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Adjunctive testing by cytology, p16/Ki-67 dual-stained cytology or HPV16/18 E6 oncoprotein for the management of HPV16/18 screen-positive women

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Abstract

High-risk human papillomavirus type 16/18 (HPV16/18) genotyping is unable to accurately discriminate nonprogressive infections from those that will progress to cervical cancer. Our study aimed to assesses if additional testing either with liquid-based cytology (LBC) or the putative progression markers p16/Ki-67 and HPV16/18 E6 oncoprotein (E6) can improve the efficiency of HPV16/18 genotyping for triaging high-risk HPV (hrHPV)-positive women through better cancer risk stratification. Women attending colposcopy after positive HPV16/18 genotyping results within the Forwarding Research for Improved Detection and Access for Cervical Cancer Screening and Triage (FRIDA) hrHPV-based screening study in Tlaxcala, Mexico, underwent further testing with LBC, p16/Ki-67 dual-stained (DS) cytology and E6. We calculated measures of test performance for detecting histologically confirmed cervical intraepithelial neoplasia grade 2 or higher (CIN2+) and grade 3 or higher (CIN3+). A number of 475 (64.3%) of 739 HPV16/18-positive women had complete results for all tests. Triage positivity rates were 14.1%, 18.5% and 24.4%, for LBC, E6 and DS,

Abbreviations: ASC-US, atypical squamous cells of undetermined significance; ASCUS+, atypical squamous cells of undetermined significance or worse; CIN2+, cervical intraepithelial neoplasia grade 2 or higher; CIN3+, cervical intraepithelial neoplasia grade 3 or higher; DS, p16/Ki-67 dual-stained cytology; E6, HPV16/18 E6 oncoprotein; HPV, human papillomavirus; HPV16, human papillomavirus type 16; HPV18, human papillomavirus type 18; hrHPV, high-risk human papillomavirus; LBC, liquid-based cytology; mAb, monoclonal antibody; NPV, negative predictive value; PPV, positive predictive value.

respectively. Compared with LBC, DS had higher sensitivity (24.4% vs 60.0%) although lower specificity (87.0% vs 79.3%) for CIN3+ ($P < .001$), whereas E6 had a sensitivity of 37.8% and a specificity of 83.5%. No invasive cancer was missed by DS or E6, but 75% were in normal cytology. DS test was associated with nearly 75% reduction of colposcopy referrals compared with the direct referral of all HPV16/18-positive women, giving the least number of colposcopies (n = 4.3) per CIN3+ detected. We show that adjunctive testing of HPV16/18-positive women with DS may greatly reduce unnecessary colposcopy referrals within HPV-based screening employing HPV16/18 genotyping while retaining acceptable sensitivity for CIN2+ and CIN3+.

KEYWORDS

cervical cancer, cytology, E6 oncoprotein, human papillomavirus, p16/Ki-67

1 | INTRODUCTION

Cervical cancer is the most amenable cancer to reduction by screening yet, is the fourth most common cause of death due to cancer in women worldwide, although 90% of cervical cancer deaths occur in developing countries.[1] The availability of human papillomavirus (HPV) testing offers an opportunity to address important obstacles of ineffective screening programs. However, the benefits of high-risk HPV (hrHPV)-based screening can be enhanced by the application of triage tests.

Over the recent years, findings from several studies have pointed out that testing cervical specimens for HPV types 16 and 18 (HPV16 and HPV18), the viral types responsible for 70% of invasive squamous cervical cancer, is a good alternative for triaging hrHPV-positive women.[2] Actually, direct colposcopy referral of women with positive results either from HPV16 and/or HPV18 (HPV16/18) has become a clinical algorithm in some HPV screening guidelines.[3,4] Nevertheless, the main shortcoming of HPV16/18 genotyping test is that we cannot distinguish between transient and persistent infections. Over 90% of infections due to HPV16/18 will be cleared spontaneously after 2 years.[5,6] Therefore, referring all HPV16/18-positive women to colposcopy may result in too many patient and systems-related adverse outcomes.

There are several novel biomarkers that are able to predict abnormalities related to neoplastic progression and thereby achieve better risk stratification for cancer. A more accurate discrimination of cancer progression in HPV16/18-positive women through novel specific biomarkers, based on viral cell-cycle or abnormal cell proliferation, would assist in the decision to direct women to immediate treatment or prevent unnecessary further testing and overtreatment.[7]

According to evidence from recent studies conducted in the United States and Europe, p16/Ki-67 dual-stained cytology (DS) may be a promising biomarker to achieve a better risk stratification of transforming infections.[8] DS test has shown a reasonable balance by maximizing specificity with a suitable sensitivity for triage of women with hrHPV-positive infections, compared with conventional cytology.[9-11] The overexpression of $p16^{INK4a}$ (p16) is associated with the control of the cell cycle,[12,13] whereas Ki-67 is a cellular proliferation marker.[14] The simultaneous overexpression of both markers is a hallmark of cell-cycle deregulation. The HPV16/18 E6 oncoprotein (E6) is part of the expressed HPV genome and plays an important role in the onset of HPV-induced carcinogenesis. E6 interferes with the regulation of cell-cycle control and apoptosis, inactivating the *p53* tumor suppressor gene.[15] Overexpression of E6 is therefore indicative of progression toward malignant cell transformation.

To our knowledge, few studies have examined the potential role of further testing in women who screen positive to HPV16/18 through triage by cytology, E6 or methylation testing, the latter with very promising results.[16-18] There is a greater dearth of evidence describing the performance of DS as triage in HPV16/18-positive samples.

Previously, we assessed the clinical performance of different strategies based on HPV16/18 and liquid-based cytology (LBC) as triage for women screened with hrHPV, and we found that triaging with HPV16/18 genotyping followed by cytology yields the highest gain in sensitivity to detect precancerous lesions although at the expense of increasing the false positives.[19] Hence, in the present study we aimed to assess if additional testing either with LBC or the putative progression markers p16/Ki-67 and E6 would improve the overall accuracy of the triage with HPV16/18 testing to detect high-grade cervical

What's new?

Testing for HPV 16/18 is currently used in many screening programs for cervical cancer. However, standard HPV genotyping cannot determine whether or not the infection is likely to progress to cancer. In this study, the authors found that when dual-stained cytology (DS) for p16/Ki-67 is added to the screening, it may reduce unnecessary colposcopy referrals by as much as 75%. Meanwhile, this combination testing is also sensitive enough that invasive lesions can be caught early. This approach to HPV triage may thus be significantly more efficient, while reducing unnecessary procedures.

intraepithelial neoplasia (N) and cervical cancer within a hrHPV screening program in Tlaxcala, Mexico.

2 | MATERIAL AND METHODS

2.1 | Study population

The data to be used in this study come from a demonstration project Forwarding Research for Improved Detection and Access for Cervical Cancer Screening and Triage, "FRIDA." The FRIDA study was conducted in women undergoing the routine cervical cancer screening provided by Tlaxcala Public Health Services at the State of Tlaxcala, Mexico.

A detailed description of the FRIDA study procedures has been described previously.[20] Briefly, from August 2013 to February 2016, the study enrolled women aged from 30 to 64 years old attending for routine screening in any of the primary healthcare clinics administered by the Ministry of Health in Tlaxcala. Pregnant women or women with a previous hysterectomy were excluded. All participants were screened for hrHPV using the Cobas 4800 HPV DNA test (Roche Diagnostics). The Cobas 4800 test is a real-time polymerase chain reaction-based assay that detects the presence of 14 HPV types, with HPV16 and HPV18 detected individually and a pool of 12 other HPV types (HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66 and HPV68). HPV16/18 genotyping was used for triaging all hrHPV-positive women and those with positive results for HPV16/18 were referred to colposcopy. Women who attended any of two colposcopy clinics from Tlaxcala Public Health Services after a positive triage result with HPV16/18 were eligible for the present study.

After explaining the study procedures, verbal consent was obtained for all study participants before enrollment at a screening visit. All assays were performed on cervical specimens collected from the baseline screening visit.

At baseline, women were asked to provide two cervical samples. The first sample was placed in a SurePath vial and cytology and DS testing were carried out on this specimen. The second sample was placed in PreservCyt for hrHPV testing. Residual material from PreservCyt samples was used for the E6 Oncoprotein test. Samples were stored at room temperature at the local healthcare services for 2 weeks on average and then transported to the laboratories and stored at 4°C until processing.

2.2 | Testing procedures

2.2.1 | Liquid-based cytology

As part of the main objective of FRIDA's study, all women who were positive for hrHPV were tested for cytological abnormalities using samples from SurePath using the PrepStain System (TriPath Imaging, Inc., Burlington, NC, USA). This means that regardless of HPV16/18 status, the samples from all hrHPV-positive women were sent for cytological examination, although this analysis was restricted to HPV16/18-positive women. Either cytology or DS were processed in the cytology lab belonging to Tlaxcala Public Health Services. Slides stained with the Papanicolaou method were interpreted by the cytotechnicians who work in the routine cervical screening program at Tlaxcala using the Bethesda 2001 criteria.[21] The threshold used to define abnormal cytology findings was atypical squamous cells of undetermined significance (ASC-US) or worse (ASCUS+).

A double reading of these slides was employed to adjudicate the cytological result, as previously described.[22] For all cytological examinations, both the cytotechnologists and the cytopathologist were aware that specimens were hrHPV positives, but they were blinded to HPV16/18 result.

2.2.2 | p16/Ki-67 dual-stained cytology

Residual cell pellets obtained after producing the Papanicolaou-stained slides were added to 2 mL of SurePath Preservative Fluid. These samples were stored at 2°C to 8°C for up to maximum 1 year when kits for processing DS were available. Dual-stain slides were prepared using the CINtec PLUS Cytology kit (Roche MTM laboratories AG, Mannheim, Germany) on a fully automated slide-stainer platform (Ventana Benchmark XT; Roche Diagnostics), according to manufacturer's instructions. The evaluation of DS was conducted independently by two trained cytotechnologists at a private Lab in Cuernavaca, Morelos, who were blinded to other triage test results. Classification of DS results was conducted according to the manufacturer's recommendations. A positive DS test was considered if one or more cells were stained simultaneously with a brown cytoplasmic stain (p16) and a nuclear red staining (Ki-67). The DS slides that did not meet the minimum squamous cellularity criteria given by the Bethesda system[21] were considered invalid. In addition, as quality control, a valid test was considered if in the same slide, a brown staining of the cytoplasm of at least one cell (p16INK4a) and a red staining of at least one nucleus (Ki-67) was observed. Furthermore, DS positive slides as well as a random subset of DS negative slides, were reviewed by a pathologist.

2.2.3 | HPV16/18-E6 oncoprotein

A 3 mL aliquot of residual PreservCyt specimen stored at 4°C from women positive for either HPV16 or HPV18 were tested for HPV16/18-E6 Oncoprotein using the OncoE6 Cervical Test (Arbor Vita Corporation, Fremont, CA).[23] Though OncoE6 test was developed for testing swab specimens, a modification of the method, previously validated by the manufacturer, was used in FRIDA study employing exfoliated cervical cells from PreservCyt rather than collecting an extra cervical swab. Briefly, this manual assay is based in an immunochromatographic test using a lateral flow format device. An aliquot of the lysed sample is mixed with alkaline phosphatase-conjugated monoclonal antibodies (mAbs) targeting HPV16 and HPV18 E6 oncoproteins. The presence of any of HPV16 or HPV18 E6 oncoproteins in the mix forms a sandwich (capture mAb/E6

oncoprotein/detector mAb) resulting in a colorimetric reaction and the appearance of a purple test line on test strips. The test result is positive (E6 oncoprotein detected in the sample) if the control line and one or more test line(s) for the E6 oncoprotein appear.

2.3 | Diagnosis confirmation

During colposcopy examination, at least four biopsies (one biopsy from what the colposcopist suspected to be the most abnormal zone of each quadrant) and an endocervical sample were taken in all women.

All histological specimens were processed at the central pathology laboratory in the Tlaxcala's General Hospital. Histological diagnosis of cervical precancer, used as our clinical endpoint, was made by a panel of pathologists blinded to each other's results. First, two independent pathologists reviewed all biopsy specimens and made the histological grading. If a consensus by the two pathologists was met, that was the definitive histological diagnosis. Otherwise, a third more experience pathologist made the end-point adjudication. Histopathological diagnosis was based on CIN grading. Women with CIN grade 2/3 or higher (CIN2/3) were treated by Loop Electrosurgical Excision Procedure and those with cervical cancer referred to receive medical care at the Mexico's National Cancer Institute.

2.4 | Statistical analysis

Our main outcome measures were sensitivity and specificity of the three adjunctive tests either with cytology, E6 Oncoprotein or DS, for detecting cervical precancerous lesions in women referred to colposcopy based on their positive results to HPV16/18 genotyping. Because the data are correlated through repeated tests for each woman, a generalized estimating equation approach with an exchangeable working correlation structure was used to calculate statistics of sensitivity and specificity along with 95% confidence intervals (95% CI). In addition, positive predictive value (PPV), and negative predictive value (NPV) was calculated. Positivity results according to histopathological diagnosis grading are reported for all tests. Our main disease endpoint was histologically confirmed CIN3+, CIN2+ was set as a secondary endpoint because, although a high proportion of CIN2 are likely to regress, this is the currently accepted clinical threshold *t* for treatment.

The probability of agreement, was calculated by the total number of results for which both tests agree, divided by the total number of samples tested. To compare the proportions from matched tests we used McNemar's test which takes into account that both tests were carried out from a single woman. We tested differences in sensitivity (CIN3+, CIN2+), and specificity (<CIN3, and <CIN2) between the three paired tests by use of McNemars χ^2 test. A McNemar *P* value of <.05 indicates that the two methods differ in their performance.[24]

As indicators of efficiency, we computed the number of tests performed by each strategy, the referral rate for colposcopy if we had applied each one of the three tests, and the numbers of colposcopies per CIN3+ detected by each triage strategy. The triage algorithm where women with positive results in HPV16/18 were directly referred to colposcopy was set as comparator.

All analyses were performed using STATA software (version 14; Stata Corporation. College Station, TX). $P < .05$ was considered significant.

3 | RESULTS

As shown in Figure 1, 739 of 4051 women with hrHPV-tested positive for HPV16/18 infection; 543 of the 739 (73.5%) women successfully attended colposcopy and had histological results available. The residual material of these 543 women with positive results for HPV16/18 were assayed for LBC, DS and E6 oncoprotein test. However, our analytic sample included only 475 participants who had complete and valid results for all three adjunctive tests. The median age of these 475 participants was 37 years (interquartile range 33-43), over three quarters of participants reported having had less than three lifetime sexual partners (77.3%). Nearly half of the participants had three to four pregnancies (44.8%) and 23% more than four pregnancies. Twenty-five percent of our participants reported never having had a Papanicolaou test. There were no significant differences in positivity for cytology ($P = .79$) and E6 oncoprotein ($P = .44$) among women excluded for the analysis because of missing DS test results (data not shown).

3.1 | Positivity of different tests among HPV16/18-infected women

The histological grading by tests result is shown in Table 1. Overall, 67 and 45 women were diagnosed as CIN2+ and CIN3+, respectively. Of these, 14 out 22 CIN2 and 25 of the 40 CIN3 were aged 40 or below. It is noteworthy that in five women diagnosed with invasive cervical cancer all tested positive for DS and E6 oncoprotein, in contrast only one of them had abnormal cytological findings. (Table 1).

The overall positive rates were 14.1% (95%CI, 11.3-17.5) for ASCUS+ in cytology, 18.5% for E6 oncoprotein (95% CI, 15.3-22.3) and 24.4% (95% CI, 20.8-28.5) for DS. DS had a significantly higher positivity rate than cytology ($P = .000$, McNemar's test) and E6 oncoprotein ($P = .0112$, McNemar's test) (data not shown).

Of 45 women with CIN3+ 19 of them had normal cytological findings but were positive for DS testing and 3 were ASCUS+ but were DS negative, yielding an agreement between the both tests of 51.1% (95% CI 35.9-66.3) (Table 2). Less difference was observed between the cytology and E6 oncoprotein tests.

Of the 408 women who tested positive for HPV16/18 with <CIN2, the pairwise agreement was 77.5% for all tests, although among discordant pairs, there were more women with normal results for cytology and positive to the other tests (Table 2).

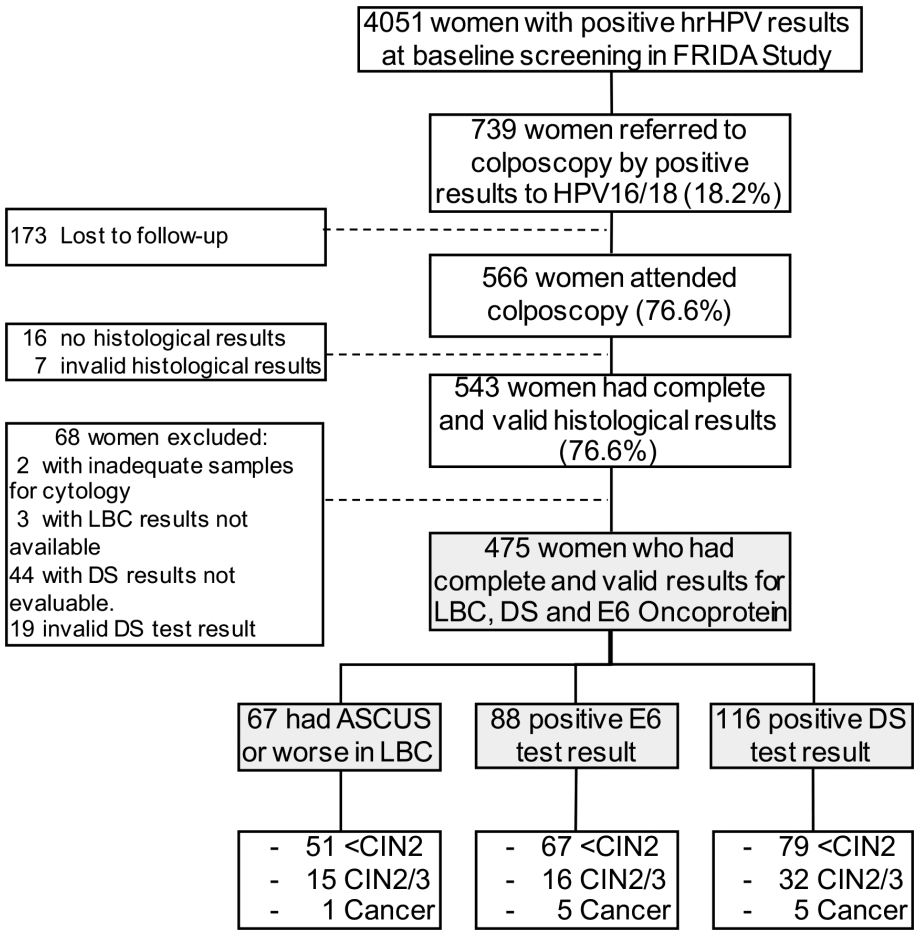

FIGURE 1 Flow diagram of participants' disposition to evaluate adjunctive testing by cytology, p16/Ki-67 dual-stained cytology, or HPV16/18 E6 oncoprotein on HPV16/18 screen-positive women using the baseline results of the first screening round of the FRIDA study trial. hrHPV, high-risk human papillomavirus; HPV16/18, Human papillomavirus type 16 or type 18; DS, p16/Ki-67 dual-stain cytology; CIN2/3, cervical intraepithelial neoplasia grade 2 or 3; ASCUS, Atypical squamous cells of undetermined significance and is the threshold for abnormal cytological finding

TABLE 1 Results of further testing either with cytology, DS or E6 oncoprotein by histopathologic categories among women referred to colposcopy after positive results to HPV16/18 genotyping

Test result	Total tested, N (%)	No biopsy, n	Inadequate, n	Normal/chronic inflammation, n	CIN1, n	CIN2, n	CIN3, n	Cancer, n	Total histologic results, N
Positive HPV16/18 result	562	81	6	180	228	22	40	5	475
LBC test result									
NILM	481 (85.6)	67	6	166	191	17	30	4	408
ASC-US	17 (3.0)	2	0	4	9	1	1	0	15
LSIL	41 (7.3)	8	0	6	20	4	3	0	33
ASC-H	0	—	—	—	—	—	—	—	—
HSIL	20 (3.6)	3	0	4	8	0	4	1	17
SCC	2 (0.4)	0	0	0	0	0	2	0	2
AGC	1 (0.2)	1	—	—	—	—	—	—	—
p16/Ki-67 dual-stain cytology									
Positive	132 (23.5)	66	1	32	47	10	22	5	116
Negative	430 (76.5)	15	5	148	181	12	18	0	359
E6 oncoprotein									
Positive	102 (18.2)	12	2	32	35	4	12	5	88
Negative	460 (81.8)	69	4	148	193	18	28	0	387

Abbreviations: AGC, atypical glandular cells; AIS, adenocarcinoma in situ; ASC-US, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; DS, p16/Ki-67 dual-stain cytology; E6, HPV16/18 E6 oncoprotein; hrHPV, high-risk human papillomavirus; HPV16/18, human papillomavirus type 16 or type 18; HSIL, high-grade squamous intraepithelial lesion; LBC, liquid-based cytology; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy; SCC, squamous cell carcinoma.

TABLE 2 Number of discordant pairs and agreement for discordant pairs between LBC, DS or E6 oncoprotein among women with HPV16/18 infections with CIN3+ and CIN2+

			Test1/Test2 result				
Test 1	Test 2	Total (n)	−/−	+/−	−/+	+/+	% Agreement (95% CI)
CIN3+							
DS	LBC	45	15	19	3	8	51.1 (35.9–66.3)
DS	E6 oncoprotein	45	12	16	6	11	51.1 (35.9–66.3)
LBC	E6 oncoprotein	45	23	5	11	6	64.4 (49.9-79.0)
<CIN3							
DS	LBC	430	306	68	35	21	76.1 (72.0-80.1)
DS	E6 oncoprotein	430	300	59	41	30	76.7 (72.7-80.7)
LBC	E6 oncoprotein	430	317	42	57	14	77.0 (73.0-81.0)
CIN2+							
DS	LBC	67	24	27	6	10	50.7 (38.5-63.0)
DS	E6 oncoprotein	67	23	23	7	14	55.2 (43.0-67.4)
LBC	E6 oncoprotein	67	37	9	14	7	65.7 (54.0-77.3)
<CIN2							
DS	LBC	408	297	60	32	19	77.5 (73.4-81.5)
DS	E6 oncoprotein	408	289	52	40	27	77.5 (73.4–81.5)
LBC	E6 oncoprotein	408	303	38	54	13	77.5 (73.4-81.5)

Abbreviations: DS, p16/Ki-67 dual-stained cytology; LBC, Liquid-based cytology; CIN2+, cervical intraepithelial neoplasia grade 2 or worst; CIN3+, cervical intraepithelial neoplasia grade 3 or worst; E6, HPV16/18 E6 oncoprotein; HPV16/18, high-risk human papillomavirus type 16 or 18; LBC, liquid-based cytology.

TABLE 3 Clinical performance of three different tests for detection of CIN2+ and CIN3+ among women referred to colposcopy after positive results to HPV16/18 genotyping

Threshold for referral to colposcopy Positivity	Women with ASCUS+ in LBC 14.1% (67/475) [11.3-17.5]	Women with results positive to E6 oncoprotein 18.5% (88/475) [15.3-22.3]	Women with results positive to DS 24.4% (116/475) [20.8-28.5]	*P* value[a]		
				LBC vs DS	LBC vs E6	DS vs E6
Detection of CIN3+ (n = 45)						
Sensitivity	24.4 (12.9–39.5)	37.8 (23.8–53.5)	60 (44.3-74.3)	.0009	.2101	.0525
Specificity	87 (83.4–90)	83.5 (79.6-86.9)	79.3 (75.2-83)	.0011	.1591	.0719
PPV	16.4 (8.49-27.5)	19.3 (11.7-29.1)	23.3 (15.9-32)			
NPV	91.7 (88.5-94.2)	92.8 (89.7-95.1)	95 (92.2-97)			
Detection of CIN2+ (n = 67)						
Sensitivity	23.9 (14.3-35.9)	31.3 (20.6-43.8)	55.2 (42.6-67.4)	.003	.4049	.0052
Specificity	87.5 (83.9-90.5)	83.6 (79.6-87)	80.6 (76.5-84.4)	.0035	.0953	.2109
PPV	23.9 (14.3–35.9)	23.9 (15.4-34.1)	31.9 (23.6-41.2)			
NPV	87.5 (83.9–90.5)	88.1 (84.5-91.2)	91.6 (88.3-94.3)			

Abbreviations: ASCUS+, Atypical squamous cells of undetermined significance or worse; CIN3+, cervical intraepithelial neoplasia grade 3 or worse; CIN2+, cervical intraepithelial neoplasia grade 2 or worse; DS, p16/Ki-67 dual-stained cytology; HPV16/18, high-risk human papillomavirus type 16 or 18; LBC, liquid-based cytology; NPV, negative predictive value; PPV, positive predictive value.

[a]*P* value comparing each paired test using McNemar test for sensitivity and specificity. Note that p-values of asymptotic McNemar's test are shown when the frequency of discordant pairs is greater than 10, otherwise McNemar exact is reported.

3.2 | Clinical test performance

DS had greater sensitivity than cytology for CIN3+, showing 60.0% (95% CI, 44.3-74.3) vs 24.4% (95% CI, (12.9-39.5) respectively, compared with a cytology result of ASCUS+ (*P* = .009) (Table 3). However, cytology had a significantly higher (*P* = .0011) specificity of 87% (95% CI, 83.4-90) than the DS test, 79.3% (95% CI, 75.2-83). Testing positive for E6 oncoprotein had a sensitivity of 37.8 (95% CI, 23.8-

TABLE 4 Diagnostic efficiency for disease detection using distinct combination of triage strategies based on HPV16/18 genotyping among women with positive results to hrHPV

Indicators	All women with positive results to HPV16/18	Triage strategies in HPV16/18-positive women		
		LBC (ASCUS+ threshold)	E6 Oncoprotein	p16/Ki-67 DS
No. of tests	0	475	475	475
Colposcopy referrals, % (n/N) [95% CI]	100%	14.1% (67/475) [11.3-17.5]	18.5% (88/475) [15.3-22.3]	24.4% (116/475) [20.8-28.5]
CIN3+ detected, no.	45	11	17	27
Number of colposcopies to detect 1 CIN3+	10.6	6.1	5.2	4.3

Abbreviations: ASCUS+, Atypical squamous cells of undetermined significance or worse; CIN3+, cervical intraepithelial neoplasia grade 3 or worse; DS, dual-stained cytology; HPV16/18, high-risk human papillomavirus type 16 and/or 18; LBC, liquid-based cytology.

53.5) for CIN3+ in women who were HPV16/18 positive, but compared with DS, there were no significant differences between sensitivity of both tests (Table 3). The performance for the three tests were similar for CIN2+, although for this endpoint, DS had a significantly higher sensitivity than E6 oncoprotein ($P = .0052$).

3.3 | Efficiency for disease detection

Compared with the strategy of referring all women with HPV16/18 to colposcopy, further testing with all three tests would lead to a substantial reduction in colposcopy referrals (Table 4). Colposcopy referrals for the three tests ranged from 14.4% for women with ASCUS+ to 23.5% for those with positive DS test.

Greater CIN3+ detection was found for DS testing with 27 CIN3+, followed by 17 among women who tested E6 oncoprotein positive and 11 in those with cytology results of ASCUS+. In addition, further testing with DS would require fewer colposcopies to detect one CIN3+ compared with the strategy of referring all HPV16/18-positive women to colposcopy (4.3 vs 10.6).

4 | DISCUSSION

The present work was encouraged by the need to avert unnecessary colposcopies and treatments associated with nonprogressive infections with HPV16/18 following HPV screening.[6,25,26] Our results showed that in women with positive results for HPV16/18 genotyping, DS test performs better than cytology and E6 oncoprotein in discriminating relevant progressive infections. DS had a substantial reduction of colposcopy referrals vs the direct colposcopy referral of all HPV16/18-positive women. DS, has high specificity with minimal loss of sensitivity for HPV16/18-positive women.

We found an increase in the positivity of both the DS and E6 progression biomarkers as the degree of cervical neoplasia increased something that was not observed for cytology testing. These results can highlight the underlying molecular mechanism of both biomarkers, which are indicative of a progression toward malignant cell transformation. In this regard, the reduction of sensitivity for CIN2/3 after using adjunctive testing compared with referring all HPV16/18-positive women to colposcopy highlights how morphologically similar neoplasia may differ in molecular processes. CIN2/3 are surrogate endpoints for cancer risk but there is compelling evidence that many CIN2/3 regress and never progress to cancer.[27] For instance, it has been reported that simply around 30% of CIN3 cases will progress to invasive cancer if left untreated.[28] If progression markers such as DS or E6 are associated with transforming infections, it might be possible that CIN3 where p16/Ki-67 is not expressed are actually lesions which will not develop cervical cancer, and thus they could be considered true negatives. The above is in line as previously suggested by Yu et al,[29] who pointed out that the overexpression of E6 is a better indicator of HPV persistence and therefore may be more useful as a predictor of cancer risk rather than a tool for classifying the existing disease using morphology based diagnosis.

Notably, the use of DS or E6 Oncoprotein was associated with a reduction of colposcopy rates by more than 75% with 100% sensitivity for detection of invasive cancer. This is crucial considering that any suitable screening test should be effective enough to detect virtually all invasive cancers to decrease the consequences provoked by missing cancers.

Our study supports previous conclusions that DS rather than cytology may be a more promising candidate for discriminating women at higher risk for progressive hrHPV infections because it is more sensitive for CIN3+ with a similar specificity.[8-11,30-35] However, our work outlines the utility of DS when it is applied in women with HPV16/18 infections, which can maximize screening efficiency, helping to increase disease prediction and further reduce unneeded colposcopies.

In recent years, overexpression of E6 has also attracted attention from some studies by its potential clinical utility to distinguish progressing infections.[16,36] Ferrera et al showed that E6 oncoprotein of HPV16/18 may be a useful marker to predict CIN3+. Interestingly, they used a more accurate definition of truly CIN3+, by using p16 immunostaining to confirm the histological outcomes, observing a sensitivity of 96.8%, substantially superior to our results.[16] As we mentioned above, these results can suggest that the greatest benefit of E6 might be as an indicator of cancer risk reflecting more advanced stages of cell transformation.

Longitudinal data from a large, well-powered study, will help to confirm if the risk of CIN3+ in women positive for both HPV16/18

and DS can justify immediate treatment at the first colposcopy visit. Besides, the risk of reproductive adverse outcomes after the overtreatment in women at reproductive age may be solved with a more accurate screening.[37] Previous screening cohort studies have suggested long-term reassurance of decreased risk for CIN3+ in women who are HPV16/18 positive but test negative for either DS or E6, which is promising toward achieving more efficient screening programs. In a recent paper by Wentzensen and colleagues,[10] they reported that the risk of CIN3+ in women positive for HPV16/18 but negative to DS is just 6%, which is below the threshold for colposcopy referral. Similarly, results from a 15-year prospective screening cohort study, reported that HPV-positive women with E6 negative test have an increased probability of regression than E6-positive women.[30] If these results are validated they may allow a safe extension of the screening intervals for 3 years in women HPV16/18 positive but negative for DS or E6 progression markers.

Our results have implications for clinical and public health practice. We acknowledge that the adoption of more disease-specific biomarkers may not be practical if the cost of these biomarkers is high. This is particularly important when limited resources need to be prioritized for large-scale population screening. However, most of the hrHPV tests provide the results of HPV16 and HPV18 individually in the same HPV assay, ergo using a stepped screening could maximize resources allocation if biomarkers, such as DS, are applied as reflex testing on a small fraction of women. For example, in our population where ~2% of all screened women had an infection with HPV16/18, this would be the target population for adjunctive testing with the progression biomarkers.

Another advantage of new biomarkers of neoplastic progression, such as DS, is the robustness of their measurement.[38] In comparison with cytology, DS does not depend of morphological evaluation and good reproducibility of DS has been consistently demonstrated.[39-41] A similar performance of DS has been observed when triaging HPV-positive women among expert and nonexpert readers.[42] Besides, the performance of subjective interpretation can be improved even further with the emerging of automated reading with a deep learning tool.[43] This constitutes a relevant research area in view that cloud-based implementation of automated DS evaluation can makes this tool more feasible to implement and possibly more affordable for developing countries.

Our study has some limitations worth noting. Our use of CIN2+ as the main cancer risk surrogate, could be debated, some experts favor the use of CIN3+ as the definitive endpoint. Nonetheless, CIN2 is the currently accepted clinical threshold to initiate immediate treatment. PPVs shown for CIN2 and CIN3 should be interpreted in the context of women referred for colposcopy by positive results to HPV16/18 and not for the general population. The sample size of CIN3+ was small and therefore, under the null hypothesis of McNemar's test, we do not have enough power (71%) to detect if there was a significant difference between DS and E6 Oncoprotein. Regarding missing data in DS, although 11% of HPV16/18-positive women were excluded from our analytic sample, these women did not have different positive rates of E6 or abnormal Pap cytology than the remaining women. In order to prevent a differential information bias, our laboratory staff, who performed each one of the tests, was blinded to HPV16/18 status as well as disease status. Gold standard disease ascertainment was one of the main strengths of our study. Our study included a systematic collection of biopsies reducing the possibility of differential misclassification related to triage results or that may arise if just colposcopy-targeted biopsies would have been used. It is worth noting that disease ascertainment was by pathology panel review, led by a highly experienced and standardized pathologist, assigning a consensus diagnosis in all cases.

Our results support the more accurate detection of cervical precancer by DS in HPV16/18-positive women, emphasizing the promising value of this test as a triage test in HPV screening programs. DS may help to identify the women who would most benefit from immediate referral to colposcopy. For many years the lack of resources has determined the use of less effective and even more aggressive procedures for cervical cancer detection and control in populations with the greatest need. Therefore, in the pathway to accomplish the benefits of screening programs based on primary HPV testing, particularly in underserved populations with the greatest cervical cancer burden, we need to move forward to determine how tests providing a more accurate risk assignment can be integrated in the most cost-effective and feasible way.

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CONFLICT OF INTEREST

Drs Salmerón and Lazcano report receiving supplies and equipment for liquid-based cytology processing from BD Diagnostics Systems, and reagents for HPV genotyping and equipment from Roche Molecular Systems and Roche/Ventana Medical Systems through their institutions. Dr. Wheeler reports receiving reagents and equipment from Roche Molecular Systems, Roche Ventana Medical Systems and Genera Biosystems through her institution and research support and personal fees from Becton Dickinson all outside of the submitted work. The rest of the authors declare no conflicts of interest.

4.1 | DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The FRIDA study was approved by the Institutional Review Boards (IRBs) of the participating institutions: National Public Health Institute (INSP) [1094] and Tlaxcala State Ministry of Health [SS.DECI-OI-13/12]. The FRIDA study is registered with ClinicalTrials.gov, number NCT02510027.

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