

## BD-ProExC as Adjunct Molecular Marker for Improved Detection of CIN2<sup>+</sup> after HPV Primary Screening

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### Abstract

**Background and Methods:** We investigated the efficacy of 8 cervical cancer screening strategies relative to cytology with emphasis on immunocytochemical detection of high-risk human papillomavirus (hrHPV)-induced cell transformation (BD-ProExC) as a tool of triage following primary cytology or hrHPV testing. 3,126 women were tested with BD-SurePath liquid-based cytology, hrHPV PCR genotyping and BD-ProExC immunostaining, and colposcopy verification to calculate sensitivity and positive predictive value (PPV) in detecting cervical intraepithelial neoplasia (CIN2<sup>+</sup>).

**Results:** Compared to cytology screening, double testing with cytology and hrHPV resulted in the same sensitivity with a significant increase in the PPV (relative PPV: 1.83). However, twice as many tests were needed. Cytology with atypical squamous cells of undetermined significance (ASC-US) triage and hrHPV testing showed comparative results to double testing requiring only a small increase in number of tests. Screening for hrHPV subtypes 16/18, and ASC-US triage with hrHPV16/18 resulted in significant reductions in sensitivity (ratio: 0.74 and 0.96, respectively). Primary hrHPV/BD-ProExC screening was significantly more sensitive (ratio: 1.63/1.33), but had a significantly lower PPV (ratio: 0.64/0.88). ASC-US triage by BD-ProExC increased the PPV (ratio: 1.90) but decreased the sensitivity (ratio: 0.96). Primary hrHPV screening followed by BD-ProExC triage, led to significant increases in sensitivity (ratio: 1.30) and PPV (ratio: 2.89), and resulted in 55% fewer referrals for colposcopy.

**Conclusions:** From the investigated screening strategies, primary hrHPV DNA-based screening followed by BD-ProExC triage was determined to be the best screening strategy.

**Impact:** Immunocytological triage could be used to perfect hrHPV primary screening. *Cancer Epidemiol Biomarkers Prev*; 20(4); 628–37. ©2011 AACR.

### Introduction

Cervical cancer is a leading cause of morbidity and mortality among women worldwide. In 2004, it was estimated that 34,300 women in the European Union (EU) developed this disease and 16,300 died from it (1). In particular, age-standardized incidence and mortality rates in Belgium were found to be 12/100,000 and 5/100,000, respectively (1). Persistent infection with high-risk (oncogenic) types of human papillomavirus (hrHPV)

has been determined to be the primary risk factor for the development of cervical cancer and its precursor lesions (1–6). The prevalence of hrHPV is especially high among women in their mid-20s with a steady decline thereafter (7, 8). However, effective screening and treatment of high-grade (2 or higher) cervical intraepithelial neoplasia (CIN2<sup>+</sup>) can substantially reduce the risk of cancer development and improve prognosis. In fact, the incidences of cervical cancer in some countries have decreased by up to 80% since the introduction of national screening programs (9).

At present, the screening situation varies considerably across Europe, with several countries still without organized programs in place. Within several countries, including Belgium, testing is largely opportunistic, based on individual initiative, and carried out by gynecologists. Consequently, the prevalence of malignant disease may be elevated in these areas. Cervical cytology alone has a highly variable sensitivity, ranging from 18.6% to 76.7%, for the detection of high-grade disease (2, 10), requiring women to undergo repeated screening to maintain high levels of protection. Currently, HPV testing is recommended for triage of women with atypical squamous

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doi: 10.1158/1055-9965.EPI-10-0818

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cells of undetermined significance (ASC-US) and after treatment of CIN, but not for primary screening. However, there is now emerging evidence for the use of hrHPV-type DNA testing as a primary screening tool, given its increased sensitivity for detecting CIN2<sup>+</sup> compared with cervical cytology (2, 10–14).

Recently, BD ProExC biomarker reagent was introduced as a mean for detecting the presence of aberrant S-phase induction when used in combination with standard immunocytochemistry techniques on cytology samples. This protein-based product consists of antibodies specific for minichromosome maintenance protein 2 (MCM-2) and topoisomerase II- $\alpha$  (TOP2A) both of which are overexpressed during this process and in cervical dysplasia and neoplasia (15–18). Studies performed using BD ProExC on BD SurePath cytology specimens have demonstrated increased analytical sensitivity for the detection of biopsy-confirmed high-grade disease compared with cytology alone, as well as improved positive predictive value (PPV) for the detection of high-grade disease compared with hrHPV DNA testing (19, 20).

In this prospective colposcopy controlled study, we investigated the potential of BD ProExC as a tool of triage following primary cytology screening or primary screening by detection of hrHPV, to assist in the identification of women with high-grade CIN. Clinical sensitivity and specificity results of 7 different algorithms of screening were compared with data from liquid-based cytology alone and to each other to evaluate their relative efficacies. In order to prevent any bias, cytology screens were carried out without knowledge of the HPV or BD ProExC results. An investigation of cytology results in knowledge of these findings will be published separately.

## Materials and Methods

### Subject enrollment

From August 2005 to February 2007, 3,126 women with a median age of 42.7 years (range 18.0–84.3) undergoing routine screening agreed to participate in this prospective, colposcopy controlled study. We evaluated samples collected in 9 gynecological practices in Flanders (Belgium). Study specific subject identification codes were assigned and transmitted in such a manner that subject confidentiality was preserved. All women gave written informed consent and this study was approved by the local ethical committee (Ziekenhuis Oost Limburg, ZOL, Genk, Belgium).

### Inclusion and exclusion criteria

Exclusion criteria included pregnancy and history of cervical disease (previous history of CIN2<sup>+</sup>); 221 women were excluded.

### Study design and assessment of study endpoints

Power-based sample size calculations were performed for the relative ratios of PPVs. Using the Inequality Tests for Two Proportions based on the

assumption of a reference proportion of 0.15, a test strategy sample of at least 80 cases and a reference strategy sample of at least 160 cases were determined sufficient to detect an effect corresponding to a ratio of at least 2.1 between Cytology (reference) and the test strategy, with  $\alpha = 0.05$ , power = 80%, and the z-test based relative risk comparison to 1.

The study algorithm is summarized in Figure 1. After the exclusion criteria were applied 2,905 women underwent liquid-based cytology, HPV DNA testing, BD ProExC immunocytochemistry (ICC), and colposcopy verification. All colposcopies at baseline were performed according to a standardized protocol by 9 different gynecologists. First a 5% acetic acid solution was applied to assist in identifying undifferentiated epithelia or inflammation as well as true CIN. After the application of the acetic acid, a judgment of the transformation zone was made. After visual inspection a cervical smear was taken with the Cervex-Brush. Biopsies were only taken from all acetowhite lesions after smear taking. Those with positive colposcopic examinations received immediate cervical biopsy with subsequent histologic analysis and were given treatment as required. All other subjects were followed up based on their HPV and cytology results and either received a second colposcopy and biopsy or were monitored based on recommended Belgian follow-up guidelines. (21)

Liquid-based cytology samples from all the eligible women were prepared for routine cervical screening. The cytology leftover was used for hrHPV detection by real-time PCR analysis, and an extra slide was prepared for BD ProExC ICC staining. All the results from cytology, ICC, biopsies, treatment/follow-up, and HPV status were entered into a database. The unique subject ID number was used to link the different cytological, histological, and virological data. For all samples included in the study the database was searched to identify cases with histology-proven CIN2<sup>+</sup> within a 24-month follow-up period.

### Cervical sample processing and classification

Cervical cells were collected using the Cervex-Brush (Rovers). After collection, the brush head of the sampling device was deposited directly into the vial containing the ethanol-based BD SurePath Preservative Fluid (BD SurePath; BD Diagnostics—TriPath). The vials were then transported to the laboratory for testing. All samples were prepared and analyzed in the laboratory of Clinical Pathology (RIATOL). A density sedimentation method (BD PrepMate; BD Diagnostics—TriPath) was used to enrich the cell samples by excluding blood and the majority of inflammatory cells, as well as necrotic debris, mucus, and other contaminants. Thin-layer slide preparations were then made with the fully robotic BD PrepStain (BD Diagnostics—TriPath) according to manufacturer instructions. Thin-layer cell dispersions with discrete staining for cytological analysis were generated with the BD PrepStain slide processor.

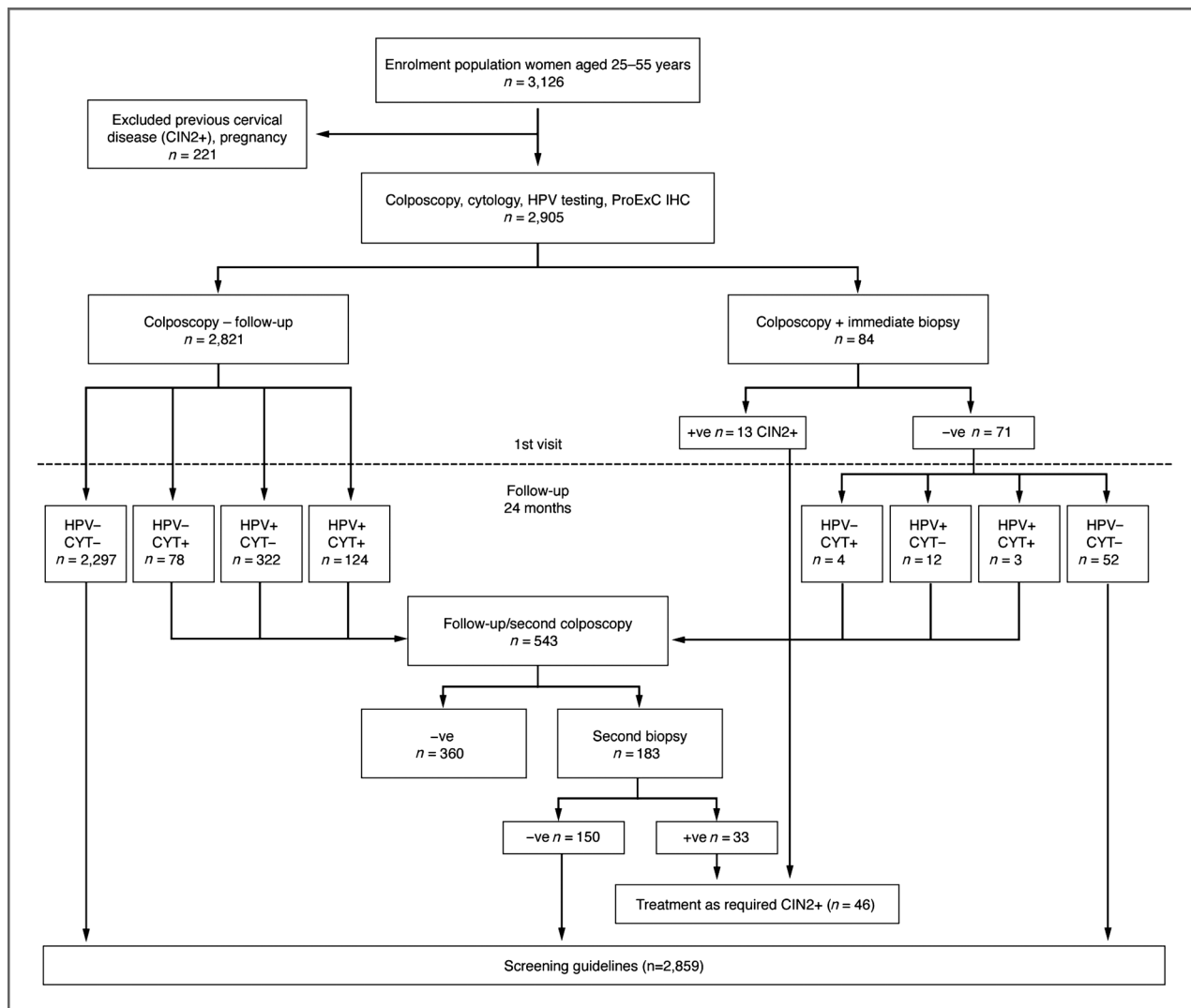


Figure 1. Study flow diagram.

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The cytological results were classified according to the Bethesda system 2001 (22): negative for intraepithelial lesion or malignancy (NILM), ASC-US, atypical squamous cells of undetermined significance, cannot exclude high-grade squamous intraepithelial lesions (ASC-H), atypical glandular cells of undetermined significance (AGC, 3 cases which were included in the ASC-US group), low-grade squamous intraepithelial lesions (LSIL), and high-grade squamous intraepithelial lesions (HSIL).

**BD FocalPoint guided screening**

All slides were scanned by the BD FocalPoint Guided Screener Imaging System according to manufacturer instructions. As a positive control, a known sample, previously diagnosed as HSIL (CIN3 biopsy confirmed) and positive for HPV 16, was loaded in between the study

samples each day. Cytology screening was performed without knowledge of the HPV status.

**Isolation of DNA from cervical cells for HPV DNA testing**

DNA isolation from the cellular pellet remaining after processing of the BD SurePath cytology specimen was performed as previously described (22–25). The DNA extracts were stored at –20°C until PCR was performed.

**Real-time type specific PCR analysis of HPV DNA**

Each sample was initially subjected to real-time PCR amplification for the detection of β-globin to confirm that the DNA quality was suitable for PCR analysis. All samples were subsequently tested for the following hrHPV types using type specific-PCR (as previously described; refs.22 and 23): 16 E7, 18 E7, 31 E6, 33 L1,

33 E6, 35 E6, 39 E7, 45 E7, 51 E7, 52 L1, 52 E7, 56 E7, 58 L1, 58 E7, 59 E7, and 68 E7. Low-risk HPV types 6 E6, 53 E6, 66 E6, and 67 L1 were also tested.

### BD ProExC immunocytochemistry

An additional thin-layer slide was prepared from the residual BD SurePath vial using the BD PrepStain instrument. Slides were treated with a pretreatment buffer for antigen retrieval (BD SureDetect; BD Diagnostics—TriPath). Slides were processed on an SMS 3600 automated stainer using the BD ProExC immunohistochemistry kit according to manufacturer instructions (BD Diagnostics—TriPath). Briefly, the liquid-based cytology slide was incubated with the BD ProExC antibody reagent, this was followed by the use of a detection reagent that included a 3,3'-diaminobenzidine tetrahydrochloride (DAB)-based chromagen (BD SureDetect detection reagent). The DAB-based chromogen imparts a brown nuclear stain to cells that over express MCM-2 and TOP2A. The specimen was then counterstained with hematoxylin—a bluing agent (BD SureDetect counterstains), and the slide was coverslipped for viewing under a light microscope. Moderate-to-intense brown nuclear staining observed in atypical epithelial cells was considered a positive result. Enlarged naked nuclei that stained positive were not considered positive. In some slides, bacilli and abundant mucus gave rise to background staining, whereas inflammatory cells did not stain. In some cases, staining of glandular cells and tubal metaplasia was seen and, for the most part, these showed a light cytoplasmic staining with occasional staining of the nuclei. They could be clearly identified as cases of tubal metaplasia or glandular cells by morphology and were not considered positive. All slides were screened by experienced cytotechnologists. The staining of the 2,905 slides was performed in 97 batches, with negative and positive controls included in each batch.

### Statistical analysis

The main analyses were calculated on intention-to-treat populations. Although CIN2<sup>+</sup> was used as the primary endpoint, we repeated the calculations with CIN3<sup>+</sup> as the endpoint. All histologically verified cases of CIN2<sup>+</sup> detected within 24 months after trial enrollment were included in this cross-sectional analysis. Women who did not have a CIN2<sup>+</sup> lesion recorded in the database were classified as not having a high-grade CIN lesion. In ASC-US triage only cytology with result ASC-US are tested (not LSIL and HSIL). Women who had a cytological diagnosis of ASC-US or worse (i.e., ASC-US, ASC-H, AGC, LSIL, or HSIL) were classified as having abnormal cytology (ASC-US<sup>+</sup>).

We evaluated the sensitivity, specificity, PPV, and negative predictive value (NPV) of HPV DNA testing and cytology. Separate analyses were performed using histologically confirmed CIN2<sup>+</sup> and histologically confirmed CIN3 or worse (i.e., CIN3<sup>+</sup>) as endpoints. We compared the efficacy—defined as the sensitivity

(proportion of CIN2<sup>+</sup> or CIN3<sup>+</sup> detected)—PPV, and number of screening tests required of 8 different screening strategies involving HPV DNA testing and ProEx C immunostaining with that of primary screening with cytology alone:

- hrHPV DNA test alone;
- ProEx C immunostaining alone;
- Double testing, both hrHPV testing and cytology screening performed independently, with only LSIL, ASC-H, and HSIL irrespective of HPV status and ASC-US/hrHPV<sup>+</sup> were considered for colposcopy;
- Cytology with ASC-US triage by hrHPV;
- Cytology with ASC-US triage by HPV 16/18;
- HPV DNA test targeting 16/18 only;
- Cytology with ASC-US triage by BD ProExC; and
- hrHPV DNA test with triage by BD ProExC.

The PPV describes the proportion of test-positive women who actually have the disease being screened for. We used the total number of screening tests and the number of tests needed to detect one case of CIN2<sup>+</sup> and CIN3<sup>+</sup> as indicators of the cost-effectiveness of a screening strategy. The following screening strategies were considered: (i) a single HPV DNA test; (ii) an HPV DNA test in combination with cytology (without knowledge of HPV results); and (iii) screening for specific hrHPV types 16 and 18, which in other cohort studies were found to confer a particularly high risk for the development of CIN3 (26, 27). All proportions were calculated with exact 95% binomial CIs. We compared the efficacy of different screening strategies by calculating risk ratios with 95% CI.

Overall numbers of CIN2<sup>+</sup>/CIN3<sup>+</sup> were calculated for primary screening and the subsequent triage. The number of referrals to colposcopy and the number of CIN2<sup>+</sup>/CIN3<sup>+</sup> lesions per referral were also determined. Statistical analysis was performed using the MedCalc programme (MedCalc Software; ref. 28).

## Results

### Histological outcomes within each primary screening method

After exclusion of 221 cases with antecedents of cervical neoplasia, 2,905 samples were included in the final study group. A cervical biopsy was taken from 9.2% ( $n = 267$ ) of these participants (84 on the baseline visit and 183 on subsequent follow-up visits) and yielded 46 histologically confirmed CIN2<sup>+</sup> cases (Fig. 1, Table 1). Of the 2,905 slides, 88 (3.0%) were excluded from automated prescreening because of failed processing and the slides had to be screened manually. Among the 2,905 women included in the study, none had an inadequate smear and 2,718 had a negative smear (93.6%; mean age 43.0 years). A total of 187 slides (6.4%) presented with ASC-US or worse (ASC-US<sup>+</sup>) results, with a mean age of 40.0 years. The histological outcomes within each detection method for the 267 biopsy specimens are summarized in Table 1. Real-time PCR testing



**Table 1.** Histological outcomes within each detection method

Histology		No biopsy	Biopsy (n = 267)					Total
			Normal	CIN1	CIN2	CIN3	INV CA	
Colposcopy		2,638	180	41	21	21	4	2,905
Cytology	NILM	2,523	153	23	7	9	3	2,718
	ASC-US	87	13	4	3	1	–	108
	LSIL	23	8	9	5	1	–	46
	ASC-H	3	2	2	–	1	–	8
	HSIL	2	4	3	6	9	1	25
Real-time PCR	hrHPV <sup>–</sup>	2,335	89	6	2	–	–	2,432
	hrHPV <sup>+</sup>	303	91	35	19	21	4	473
ProExC	Neg	2,416	164	32	8	2	–	2,622
	Pos	222	16	9	13	19	4	283

NOTE: The following HPV types were included as high risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68.

revealed 473 hrHPV-positive samples (16.3%) for one of the following high-risk types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68; the mean age in this group of subjects was 38.7 years.

#### Cytology alone is less sensitive but more specific than HPV DNA testing alone in detecting CIN2<sup>+</sup> and CIN3<sup>+</sup>

Primary screening with cytology detected 27 of the 46 CIN2<sup>+</sup> cases giving a sensitivity of 58.7% and a specificity of 94.4%, with a PPV of 14.4% for the detection of high-grade disease (Table 2). Sensitivity and specificity for detecting CIN3<sup>+</sup> using this testing method were 52.0% and 94.0%, respectively. In contrast, with HPV DNA test alone, 44 of the 46 biopsy proven CIN2<sup>+</sup> cases were detected by hrHPV screening, resulting in a sensitivity of 95.7% and a specificity of 85.0%, with a PPV of 9.3% for the detection of high-grade disease (Table 2). A sensitivity of 100.0% and a specificity of 84.4% were determined for detecting CIN3<sup>+</sup>. It was therefore demonstrated that cytology primary screening yielded better specificity but inferior sensitivity compared with HPV testing alone.

#### BD ProExC primary screening or as a tool for triage following primary screening

BD ProExC immunostaining as a primary screening test provided an improved sensitivity for both CIN2<sup>+</sup> and CIN3<sup>+</sup>, but was less specific compared to cytology (Table 2).

With regards to ASC-US triage by BD ProExC after primary screening by cytology, 26 out of the 46 cases were detected, giving a sensitivity of 56.5% and a PPV of 27.4% for CIN2<sup>+</sup> (Table 2). For CIN3<sup>+</sup> detection, the sensitivity was 52% (13/25 cases) and the PPV was 13.7%. These findings indicate similar sensitivities but superior PPVs for this strategy relative to cytology screening alone.

Triage by BD ProExC after primary screening by hrHPV detection revealed better outcomes in term of

sensitivity and PPV for both CIN2<sup>+</sup> and CIN3<sup>+</sup> detection (Table 2). A sensitivity of 76% and a PPV of 41.7% were found for CIN2<sup>+</sup>, and the corresponding results for CIN3<sup>+</sup> were 92% and 27.4%, respectively. These values were significantly higher than found for cytology alone ( $P < 0.005$ ) and the PPVs were the highest among all the strategies tested, indicating the potential effectiveness of hrHPV primary DNA screening followed by BD ProExC triage in cervical cancer screening.

#### Relative efficacies of various screening methods compared to cytology alone

Various tests for triaging abnormal cytology (ASC-US<sup>+</sup>) or positive hrHPV primary screening were compared for their ability to improve the detection rate of women with CIN2<sup>+</sup> disease, without increasing the number of women sent for unnecessary colposcopic procedures (Table 2). Triage by BD ProExC after primary screening by hrHPV was the only algorithm that was more sensitive and had a better PPV compared to cytology alone to detect CIN2<sup>+</sup> (ratio 1.30 and 2.89, respectively) and CIN3<sup>+</sup> (ratio 1.77 and 3.94, respectively). The total number of colposcopy procedures would decrease from 187 after cytology to 84 with BD ProExC triage after a hrHPV primary screening (55% decrease).

There was 1 woman diagnosed with an ovarian carcinoma with a normal cytology result and negative for all HPV types tested, her BD ProExC test was positive.

#### Discussion

There has been an immense evolution in the primary screening of cervical cancer. With the introduction and combination of such innovations as liquid-based cytology, automated image analysis and HPV testing, considerable efforts have been made to improve the quality and reliability of primary screening (29). Pap testing is affected by a substantial rate of false-negative results and

**Table 2.** Sensitivity and PPV of 8 HPV DNA and ProExC test based screening algorithms compared with primary screening by cytology only

Screening strategy	CIN2 <sup>+</sup> (n = 46)					CIN3 <sup>+</sup> (n = 25)									
	Sensitivity % (95% CI)	Relative sensitivity (95% CI)	PPV % (95% CI)	Relative PPV (95% CI)	Specificity % (95% CI)	NPV % (95% CI)	Sensitivity % (95% CI)	Relative sensitivity (95% CI)	PPV % (95% CI)	Relative PPV (95% CI)	Specificity % (95% CI)	NPV % (95% CI)	Tests used (n)	Screenests/Colpo/CIN3 <sup>+</sup> (n)	Colpo/CIN3 <sup>+</sup> (n)
Cytology only	27/46		27/187		13/25		13/187						2,905	223.5	14.4
	58.7 (43.3–72.7)	1 (referent)	14.4 (9.9–20.5)	1 (referent)	94.4 (93.5–95.2)	99.3 (98.9–99.6)	52.0 (31.8–71.7)	1 (referent)	7.0 (3.9–11.9)	1 (referent)	94.0 (93.0–94.8)	99.6 (99.2–99.8)			
HPV DNA test only	44/46		44/473		25/25		25/473								
	95.7 (84.0–99.2)	1.63 (1.37–1.94)	9.3 (6.9–12.4)	0.64 (0.60–0.70)	85.0 (83.6–86.3)	99.9 (99.7–100)	100.0 (83.4–100)	1.92 (1.40–2.63)	5.3 (3.5–7.8)	0.76 (0.66–0.90)	84.4 (83.1–85.8)	100	2,905	116.2	18.9
ProExC only	36/46		36/283		23/25		23/283								
	78.3 (63.2–88.6)	1.33 (1.22–1.46)	12.7 (9.2–17.3)	0.88 (0.84–0.93)	91.4 (90.3–92.4)	99.6 (99.3–99.8)	92.0 (72.5–98.6)	1.77 (1.38–2.28)	8.1 (5.3–12.1)	1.17 (1.02–1.36)	91.0 (89.9–92.0)	99.9 (99.7–100)	2,905	126.3	12.3
Double testing, hrHPV, and cytology	27/46		27/102		13/25		13/102								
	58.7 (43.3–72.7)	1.00 (1.00–1.00)	26.5 (18.5–36.3)	1.83 (1.77–1.87)	97.4 (96.7–97.9)	99.3 (98.9–99.6)	52.0 (31.8–71.7)	1.00 (1.00–1.00)	12.8 (7.2–21.2)	1.83 (1.79–1.85)	96.9 (96.2–97.5)	99.6 (99.2–99.8)	5,810	446.9	7.9
Cytology with ASC-US triage by hrHPV	27/46		27/102		13/25		13/102								
	58.7 (43.3–72.7)	1.00 (1.00–1.00)	26.5 (18.5–36.3)	1.83 (1.77–1.87)	97.4 (96.7–97.9)	99.3 (98.9–99.6)	52.0 (31.8–71.7)	1.00 (1.00–1.00)	12.8 (7.2–21.2)	1.83 (1.79–1.85)	96.9 (96.2–97.5)	99.6 (99.2–99.8)	3,013	231.8	7.9

(Continued on the following page)

Table 2. Sensitivity and PPV of 8 HPV DNA and ProExC test based screening algorithms compared with primary screening by cytology only (Cont'd)

Screening strategy	CIN2 <sup>+</sup> (n = 46)					CIN3 <sup>+</sup> (n = 25)								
	Sensitivity % (95% CI)	Relative sensitivity (95% CI)	PPV % (95% CI)	Relative PPV (95% CI)	Specificity % (95% CI)	NPV % (95% CI)	Sensitivity % (95% CI)	Relative sensitivity (95% CI)	PPV % (95% CI)	Relative PPV (95% CI)	Specificity % (95% CI)	NPV % (95% CI)	Tests Screened/used (n)	Colpo/CIN3 <sup>+</sup> (n)
Cytology with ASC-US triage by HPV 16/18	26/46		26/89		13/25		13/89							
	56.5 (41.2–70.8)	0.96 (0.95–0.97)	29.2 (20.3–40.0)	2.02 (1.95–2.05)	97.8 (97.2–98.3)	99.3 (98.9–99.6)	52.0 (31.8–71.7)	1.00 (1.00–1.00)	14.6 (8.3–24.1)	2.10 (2.03–2.13)	97.4 (96.7–97.9)	99.6 (99.2–99.8)	3,013	231.8
HPV DNA test targeting 16 and 18 only	20/46		20/144		11/25		11/144							6.9
	43.5 (29.3–58.8)	0.74 (0.68–0.81)	13.9 (8.9–20.9)	0.96 (0.90–1.02)	95.7 (94.8–96.4)	99.1 (98.6–99.4)	44.0 (25.0–64.7)	0.85 (0.79–0.90)	7.6 (4.1–13.6)	1.10 (1.05–1.15)	95.4 (94.5–96.1)	99.5 (99.1–99.7)	2,905	264.1
Cytology with ASC-US triage by ProExC	26/46		26/95		13/25		13/95							13.1
	56.5 (41.2–70.8)	0.96 (0.95–0.97)	27.4 (19.0–37.6)	1.90 (1.84–1.92)	97.6 (96.9–98.1)	99.3 (98.9–99.6)	52.0 (31.8–71.7)	1.00 (1.00–1.00)	13.7 (7.8–22.6)	1.97 (1.91–1.99)	97.2 (96.5–97.7)	99.6 (99.2–99.8)	3,013	231.8
HPV DNA test with ProExC triage	35/46		35/84		23/25		23/84							7.3
	76.1 (60.9–86.9)	1.30 (1.20–1.41)	41.7 (31.2–52.9)	2.89 (2.58–3.15)	98.3 (97.7–98.7)	99.6 (99.3–99.8)	92.0 (72.5–98.6)	1.77 (1.38–2.28)	27.4 (18.5–38.4)	3.94 (3.24–4.73)	97.9 (97.3–98.4)	99.9 (99.7–100)	3,378	146.9

NOTE: Cytology cutoff for test positivity: atypical squamous cells of undetermined significance or worse (i.e., atypical glandular cells of uncertain significance; atypical squamous cells of undetermined significance, cannot exclude high-grade squamous intraepithelial lesions, low-grade squamous intraepithelial lesions and high-grade squamous intraepithelial lesions). Cytology was performed independently, without knowledge of HPV or ProExC status. Abbreviations: CIN2<sup>+</sup>, cervical intraepithelial neoplasia grade 2 or worse; CIN3<sup>+</sup>, cervical intraepithelial neoplasia grade 3 or worse; HPV, human papillomavirus (i.e., HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68).

the specificity of HPV testing is not high enough to perform well in a primary screening setting. There is a strong demand for additional, more sensitive and more specific, markers to improve screening programs. Objective and clearly decisive biomarkers could also improve standardization and vigorous quality control of the histological diagnosis. Several biomarker candidates have been proposed for this application, e.g., HPV mRNA, p16, and BD ProExC. Alternatively, screening could also be improved by repeat HPV testing or repeat cytology. In the current study, we wanted to analyze reflex testing from the initial vial, and therefore repeat testing was not included in the comparison.

In this study, all included women were initially tested via Colposcopy, Cytology, HPV, and ProEx C IHC. The study design of cotesting allows for direct evaluation of the performance in a primary screening setting as well as for simulation of triage algorithms. We report extrapolated sensitivities, specificities, predictive values of various triage scenarios and provide an estimate of the necessary number of diagnostic tests. However, this study is not a randomized controlled trial, which constitutes the major limitation, and does not allow direct conclusions on the clinical applicability of the results. A next step would be to conduct a randomized controlled trial to translate the conclusions of this study into clinical practice.

To address the need for additional triage markers for cervical cytology screening, the use of the biomarker-cocktail BD ProExC as a tool for triage after primary BD SurePath cytology or primary hrHPV screening to assist in the identification of women with high-grade cervical disease was evaluated. The data from this prospective colposcopy controlled study support a screening algorithm of a primary hrHPV screen followed by BD ProExC triage. Although BD ProExC is mostly used as an adjunct test for abnormal cytology, our study design allowed for evaluation of the performance of BD ProExC as a primary screening test. For CIN3<sup>+</sup> BD ProExC immunostaining as a single test provided an improved sensitivity of 92% compared to 52% for cytology. However, test specificity for CIN3<sup>+</sup> decreased from 94.4% for cytology to 92% for ProEx C, which would result in a significant increase in necessary colposcopies. Previous studies using BD ProExC have shown increased sensitivity and specificity for the detection of high-grade lesions similar to our study (19, 20). In contrast to the present study, these previous experiments were performed using a cytology endpoint, without colposcopic verification of normal samples. In the present study, the screening algorithm starting from routine samples using primary screening with hrHPV and triaging using BD ProExC was investigated.

The sensitivity (95.4%) and specificity (85.0%) of hrHPV testing for the detection of CIN2<sup>+</sup> disease found in this study were similar to previously reported findings (10, 30). BD ProExC triage after hrHPV testing was found to increase the specificity (98.3% vs. 85.0%) and PPV

(41.7% vs. 9.3%) of screening compared to hrHPV alone, resulting in an 82% decrease in colposcopy procedures (473 vs. 84).

The benefits of a hrHPV primary screening strategy are illustrated by the group of cytology negative/hrHPV-positive subjects with CIN2<sup>+</sup>, representing 41.9% of all CIN2<sup>+</sup> cases detected. Included in this group of 18 women were 6 CIN2, 9 CIN3, and 3 invasive carcinomas. The use of a cytology primary screen would have resulted in the return of these women to routine screening, which, depending on the screening interval and patient compliance, could result in the development of more severe disease prior to detection.

The sensitivity (58.1%) of cervical cytology for the detection of CIN2<sup>+</sup> was comparable to other published results (31, 32). BD ProExC and hrHPV triage procedures from cytology testing resulted in fewer referrals for colposcopy, with 73% and 56% reductions, respectively, compared to cytology alone, and were more specific for the detection of CIN2<sup>+</sup>. BD ProExC triage from cytology increased the PPV (46.0% vs. 13.4%) for the detection of CIN2<sup>+</sup>. These results were an improvement on the hrHPV triage from cytology algorithm, which gave a PPV of 28.9%.

Of the 7 CIN2<sup>+</sup> BD ProExC-negative specimens, 5 were reported with normal cytology. One of these subjects, with a CIN1–2 histology (case 2,211) had negative cytology and tested negative for hrHPV. Among the 5 CIN2<sup>+</sup> cases positive for hrHPV and negative for BD ProExC, 3 were CIN2 cases (1 NILM, 1 LSIL, and 1 HSIL cytology) and 2 were CIN3 cases from subjects with NILM cytology, which may represent a sampling error. All but 2 of the CIN3 lesions, and all of the invasive cancers cases, were positive for BD ProExC staining. The 2 CIN3 lesions with a negative BD ProExC result also had a NILM cytology finding, which may also represent a sampling error. Longitudinal studies are required to determine if hrHPV patients negative for BD ProExC are less likely to have disease progression compared to BD ProExC-positive patients.

The goal of a successful cervical cancer screening program is to develop a testing strategy that provides high sensitivity for detecting women likely to harbor high-grade cervical disease while also having sufficient specificity to minimize the number of unnecessary colposcopy/biopsy procedures performed. With this as a goal, and within the limitations of this study, the use of BD ProExC triage after a hrHPV primary screening was the most effective strategy, with a predicted 55% decrease in colposcopy procedures. Only 2.4 colposcopies per detected CIN2<sup>+</sup> case would be needed after primary hrHPV testing followed by triage with BD ProExC compared to 6.9 colposcopic procedures after cytology. Not only less colposcopic procedures would be needed but also more CIN2<sup>+</sup>/CIN3<sup>+</sup> cases would be detected.

In conclusion, primary hrHPV testing followed by BD ProExC triage of positive cases was found to be the best



cervical cancer screening algorithm in this comparative study. It demonstrates that more studies are required to develop our strategy for detecting and guiding treatment in this prevalent cancer.

### Disclosure of Potential Conflicts of Interest

All other authors have no conflicts of interest to declare. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

### Acknowledgments

The authors thank the cytotechnologists, Els Dewulf, Kristin Van Belle, and Sabrina Van Belle, and lab technicians, Rachel Baveco, Ludo Boels, Carmen De Maesschalck, Brenda Gabriels, Inge Goegebeur, Karen Illegems, and Miranda Vervoort for excellent technical support. They especially thank Drs. Kristl Claeys and Arnold Criel for their invaluable

comments on the article, and the gynecologists Drs. G. Benijts, P. Cryns, AM. Hendrickx, F. Stroef, P. Van Dam, G. Vlaemyncx, V. Verhulst, and R. Weckhuysen for collecting the cervical smear samples and for performing the colposcopies. Technical editing of the article was provided by Touch Medical Communications.

### Grant Support

Funding for the colposcopy procedures was provided, in part, by a grant from BD Diagnostics—TriPath. The funding source had no role in study design, data collection, data analysis, data interpretation, or writing of the report. C.E. Depuydt and J.J. Bogers received traveling grants from BD Diagnostics—TriPath.

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Received July 29, 2010; revised December 10, 2010; accepted January 12, 2011; published OnlineFirst February 4, 2011.

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