV16, 18 and 45 E6 positives at baseline tested positive again at the 1-year follow-up.

Most newly diagnosed CIN2+ cases tested hrHPV DNA positive on the clinician-collected specimens at both time points for both HC2 and *care*HPV^{TM} (88% for both tests). Fewer cases of CIN2+ and CIN3+ tested positive by HC2 (72.0%) and *care*HPV^{TM} (56%) on self-collected specimens; there were nonsignificantly more cases of CIN2+ that were negative at baseline and positive at the 1-year follow-up (negative/positive) than the converse (positive/negative) for hrHPV DNA testing of self-collected specimens. The risks of

Table 1. Percent test positive by histologic diagnoses.

		*care*HPV (HPV DNA)		HC2 (HPV DNA)		
	HPV E6 (HPV16/18/45)	Clinician	Self	Clinician	Self	VIA
(A)						
Negative[1] ($N = 6,981$)	0.6	9.9	10.6	9.9	13.5	5.8
CIN1 ($N = 271$)	6.3	78.6	73.8	83.0	87.1	24.4
CIN2 ($N = 55$)	16.4	92.7	74.6	92.7	87.3	34.6
CIN3 ($N = 100$)	51.0	96.0	81.0	96.0	90.0	41.0
Cancer ($N = 14$)	78.6	100.0	85.7	100.0	85.7	78.8
<CIN2 ($N = 7,252$)	0.8	12.5	13.0	12.6	16.3	6.5
CIN2+ ($N = 169$)	42.0	95.3	79.3	95.3	88.8	42.0
<CIN3 ($N = 7,307$)	0.9	13.1	13.4	13.2	16.8	6.7
CIN3+ ($N = 114$)	54.4	96.5	81.6	96.5	89.5	45.6
(B)						
Negative[1] ($N = 2,479$)	0.6	15.9	16.2	17.4	21.1	3.8
CIN1 ($N = 121$)	9.9	87.6	78.5	93.4	90.1	23.1
CIN2 ($N = 13$)	30.8	92.3	76.9	92.3	84.6	53.9
CIN3 ($N = 12$)	50.0	100.0	75.0	100.0	91.7	16.7
Cancer[2] ($N = 0$)	0	0	0	0	0	0
<CIN2 ($N = 2,600$)	1.0	19.3	19.1	20.9	24.4	4.7
CIN2+ ($N = 25$)	40.0	96.0	76.0	96.0	88.0	36.0
<CIN3 ($N = 2,613$)	1.2	19.6	19.4	21.3	24.7	5.0
CIN3+ ($N = 12$)	50.0	100.0	75.0	100.0	91.7	16.7

(A) For baseline screening results and worst 1-year diagnoses ($n = 7,421$); and (B) for 1-year follow-up screening results and 1-year diagnoses among women with a 1-year follow-up visit and who had <CIN2 at baseline ($n = 2,625$).
[1]Includes women who did not have biopsies and biopsies that were diagnosed as negative.
[2]Excludes one CIN3 diagnosis at baseline which progressed to cancer diagnosis at 1-year follow-up.

CIN2+ and CIN3+ were low, ~6% for CIN2+ and 3% for CIN3+ (data not shown), among the repeat hrHPV DNA positives for either specimen using either test despite evidence of hrHPV persistence.

By comparison, 62.5% of the newly diagnosed cases of CIN2+ were VIA negative at both time points. Only 24% of CIN2+ were repeat HPV16, 18 and 45 E6 positive but 58.3% of CIN2+ were positive at any time point. Repeat HPV16, 18 and 45 E6 positive was risky for CIN2+ (33.3%) and CIN3+ (22.2%; data not shown).

Discussion

We present the follow-up data of our first report[15] on comparison of several lower cost screening tests for detection of cervical precancer and cancer. As the primary end point, we used 1-year cumulative diagnoses, *i.e.*, baseline diagnoses plus 1-year follow-up diagnoses in high-risk women, to account for missed prevalent or early, newly diagnosed disease that should have been detected by the screening tests in the first round. As observed here and previously,[15,17] there was a sharp increase in the percentage of E6 positive with increasing severity of disease, with only 0.6% of negative women and 6.3% with CIN1 testing E6 positive. By comparison, most women with CIN1 were HPV DNA positive.

HPV DNA detection was more sensitive and less specific for cervical precancer and cancer than E6 detection, even after controlling for HPV genotypes as discussed below, demonstrating that the HPV tests and the E6 biomarker are two different yet complementary tests serving two different purposes in secondary cervical cancer prevention. Testing hrHPV negative is very effective in ruling out disease, identifying a large subset of women who do not have clinically important infection and are therefore not at risk. Thus, they do not need to be screened for 5 or more years.[8,10,11,18] Although HPV DNA detection does not differentiate between benign and clinically important infections, it does identify a subset of at-risk "symptomatic" women within whom a secondary diagnostic can be applied more effectively[19] and predicts who will develop CIN3+ in up to almost 20 years.[11]

By contrast, detection of elevated E6 oncoprotein identifies women most likely to have or develop true precancer, *i.e.*, those with malignant potential. Of note, as shown in Table 3, only 41 of 62 (66%) HPV16, 18 and 45 DNA-positive CIN3 *vs.* 11 of 12 (92%) HPV16, 18 and 45 DNA-positive cervical cancers were also E6 positive for the same types ($p = 0.09$).

Table 2. Sensitivity and specificity for cervical intraepithelial neoplasia Grade 2 (CIN2) or more severe (CIN2+) and Grade 3 (CIN3) or more severe (CIN3+) diagnoses of baseline test results and 1-year cumulative CIN2+ and CIN3+ diagnoses ($n = 7{,}421$)

	*care*HPV		Hybrid Capture 2		VIA
HPV16/18/45 E6	Clinician	Self	Clinician	Self	
CIN2+ ($N = 169$)					
Sensitivity	42.0 (34.5, 49.8)	95.3 (90.9, 97.9)	79.3 (72.4, 85.1)	95.3 (90.9, 97.9)	88.8 (83.0, 93.1)	42.0 (34.5, 49.8)
Specificity	99.2 (99.0, 99.4)	87.5 (86.8, 88.3)	87.0 (86.2, 87.8)	87.4 (86.6, 88.1)	83.7 (82.9, 84.6)	93.5 (92.9, 94.1)
PPV	54.6 (45.7, 63.4)	15.1 (13.0, 17.4)	12.5 (10.6, 14.6)	15.0 (12.9, 17.2)	11.3 (9.63, 13.1)	13.1 (10.4, 16.3)
NPV	98.66 (98.36, 98.91)	99.87 (99.75, 99.95)	99.45 (99.23, 99.62)	99.87 (99.75, 99.95)	99.69 (99.51, 99.81)	98.58 (98.27, 98.84)
CIN3+ ($N = 114$)						
Sensitivity	54.4 (44.8, 63.7)	96.5 (91.3, 99.0)	81.6 (73.2, 88.2)	96.5 (91.3, 99.0)	89.5 (82.3, 94.4)	45.6 (36.6, 55.2)
Specificity	99.1 (98.8, 99.3)	86.9 (86.1, 87.7)	86.6 (85.8, 87.3)	86.8 (86.0, 87.5)	83.2 (82.3, 84.1)	93.3 (92.7, 93.9)
PPV	47.7 (38.9, 56.6)	10.3 (8.6, 12.3)	8.7 (7.1, 10.5)	10.2 (8.5, 12.2)	7.7 (6.3, 9.24)	9.6 (7.3, 12.4)
NPV	99.29 (99.07, 99.47)	99.94 (99.84, 99.98)	99.67 (99.49, 99.80)	99.94 (99.84, 99.98)	99.80 (99.7, 99.9)	99.10 (98.85, 99.31)

Table 3. Relationship of paired baseline detection of HPV16, HPV18 and/or HPV45 E6 and DNA among baseline *care*HPV^{TM}-positive women (clinician-collected specimen) with the worst 1-year cumulative diagnoses

Baseline HPV16, 18 and 45 status		One-year cumulative diagnoses						
E6	DNA		*N* (total)	Neg	CIN1	CIN2	CIN3	Cancer
Pos	—	*N*	112	29	15	8	49	11
		row%	100	25.9	13.4	7.1	43.8	9.8
—	Pos	*N*	302	137	55	25	72	13
		row%	100	45.4	18.2	8.3	23.8	4.3
Pos	Pos	*N*	106	23	15	8	49	11
		row%	100	21.7	14.2	7.6	46.2	10.4
Pos	Neg	*N*	6	6	0	0	0	0
		row%	100	100	0.0	0.0	0.0	0.0
Neg	Pos	*N*	196	114	40	17	23	2
		row%	100	58.2	20.4	8.7	11.7	1.0
Neg	Neg	*N*	757	548	158	26	24	1
		row%	100	72.4	20.9	3.4	3.2	0.1

However, E6 detection alone is unlikely to provide long-term negative prediction of disease because it does not effectively rule out or detect most hrHPV infections, some of which could subsequently persist and develop into cancer.

We also examined patterns of baseline E6 and DNA detection for HPV16, 18 and 45 in relationship to CIN2, CIN3 and cancer. Importantly, even when restricted to HPV16, 18 and 45 DNA positives, only 33.3% of baseline diagnosed CIN2 were positive for HPV16, 18 and 45 E6, which was similar to the fraction for CIN1 (34.9%, $p = 1.0$) and much lower than the fraction for CIN3 (66.3%, $p = 0.01$).

It is worth noting that a substantial percentage of CIN3 will never become invasive. In the 30-year follow-up of women with CIN3 (carcinoma *in situ*), approximately 30% became invasive cervical cancer.[20] The lifetime probabilities that CIN3 will become invasive or possibly regress are not known. While E6-negative CIN3 may indicate a low risk of becoming invasive cervical cancer immediately, it may subsequently develop invasive potential (and become E6 positive) in the interim until the next screen. Thus, while not all CIN3 diagnoses are truly precancer, *i.e.*, has invasive potential, we to date rely on colposcopy/biopsy to identify women with CIN3 as our best proxy for cervical precancer and treat immediately. Until an alternative method of defining cervical precancer is identified, CIN3 will be used as the cervical precancer end point for evaluating new screening modalities.

Our analysis revealed that the greater specificity of E6 detection for CIN3 and cancer compared to DNA detection was not primarily due to restriction to the most carcinogenic HPV genotypes as has been observed for qualitative mRNA

Table 4. Percentage of women diagnosed with cervical intraepithelial neoplasia Grade 2 or more severe (CIN2+) and Grade 3 or more severe (CIN3+) at the 1-year follow-up after paired baseline and 1-year follow-up test results in the high-risk cohort of women undergoing follow-up and with <CIN2 at baseline ($n = 2{,}625$)

	Positive/positive[1]				Positive/negative[1]				Negative/positive[1]				Negative/negative[1]		
	n	%	Risk	p^2	*n*	%	Risk	p^2	*n*	%	Risk	p^2	*n*	%	Risk
E6															
All	18	0.7			40	1.5			19	0.7			2548	97.1	
CIN2+	6	24.0	33.3%	<0.0001	4	16.0	10.0%	0.0002	4	16.0	21.1%	<0.0001	11	44.0	0.4%
CIN3+	4	33.3	22.2%	<0.0001	4	33.3	10.0%	<0.0001	2	16.7	10.5%	<0.0001	2	16.7	0.1%
VIA[3]															
All	53	2.0			341	13.0			79	3.0			2150	82.0	
CIN2+	2	8.0	3.8%	0.2	1	4.0	0.3%	0.2	7	28.0	8.9%	0.004	15	60.0	0.7%
CIN3+	0	0.0	0.0%	1	1	8.3	0.3%	0.7	2	16.7	2.5%	0.1	9	75.0	0.4%
HC2[4]															
All	400	15.2			374	14.2			168	6.4			1683	64.1	
CIN2+	22	88.0	5.5%	<0.0001	1	3.6	0.3%	0.3	2	7.4	1.2%	0.1	0	0.0	0.0%
CIN3+	11	91.7	2.8%	<0.0001	0	0.0	0.0%	n/a	1	7.7	0.6%	0.1	0	0.0	0.0%
***care*HPV[4]**															
All	367	14.0			400	15.2			158	6.0			1700	64.8	
CIN2+	22	88.0	6.0%	<0.0001	1	3.6	0.3%	0.3	2	7.4	1.3%	0.1	0	0.0	0.0%
CIN3+	11	91.7	3.0%	<0.0001	0	0.0	0.0%	n/a	1	7.7	0.6%	0.1	0	0.0	0.0%
HC2[5]															
All	493	18.8			504	19.2			162	6.2			1466	55.8	
CIN2+	18	72.0	3.7%	0.0001	0	0.0	0.0%	0.5	4	13.8	2.5%	0.004	3	12.0	0.2%
CIN3+	9	75.0	1.8%	0.007	0	0.0	0.0%	1	2	14.3	1.2%	0.05	1	8.3	0.1%
***care*HPV[5]**															
All	352	13.4			448	17.1			164	5.9			1661	63.3	
CIN2+	14	56.0	4.0%	0.0001	1	4.0	0.2%	0.7	5	20.0	3.0%	0.003	5	20.0	0.3%
CIN3+	7	58.3	2.0%	0.006	1	8.3	0.2%	1	2	16.7	1.2%	0.08	2	16.7	0.1%

[1]These are paired comparisons where the former is the baseline test result and the latter is the 1-year follow-up result.
[2]Versus negative/negative.
[3]Two missing follow-up results.
[4]Clinician-collected specimen.
[5]Self-collected specime.

tests that target only HPV16, 18, 31, 45 and 52.[21] Rather, it was a consequence of the ability of the E6 marker to biologically differentiate between HPV-related lesions and infection with high (E6 positive) *vs.* low (E6 negative) progressive potential. The pattern was similar for the newly diagnosed cases of CIN2/3 at the 1-year follow-up. Notably, there was significantly elevated risk of a 1-year CIN3 diagnosis among E6 positive/DNA positive *vs.* E6 negative/DNA positive for HPV16, 18 and 45 (odds ratio = 12.8, 95% CI = 2.25–130).

To further enhance applicability of the Onco***E6***^{TM} Cervical Test, we suggest several adaptations. First, the number of HPV types targeted by the assay needs to be expanded. Indeed, detection of E6 as a biomarker of malignant potential may also be more important in weaker carcinogenic HPV genotypes since an even higher proportion of those infections will be benign and will clear spontaneously. An important application of detection of E6 for these "weaker" carcinogenic HPV genotypes will be screening in populations that have been vaccinated against HPV16 and HPV18.

Second, as previously discussed,[15] E6 detection from the same buffers used for HPV DNA detection would eliminate the need for collecting and handling a second specimen. Finally, as observed in Table 4, there was evidence of "skip" patterns, *i.e.*, positive and then negative prior to the diagnosis of CIN3+, which might suggest that a slightly lower analytic threshold might improve its performance and reliability in those HPV-positive women who will develop CIN3+. We did not quantitate the E6 signal on the detection strip to ascertain whether skip patterns were more apt to have lower signal positives than the positives followed by a second positive result.

We noted that VIA detected only about half of the baseline cases of CIN3 and missed most of the few cases of newly detected CIN3 at the 1-year follow-up. In fact, VIA demonstrated only good sensitivity for baseline cervical cancers, which is consistent with what was observed in a recent study in India that found VIA only down-staged cancers.[22] Although the number of newly detected CIN3 cases was too small to come to any strong conclusions, the implication is that following sensitive screening, VIA may perform less well. We hypothesize that the first round of sensitive screening effectively removed all large CIN3 lesions, leaving only the smaller (missed) prevalent or newly diagnosed CIN3 lesions to be found in the next round of screening, which may be harder to visualize. While 11 of the 12 newly diagnosed CIN3 cases in Year 1 were HPV DNA positive at baseline, none was VIA positive at baseline.

The main limitation for this or any study of screening performance longitudinally is that we cannot differentiate between missed prevalent and truly newly diagnosed disease. The protocol, with sensitive screening and colposcopy, likely detected the vast majority of disease at baseline, inferring that most of the 25 cases of CIN2+ at the 1-year follow-up were truly newly diagnosed disease. These cases likely progressed from HPV infection to CIN3 in the 12 months of follow-up, as we noted most newly diagnosed CIN3 were HPV DNA positive at baseline. But we cannot rule out the possibility that small lesions were missed at baseline but enlarged sufficiently to be detected at the 1-year follow-up. Another limitation is that we did not evaluate E6 detection on self-collected specimens. However, such an evaluation would have required an additional self-collected specimen as a dry swab, which may have impacted the interpretation of any tests done on the second self-collected specimen.

We conclude that there is now a menu of options for cervical cancer screening and management,[7,23,24] options that extends beyond and complement the traditional cytology-based approach and might overcome some of its limitations. These numerous options allow screening programs to be tailored to meet local demands, *i.e.*, choice of screening test and algorithm should be based on the desired programmatic sensitivity and specificity, the capacities to implement the programs and the different tests within the programs, and immediate and follow-up costs. More data are needed on the effectiveness and cost-effectiveness of these different approaches, including the impact of multiple rounds of screening on performance.

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References

1. International Agency for Research on Cancer (IARC). IARC Handbooks of Cancer Prevention: Cervix Cancer Screening, vol. 10. Lyon: IARCPress, 2005.
2. de Martel C, Ferlay J, Franceschi S, et al. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol.* 2012;13:607–15.
3. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer.* 2010;127:2893–917.
4. Sankaranarayanan R, Budukh AM, Rajkumar R. Effective screening programmes for cervical cancer in low- and middle-income developing countries. *Bull World Health Organ.* 2001;79:954–62.
5. Kitchener HC, Castle PE, Cox JT. Chapter 7: achievements and limitations of cervical cytology screening. *Vaccine.* 2006;24:S3/63–70.
6. Yang BH, Bray FI, Parkin DM, et al. Cervical cancer as a priority for prevention in different world regions: an evaluation using years of life lost. *Int J Cancer.* 2004;109:418–24.
7. World Health Organization (WHO). WHO guidelines for screening and treatment of precancerous lesions for cervical cancer prevention. Geneva: WHO, 2013.
8. Ronco G, Dillner J, Elfstrom KM, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet.* 2014;383:524–32.
9. Sankaranarayanan R, Nene BM, Shastri SS, et al. HPV screening for cervical cancer in rural India. *N Engl J Med.* 2009;360:1385–94.
10. Dillner J, Rebolj M, Birembaut P, et al. Long term predictive values of cytology and human papillomavirus testing in cervical cancer screening: joint European cohort study. *BMJ* 2008;337:a1754.
11. Castle PE, Glass AG, Rush BB, et al. Clinical human papillomavirus detection forecasts cervical cancer risk in women over 18 years of follow-up. *J Clin Oncol.* 2012;30:3044–50.
12. Kjaer SK, Frederiksen K, Munk C, et al. Long-term absolute risk of cervical intraepithelial neoplasia grade 3 or worse following human papillomavirus infection: role of persistence. *J Natl Cancer Inst.* 2010;102:1478–88.
13. Qiao YL, Sellors JW, Eder PS, et al. A new HPV-DNA test for cervical-cancer screening in developing regions: a cross-sectional study of clinical accuracy in rural China. *Lancet Oncol.* 2008;9:929–36.
14. Schweizer J, Lu PS, Mahoney CW, et al. Feasibility study of a human papillomavirus e6 oncoprotein test for diagnosis of cervical precancer and cancer. *J Clin Microbiol.* 2010;48:4646–48.
15. Zhao FH, Jeronimo J, Qiao YL, et al. An evaluation of novel, lower-cost molecular screening tests for human papillomavirus in rural China. *Cancer Prev Res (Phila).* 2013;6:938–48.
16. Pretorius RG, Zhang WH, Belinson JL, et al. Colposcopically directed biopsy, random cervical biopsy, and endocervical curettage in the diagnosis of cervical intraepithelial neoplasia II or worse. *Am J Obstet Gynecol.* 2004;191:430–4.
17. Qiao YL, Jeronimo J, Zhao FH, et al. Lower cost strategies for triage of human papillomavirus DNA-positive women. *Int J Cancer.* 2014;134:2891–901.
18. Sherman ME, Lorincz AT, Scott DR, et al. Baseline cytology, human papillomavirus testing, and

risk for cervical neoplasia: a 10-year cohort analysis. *J Natl Cancer Inst.* 2003;95:46–52.
19. Wentzensen N, Wacholder S. From differences in means between cases and controls to risk stratification: a business plan for biomarker development. *Cancer Discov.* 2013;3:148–57.
20. McCredie MR, Sharples KJ, Paul C, et al. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. *Lancet Oncol.* 2008;9:425–34.
21. Castle PE, Follansbee S, Borgonovo S, et al. A comparison of human papillomavirus genotype-specific DNA and E6/E7 mRNA detection to identify anal precancer among HIV-infected men who have sex with men. *Cancer Epidemiol Biomarkers Prev.* 2013;22:42–9.
22. Shastri SS, Mittra I, Mishra GA, et al. Effect of VIA screening by primary health workers: randomized controlled study in Mumbai, India. *J Natl Cancer Inst.* 2014;106:dju009. doi:10.1093/jnci/dju009
23. Gage JC, Castle PE. Preventing cervical cancer globally by acting locally: if not now, when? *J Natl Cancer Inst.* 2010;102:1524–27.
24. Schiffman M, Wentzensen N, Wacholder S, et al. Human papillomavirus testing in the prevention of cervical cancer. *J Natl Cancer Inst.* 2011;103:368–83.