ONCOLOGY

Prior knowledge of HPV status improves detection of CIN2+ by cytology screening

Ina H. Benoy, MSc, PhD; Davy Vanden Broeck, MSc, PhD; Maya J. Ruymbeke, MD; Shaira Sahebali, MD, PhD; Marc Arbyn, MD, PhD; Johannes J. Bogers, MD, PhD; Marleen Temmerman, MD, PhD; Christophe E. Depuydt, MSc, PhD

OBJECTIVE: The objective of the study was to investigate whether knowledge of human papillomavirus (HPV) deoxyribonucleic acid test results increases sensitivity of guided cytology screening for the detection of cervical intraepithelial neoplasia (CIN)-2 or higher-grade cervical lesions.

STUDY DESIGN: This was a prospective colposcopy-controlled study of 2905 BD SurePath samples to identify cases with CIN2+ within a 24 month follow-up period. Sensitivity and specificity to detect CIN2+ was evaluated, comparing guided cytology screening with and without prior knowledge of HPV status.

RESULTS: Prior knowledge of HPV status resulted in significantly higher detection rate of CIN2+ compared with screening blinded to HPV status ($P = .005$) with limited loss of specificity ($P = .026$). Gain in sensitivity is higher in older women (43.8%, $P = .008$) vs in younger women (10.2%, $P = .317$), whereas loss of specificity is more pronounced in younger women ($P < .001$) vs older women ($P = .729$).

CONCLUSION: Guided cytological screening performed with prior knowledge of HPV status results in an improved detection of CIN2 or higher-grade lesions.

Key words: cervical cancer, cervical cytology, human papillomavirus, human papillomavirus genotypes, real-time polymerase chain reaction

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The recognition of the strong causal relationship between persistent infection with high-risk (HR) human papillomavirus (HPV) (HR-HPV) types, and cervical cancer and its precursors, has resulted in the development assays that detect viral nucleic acids as an alternative for or as an adjunct to cervical cytology.[1] One can distinguish assays that detect all HR-HPV types as a group and genotyping tests that distinguish individual HPV types.[2] Liquid-based cytology is now gradually replacing conventional cytological testing, because of practical advantages (quicker interpretation, easy ancillary molecular testing, and possibility of computerized guided screening), in spite of the lack of evidence that it increases the detection of cervical intraepithelial neoplasia (CIN)-2 or higher-grade cervical abnormalities.[3-5]

Cytological screening combined HR-HPV testing and HPV-based screening followed by cytology triage have been evaluated as more sensitive than cytology screening alone.[6] Compared with cytology alone, this screening strategy improves detection of precancerous growths but with a certain increase in the number of false-positive tests.[6-8] Recent randomized trials have confirmed that HPV-based screening, in women older than 30-35 years followed by cytology triage results in detection of more CIN2 or worse lesions compared with cytology screening. Moreover, longitudinal results of these trials have demonstrated that women with a negative HPV test have a lower risk of CIN3 and even invasive cancer.[9,10]

This study aimed to evaluate the influence of knowing the different HR-HPV genotypes present in cervical specimens before performing guided cytological screening.

Materials and Methods

Study population

In this prospective, colposcopy-controlled study, we enrolled 3126 voluntary participants from August 2005 until February 2007 (Figure 1). Samples were collected during opportunistic routine health checks by 11 selected gynecologists in Flanders

From the Department of Molecular Pathology (RIATOL) Sonic Healthcare, Antwerp, Belgium (Drs Benoy, Sahebali, Bogers, and Depuydt) and Department of Applied Molecular Biology Research (AMBIOR), University of Antwerp (Dr Bogers), Antwerp; the International Centre for Reproductive Health (ICRH), Ghent University (Drs Vanden Broeck and Temmerman), and Jan Palfijn Hospital and Department of Obstetrics and Gynaecology, University Hospital (Dr Ruymbeke), Ghent; and the Unit of Cancer Epidemiology/Belgian Cancer Centre, Scientific Institute of Public Health, Brussels (Dr Arbyn), Belgium.

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The first 2 authors contributed equally to the study and manuscript.

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Reprints: Ina Benoy, PhD, Emiel Vloorsstraat 9, 2020 Antwerp, Belgium. ina.benoy@riatol.be.

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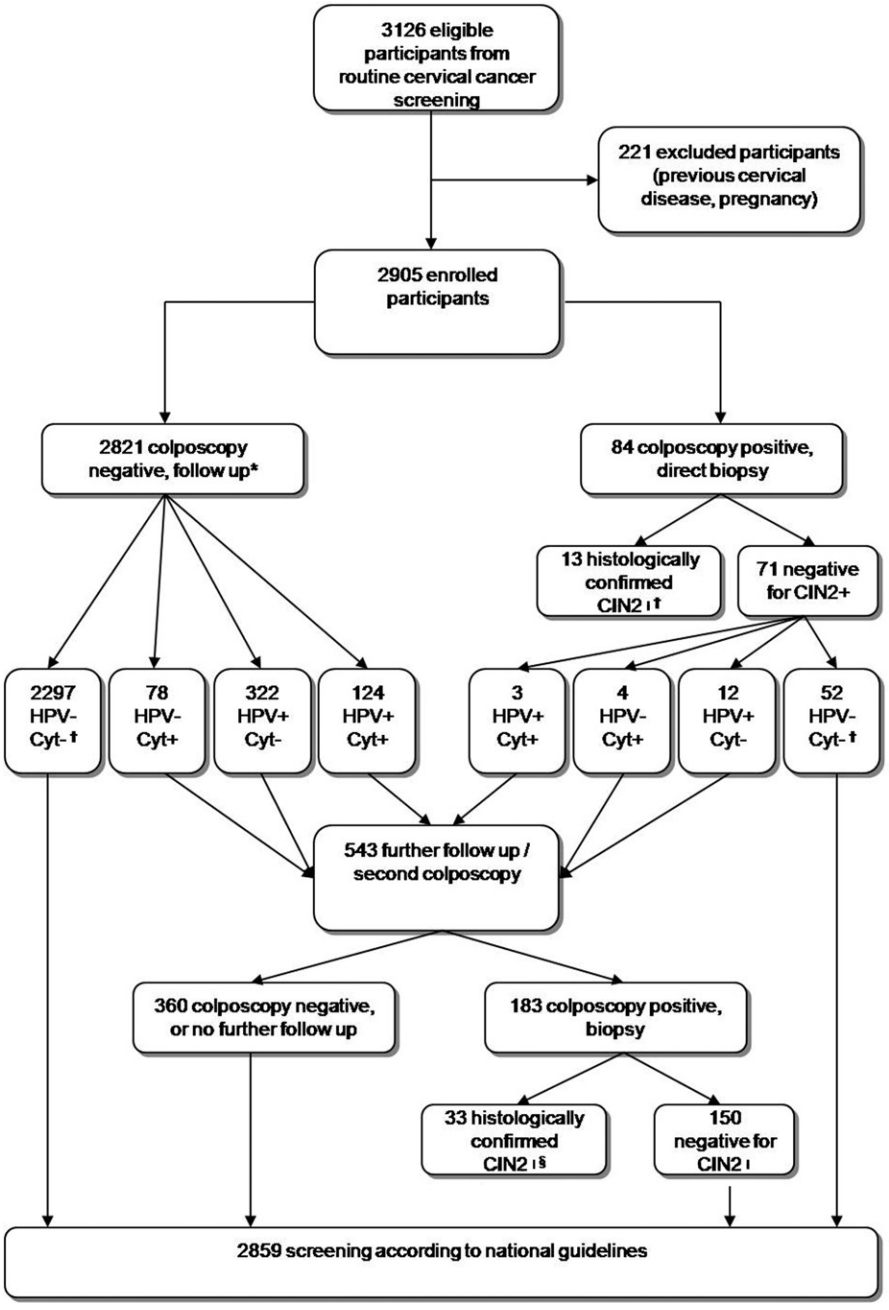
FIGURE 1
Enrolled women classified by colposcopy test results, screening results, and detection of CIN 2 or higher



Asterisk indicates follow-up defined as HPV genotyping by real-time PCR analysis and cytology screening (with and without prior knowledge). *Dagger* indicates HPV DNA test negative and cytology screening test negative defined as no further referral for study procedures; screening was according to national guidelines applicable. *Double dagger* indicates that of which 5 women had CIN2 lesions and 8 women had CIN3+ lesions. *Section mark* indicates that this includes 16 women with CIN2 and 17 women with CIN3+ lesions.

CIN, cervical intraepithelial neoplasia; *Cyt-,* cytology negative; *Cyt+,* cytology positive; *HPV,* human papillomavirus; *PCR,* polymerase chain reaction.

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(Belgium). All women gave written informed consent.

Exclusion criteria included pregnancy and history of cervical disease (previous history of CIN2+); 221 women were excluded. At enrollment, participants underwent a colposcopy after smear taking. Colposcopy was performed in the framework of this study to obtain a gold standard. Study-specific patient identification codes were assigned and transmitted in such a manner that patient confidentiality was preserved and linked with follow-up and histology results. This study was approved by the local ethical committee (Ziekenhuis Oost Limburg, Genk, Belgium).

Cervical sample processing

Slide preparation

Cervical cells were collected using the Cervex-Brush (Rovers, Oss, The Netherlands).

After collection, brush heads were transferred directly into alcohol-based preservative (SurePath; Tripath Imaging Inc, Burlington, NC), and the vials were transported to the Laboratory for Clinical Pathology (labo RIATOL, Antwerp, Belgium). Thin-layer slide preparations were made with the fully robotic AutoCyte PREP System (AutoCyte; Tripath Imaging) and were prepared as described elsewhere.[11]

High-risk HPV testing

All specimens from the screening visit were tested for HPV deoxyribonucleic acid (DNA) by polymerase chain reaction (PCR) amplification.DNA isolation from liquid-based cytology was performed as previously described.[12,13] Briefly, HPV DNA was extracted from cervical cells using standard proteinase K-based digestion according to the manufacturers' protocol. Washed cell pellets were incubated with proteinase K solution (100 μg/mL) for 3 hours at 55°C. First, each sample was subjected to quantitative PCR (qPCR) amplification for the detection of β-globin to confirm that the DNA quality was suitable for PCR analysis.

All samples were tested for the presence of 14 different HR-HPV genotypes using TaqMan-based real-time qPCR, targeting type-specific sequences of viral genes: 16 E7, 18 E7, 31 E6, 33 E6, 35 E6,

39 E7, 45 E7, 51 E6, 52 E7, 56 E7, 58 E6, 59 E7, 66 E6, and 68 E7. Presence of low-risk HPV types, for instance, HPV6, HPV53, and HPV67, were considered negative for HPV infection.

Slide classification

BD FocalPoint reading

All liquid-based cytology samples were first scanned with the BD-FocalPoint system followed by guided assisted screening with BD-SlideWizard, according to the manufacturer's instructions (BD Diagnostics-TriPath, Burlington, NC).[14] The FocalPoint system classifies 25% of all slides as no further review (NFR) in which the probability of intraepithelial lesions is extremely low. The other 75% are categorized in quintile 1-5, in which quintile 1 has the highest probability of abnormality, based on slide scores. In this study, also, the NFR slides were screened cytologically.

BD SurePath liquid-based cytology using guided screening with a BD-SlideWizard was used to compare screening with and without prior knowledge of HR-HPV status for each selected sample.

Bethesda classification

The cytological results were classified according to the Bethesda system 2001,[15] using the classes negative for intraepithelial lesions or malignancy, atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells of undetermined significance that cannot exclude high-grade squamous intraepithelial lesions (ASC-H), low-grade squamous intraepithelial lesions (LSIL), and high-grade squamous intraepithelial lesions (HSIL). Slides were read by 2 independent cytologists in arbitrary order so that for any sample, it was equally likely that the cytology with or without prior knowledge would take the first reading. High-grade cervical disease was considered to comprise histological grade CIN2 or higher (CIN2, CIN3, adenocarcinoma in situ, invasive squamous cell carcinoma).

Cytology with/without prior knowledge

Cytology was performed twice on every sample, with and without knowledge of HPV DNA test results. Reading was done

TABLE 1
Characteristics of the population

Characteristic	Total population (n = 2905)
General	
Age, y	Median = 42.7 (first quartile = 32.9; third quartile = 51.9)
<30 y	562 (19.3%)
≥30 y	2343 (80.7%)
Viral characteristics	
HR-HPV positive	473 (16.3%)
HR-HPV (<30 y)	143 (25.4%)
HR-HPV (≥30 y)	329 (14.0%)
Type 16	110 (23.3%)
Type 51	98 (20.7%)
Type 31	75 (15.9%)
Type 59	50 (10.6%)
Type 39	47 (9.9%)
Type 52	44 (9.3%)
Type 56	44 (9.3%)
Type 18	41 (8.7%)
Type 66	28 (5.9%)
Type 33	321 (4.4%)
Type 35	20 (4.2%)
Type 58	20 (4.2%)
Type 53	17 (3.6%)
Type 45	9 (1.9%)
Single HR-HPV infection	372 (78.6%)
Multiple HR-HPV infection	3101 (21.4%)
Median number of infections	1
Histology	256 (8.8%)
Normal/CIN 1	210 (7.2%)
CIN 2	21 (0.7%)
CIN 3	25 (0.9%)
Cytology	n = 2905

	Without prior knowledge, n (%)	With prior knowledge, n (%)	*P* value
Normal	2718 (93.6)	2685 (92.4)	.10
ASC-US	108 (3.7)	110 (3.8)	.94
LSIL	46 (1.6)	67 (2.3)	.05
ASC-H	8 (0.3)	15 (0.5)	.21
HSIL	25 (0.9)	27 (0.9)	.89

ASC-US, atypical squamous cells of undetermined significance; *CIN*, cervical intraepithelial neoplasia; *HPV*, human papillomavirus; *HR-HPV*, high-risk human papillomavirus; *HSIL*, high-grade squamous intraepithelial lesions; *LSIL*, low-grade squamous intraepithelial lesions.

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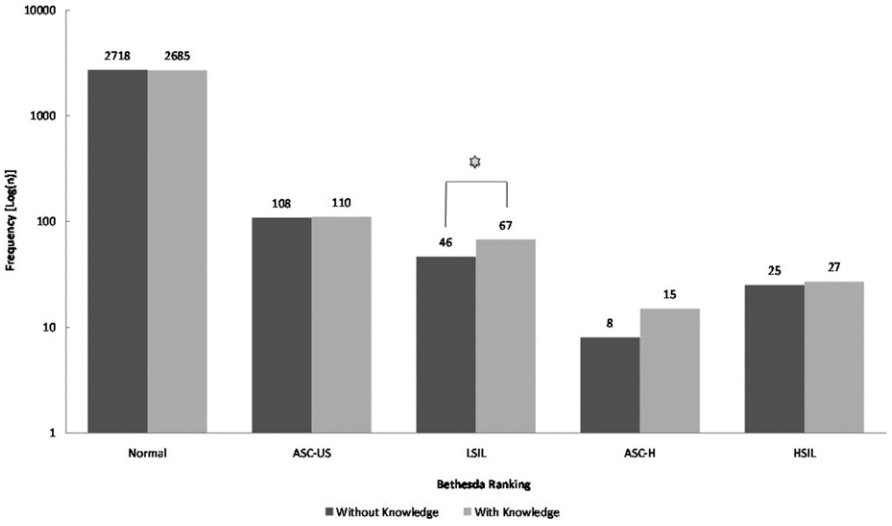
FIGURE 2
Cytology according to prior knowledge



Asterisk indicates that the significant difference was found for LSIL when comparing reading with and without prior knowledge of HPV ($P = .05$).

HPV, human papillomavirus; *LSIL*, low-grade squamous intraepithelial lesions.

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in arbitrary order, and it was ensured that every slide was read by different cytologists, arbitrarily selected from a pool of cytologists. In both cytological readings, demographic and clinical information was provided. Cytological interpretation with knowledge of HPV status included information on the type and the type-specific viral load. All slides were reviewed by cytopathologists, blinded from HPV DNA tests results but aware of the screening results of the cytologists.

Follow-up and assessment of study endpoints

All women underwent colposcopy at enrollment and participants with cervical abnormalities were referred for biopsy (Figure 1). Women with a normal colposcopy result (or a negative histology result if a biopsy was taken) were considered as free of CIN. Women with a precancerous lesion or cervical cancer were referred for further management. The number of detected CIN 2 or higher grade cases in a 24 month follow-up period was used as study endpoint.

Data management

All results from cytology, histology, treatment/follow-up, and HPV status were entered in a database. Each patient was allocated a unique patient identification (ID) number. This patient ID number was used to link the different cytological, histological, and virological data.

Statistical analysis

The statistical package R version 2.10.1 was used for data analysis.[16] HR-HPV positivity was defined as the presence of 1 or more of the following 14 HPV types: HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, and HPV68. Estimates of sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) for detection of CIN 2+ were obtained for both screening strategies: cytology with prior knowledge of HPV types and cytology without prior knowledge. Confidence intervals (CIs) for proportions were calculated by Wilson's method.[17] Differences in proportions were tested using McNemar statistics for paired comparison.[18] The 95% CIs were computed for proportions, differences, and ratios of proportions. All statistical tests were 2 sided, and $P \leq .05$ was considered statistically significant.

Women were divided by age into 2 categories; younger (<30 years old) and older (≥30 years old). The difference in sensitivity to detect CIN2+ in the 2 age groups and corresponding specificities and predictive values were assessed. A receiver operating characteristics (ROC) plot was used to evaluate/visualize the true positive rate vs the false-negative rate.

Histologically verified CIN 2+ cases that were detected within 24 months after enrollment at routine screening were included. Women who had a cytological diagnosis of ASC-US or worse were classified as having abnormal cytology. Separate analyses were performed to compare the sensitivity in the detection of CIN 2 or worse.

RESULTS

Characteristics of the population

In total, 3126 women were eligible for inclusion of the current study. After exclusion of 221 subjects with antecedents of cervical neoplasia and/or pregnancy, 2905 participants were included in the final study group. The median age was 42.7 years (first quartile, 32.9; third quartile, 51.9). Eighty-four percent of the study population belonged to the target age group of 25-64 years, for whom screening is recommended in Belgium.[16] The proportion of younger (<30 years) women in the study population was 19.3%, with HPV prevalence of 25.4%, whereas 80.7% of women were 30 years old or older with HPV prevalence of 14.0% ($P < .001$) (Table 1).

Of the 2905 women in the study, 473 (16.3%) tested positive for at least 1 HR-HPV type. Single HPV infection was found in 78.6% of the HR-HPV-infected women and 21.4% were infected with 2 or more HR-HPV types. The maximum number of multiple HR-HPV infection per woman was 5. The most common type was HPV16 (23.2%), followed by HPV51 (20.7%), HPV31 (15.9%), and HPV59 (10.6%), respectively. A high prevalence of HR-HPV infections was observed in women with cytological abnormalities (Table 1).

Forty-six histologically confirmed CIN 2 or worse cases were found. Five CIN 2 and 8 CIN 3 subjects were detected during the first colposcopy visit and 16 CIN 2 and 17 CIN3 subjects during subsequent visits (Figure 1). Two (9.5%) of 21 women with

CIN 2 were HR-HPV negative. All CIN 3+ were HR-HPV positive.

Figure 2 shows a decrease in the number of slides ranked as normal when cytology was performed with prior knowledge; nevertheless, this decrease was not statistically significant ($P = .10$). Concurrently, when prior knowledge was available, more slides were ranked as abnormal in comparison with blinded cytology, but this difference was also not statistically significant ($P = .06$). Of all the slides ranked abnormal, a significant increase in the number of LSIL rated slides was noted ($P = .05$) when prior knowledge was available. No significant differences in cytological results between blinded cytology and reading with prior knowledge of HPV DNA could be found for the remaining cytological categories, that is, ASC-US, ASC-H, and HSIL ($P = .47$; $P = .11$; and $P = .44$ respectively). Figure 3 shows more detailed shifts in cytological categories.

When prior knowledge of HPV DNA was available, the sensitivity of guided cytology to detect CIN 2+ was significantly higher as compared with detection without prior knowledge ($P = .005$). Guided cytology, with and without prior knowledge of HPV status, showed a sensitivity of 76.1% (95% CI, 62.1–86.1%) and 58.7% (95% CI, 44.3–71.7%), respectively (Table 2). For CIN 3+, the sensitivities were 64.0% (95% CI, 44.5–79.7%) with 52.0% (95% CI, 33.5–69.9%) without prior knowledge.

The positive predictive value of cytology for CIN2+ and CIN3+ were not statistically significantly higher with revealed HPV status than those for blinded screening. Relative PPV equals 1.11 (95% CI, 0.70–1.75) and 1.05 (95% CI, 0.53–2.10, for CIN2+ and CIN 3+, respectively. There was a significant decrease in specificity when prior knowledge was involved ($P = .026$), 93.6% (95% CI, 92.7%–94.5%) vs 94.4% (95% CI 93.6%–95.2%) for screening with and without knowledge, respectively (Table 2).

The prevalence of CIN 2+ varied by age: 2.5% and 1.3%, respectively, younger than 30 years or 30 years or older. Overall, cytology screening showed higher sensitivity in younger than in older women. The gain in sensitivity induced by knowing the HPV status was observed in both age groups but was highest in women aged 30 years old or older: +43.8%, whereas +10.2% in younger women) (Figure 4).

No loss in specificity was observed for older women ($P = .729$), whereas in younger women an important drop in specificity could be found ($P < .001$). Knowledge of HPV status yielded higher PPVs in older ($P = .347$) but not younger women ($P = .511$). Overall, there were no remarkable differences in the NPVs by screening procedure or by age strata (Table 2).

FIGURE 3
Prior knowledge of HPV DNA induced shifts in cytology outcome

Screening with prior knowledge

Screening without prior knowledge	NILM	ASC-US	LSIL	ASC-H	HSIL	*Total*
NILM	2640	41	25	8	4	2718
ASC-US	38	65	4	1	0	108
LSIL	4	2	37	0	3	46
ASC-H	3	0	0	4	1	8
HSIL	1	2	1	2	19	25
Total	2686	110	67	15	27	2905

ASC-H, high-grade squamous intraepithelial lesions; *ASC-US*, atypical squamous cells of undetermined significance; *HPV*, human papillomavirus; *HSIL*, high-grade squamous intraepithelial lesions; *LSIL*, low-grade squamous intraepithelial lesions; *NILM*, negative for intraepithelial lesion and malignancy.

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Comment

This study demonstrates that prior knowledge of presence of HR-HPV types

TABLE 2
Test protocol comparison with detect histologically confirmed CIN2+ lesions

Variable	Cytology without prior knowledge (%)	Cytology with prior knowledge (%)	*P* value
Total population			
Sensitivity	27/46 (58.7)	35/46 (76.1)	.005
Specificity	2699/2859 (94.4)	2675/2859 (93.6)	.026
NPV	2699/2718 (99.3)	2675/2686 (99.6)	.211
PPV	27/187 (14.4)	35/219 (16.0)	.771
Age (<30 y)			
Sensitivity	11/14 (78.5)	12/14 (85.7)	.317
Specificity	506/548 (92.3)	479/548 (87.4)	< .001
NPV	506/509 (99.4)	479/481 (99.6)	.943
PPV	11/53 (20.75)	12/81 (14.8)	.511
Age (≥30 y)			
Sensitivity	16/32 (50.0)	23/32 (71.9)	.008
Specificity	2192/2310 (94.9)	2195/2310 (95.0)	.729
NPV	2192/2208 (99.3)	2195/2204 (99.6)	.242
PPV	16/134 (11.9)	23/138 (16.7)	.347

All comparisons evaluate the difference between cytological screening without vs with prior knowledge. McNemar statistics for comparison of 2 matched proportions was used.

CIN, cervical intraepithelial neoplasia; *NPV*, negative predictive value; *PPV*, positive predictive value.

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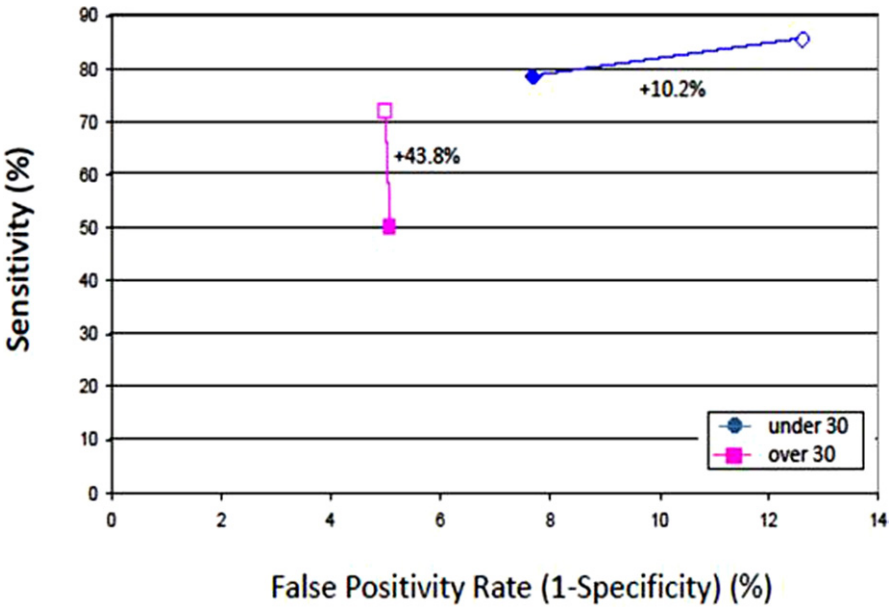
FIGURE 4
ROC plot



In both young women (*diamonds*), a limited gain in sensitivity is combined with a loss of specificity. In older women (*squares*), a substantial gain in sensitivity is paralleled by virtually no loss of specificity. *Closed symbols* represent screening without prior knowledge and *open symbols* when prior knowledge was applied.

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increased cytological detection of CIN 2 grade lesions or worse. Compared with cytology alone, knowledge of the HPV status resulted in a gain in sensitivity of 30%, a slight loss in specificity, however, without loss of positive predictive value.

The prevalence of HR-HPV genotypes in our study population was similar to the national distribution, indicating that a representative sample was taken.[11] It remains to be elucidated how to utilize HPV DNA testing: as a complementary screening method to cytology or as a stand-alone screening followed by cytology-based triage of HR-HPV-positive women. A major concern regarding the use of HPV DNA tests in cervical cancer screening has been their lower specificity compared with cytology, which results in more women needing to go through repeat testing and diagnostic procedures.[7]

Overall, cytological screening with prior knowledge resulted in an increased number of abnormal smears, in particular LSIL.

Our observations are in concordance with results from certain randomized trials indicating that HPV screening followed by cytology triage identifies more CIN2+ than cytology screening alone.[19] These findings can be explained only by a more cautious evaluation of HPV-positive slides by the cytotechnologist alerted by knowledge of HPV status.

A striking finding was that knowing the HPV status prior to cytological screening was more sensitive in both age group, but the gain was higher in women 30 years old or older (young women: 78.5% vs 85.7%; older women: 50.0% vs 71.9%). Prior knowledge of HPV status resulted in a loss in specificity in younger (92.3% vs 87.4%) but not in older women: (94.9% vs 95.0%).

HR-HPV infection is very common, especially in younger women.[19] Among women, whose smears are processed in our laboratory, HR-HPV prevalence reaches a peak of at least 20% among women between the ages of 18 and 30 years of age, with a subsequent decline to approximately 10% among women in their 30s and less thereafter.[11]

The lower specificity and PPV (albeit not significant in our study) in women younger than 30 years old is not unexpected because infections usually are transient and are not accompanied by high-grade neoplasia at this age group. Moreover, HPV-induced increase in sensitivity for CIN2+ has to be interpreted with caution because of the high probability of overdiagnosis in this age group.[10]

As illustrated in Figure 4, gain in sensitivity is most substantial in older women, this combined with virtually no loss of specificity. In contrast, a gain in sensitivity is accompanied by an increased false positivity rate. Hence, our findings suggest that prior knowledge of HPV is particularly useful in older women and support the guidelines of the American Cancer Society recommending women above 30 cytological screening (conventional or liquid-based) every 3 years in combination with an HPV test.

Diagnostic accuracy studies in which verification of the outcome is incomplete and mainly restricted to screen-positive subjects may be vulnerable to verification bias, resulting in overestimatation of sensitivity and underestimation of specificity.[20]

In our study, colposcopy was incorporated in the enrollment phase and in eventual follow-up visits to avoid verification bias.

From this study, no relevant conclusions could be drawn toward test performance with CIN3+ as endpoint because of a lack of statistical power to determine sensitivity and specificity of the 2 screening strategies. Further research in larger cohorts needs to be conducted to assess the significant impact of prior knowledge as a complementary screening strategy. A woman's HPV status together with information on the exact type and perhaps viral load involved in the infection may have far more important clinical significance. It will allow more close monitoring of the natural history of the disease and identify the all-important persistent infections.

More research is required to investigate the influence of the type of knowledge that should be provided prior to cytological reading. The criteria for defining abnor-

malities, because of the subjectivity of both cytology and histology in cervical screening programs, could be more quantitative to ensure more objective and accurate measurements.

An important limitation of this study was the sample size, although more than 3000 women were screened, conclusions could be formulated only with CIN2 as an endpoint. No conclusive answers were formulated toward the question of whether screening with prior knowledge of HPV improves sensitivity to detect CIN3 or higher grade lesions. Extra sample size could also allow for analysis for more information on the nature of the prior knowledge (HPV type, viral load, etc).

From a methodological point of view, the use of colposcopy/biopsy has inherent limitations, and imperfections toward disease certification could have been overcome by using random biopsy. Women were recruited from the normal Belgian screening population, based on opportunistic screening scheme. Here a potential bias could be recognized.

Summarizing, the real home message is that sensitivity for CIN2+ markedly increased with minimal effect on specificity in both young and old but most importantly those greater than or equal to 30. The loss in specificity was limited to the younger age group. No loss of PPV was noted between the 2 strategies. There was no decline in NPV with both groups or by age, which is the most important factor for a screening test.

The decrease in specificity in young patients is to be expected and supports the use of American Society for Colposcopy and Cervical Pathology guidelines, which advocate avoiding intervention with colposcopy in the young to allow regression of HPV infection and low-grade lesions. ■

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