

p16/Ki-67 dual-stained cytology for detecting cervical (pre)cancer in a HPV-positive gynecologic outpatient population

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Women who test positive for a high-risk type of the human papillomavirus (HPV) require triage testing to identify those women with cervical intraepithelial neoplasia grade 3 or cancer (\geq CIN3). Although Pap cytology is considered an attractive triage test, its applicability is hampered by its subjective nature. This study prospectively compared the clinical performance of p16/Ki-67 dual-stained cytology to that of Pap cytology, with or without HPV16/18 genotyping, in high-risk HPV-positive women visiting gynecologic outpatient clinics ($n=446$ and age 18–66 years). From all women, cervical scrapes (for Pap cytology, HPV16/18 genotyping, and p16/Ki-67 dual-stained cytology) and colposcopy-directed biopsies were obtained. The sensitivity of p16/Ki-67 dual-stained cytology for \geq CIN3 (93.8%) did neither differ significantly from that of Pap cytology (87.7%; ratio 1.07 and 95% confidence interval (CI): 0.97–1.18) nor from that of Pap cytology combined with HPV16/18 genotyping (95.1%; ratio 0.99 and 95% CI: 0.91–1.07). However, the specificity of p16/Ki-67 dual-stained cytology for \geq CIN3 (51.2%) was significantly higher than that of Pap cytology (44.9%; ratio 1.14 and 95% CI: 1.01–1.29) and Pap cytology combined with HPV16/18 genotyping (25.8%; ratio 1.99 and 95% CI: 1.68–2.35). After exclusion of women who had been referred because of abnormal Pap cytology, the specificity of p16/Ki-67 dual-stained cytology for \geq CIN3 (56.7%) remained the same, whereas that of Pap cytology (60.3%) increased substantially, resulting in a similar specificity of both assays (ratio 0.94 and 95% CI: 0.83–1.07) in this sub-cohort. In summary, p16/Ki-67 dual-stained cytology has a good clinical performance and is an interesting objective microscopy-based triage tool for high-risk HPV-positive women.

Modern Pathology (2016) 29, 870–878; doi:10.1038/modpathol.2016.80; published online 6 May 2016

Persistent infection with a high-risk type of the human papillomavirus (HPV) is essential for the development of almost all cervical cancers.^{1,2}

Testing for high-risk HPV has been shown to provide superior protection against cervical intraepithelial neoplasia grade 3 and cervical cancer (together referred to as \geq CIN3) compared with cervical cytology (Pap cytology).³ However, most women who test positive for high-risk HPV clear the virus spontaneously and do not develop clinically relevant cervical disease. Therefore, additional triage testing is required to identify the subgroup of high-risk HPV-positive women who actually have \geq CIN3, thereby

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Received 7 January 2016; revised 11 March 2016; accepted 12 March 2016; published online 6 May 2016

reducing the risk of overdiagnosis, unnecessary colposcopy referral, and treatment.

Pap cytology is considered an effective triage strategy for high-risk HPV-positive women.^{4,5} Yet, the performance of Pap cytology as a triage test is limited by its subjective nature and thus its dependence on a high level of expertise.^{4,5} In search of a reproducible, objective, and direct triage method with a higher sensitivity, several molecular assays have been proposed as valuable additions to Pap cytology. Among them is the assessment of the presence of HPV16 and/or HPV18 (HPV16/18 genotyping), a method to identify women with the most carcinogenic HPV types that together account for the majority of \geq CIN3.^{6–8} The combined use of Pap cytology and HPV16/18 genotyping yields a substantially lower \geq CIN3 risk after a negative test result compared with Pap cytology alone. However, this is at the cost of a lower specificity, leading to unnecessarily high referral rates.^{4,5,7}

Another triage test that has been described as a promising alternative to Pap cytology is the combined p16 and Ki-67 immunostaining of cervical cytology specimens. Simultaneous co-expression of the anti-proliferative p16 protein and the proliferation marker Ki-67 in the same cervical epithelial cell is a biomarker combination indicative of high-risk HPV-induced cell cycle deregulation and transforming HPV infection.^{9,10} The use of p16/Ki-67 dual-stained cytology has been shown to yield a high specificity for \geq CIN3.^{11–14} In addition, p16/Ki-67 dual-stained cytology has been reported to decrease the inter-observer variability of cytology scoring.¹⁵

In this study, we compared the performance of p16/Ki-67 dual-stained cytology with that of Pap cytology, with or without HPV16/8 genotyping, on cervical liquid-based cytology specimens for the detection of \geq CIN3 in high-risk HPV-positive women from a gynecologic outpatient population.

Materials and methods

Study Design, Participants, and Procedures

The present study was conducted within the COMETH study, of which the design, participants, and procedures have been previously described in detail.¹⁶ From December 2010 till December 2013, women aged 18–70 years were asked to participate in a prospective observational multi-center cohort study during their visit to the gynecologic outpatient clinics of six hospitals in The Netherlands. The study was approved by the Medical Ethical Committee of all participating hospitals (METc-VUmc2009-/178) and registered in the Dutch National Trial Registry (NTR2447). Women with a history of treatment for cervical dysplasia or cancer, current cancer, pregnancy, or lactation were excluded from participation.¹⁶ Women could participate in the study regardless of their reason for referral to the

gynecology outpatient clinic. After providing informed consent, participants collected cervicovaginal lavage material (using a Delphi screener, Delphi Bioscience, The Netherlands) for high-risk HPV testing. Women who were eligible for the study and tested positive for high-risk HPV on the cervicovaginal lavage were invited for a physician-taken cervical scrape and a colposcopy.¹⁶ Cervical scrapes were stored in Thinprep PreservCyt solution (Hologic, USA). Each cervical liquid-based cytology sample was used to perform a high-risk HPV test with subsequent HPV16/18 genotyping, to prepare one slide for Pap cytology testing and one separate cytology slide for p16/Ki-67 dual-stained cytology testing. Additional aliquots have been removed for other molecular tests (as described recently)¹⁶ before the vials have been used to prepare slides for p16/Ki-67 dual-stained cytology testing.

Figure 1 shows the composition of the study population. As described before,¹⁶ among a total of 2970 women who gave informed consent and participated in the study, 717 women (24%) tested positive for high-risk HPV on self-collected material. After exclusion of 78 women (11%) for various reasons described in Figure 1, cervical scrapes were obtained from the remaining 639 women. Cervical scrapes that tested positive for high-risk HPV and contained sufficient liquid-based cytology material ($n=535$) were tested for Pap cytology, p16/Ki-67 dual-stained cytology, and HPV16/18 genotyping. No invalid HPV16/18 genotyping results were recorded. Cases with invalid test results for Pap cytology (6/535; 1%) or p16/Ki-67 dual-stained cytology (88/535; 16%) were excluded from further analyses. The remaining cohort comprised the final study population ($n=446$; ages 18–66 years). All of these women had a valid histological endpoint obtained by colposcopy-directed biopsy.

In the final study population, 44% (197/446) of women had been referred to the gynecologist because of a recent abnormal Pap cytology result. The remaining women (56%; 249/446) were visiting the gynecologist for other, non-cervix-related gynecologic complaints. For logistic reasons, 44% (196/446) of cervical scrapes were obtained directly before colposcopy, whereas the remaining scrapes (56%; 250/446) were collected in a separate visit 2–3 weeks before colposcopy.

HPV Testing and Genotyping

DNA isolation of 1/10th of the liquid-based cytology material was performed with the Nucleo-Spin 96 Tissue kit (Macherey-Nagel, Germany) and a Micro-lab Star robotic system (Hamilton, Germany) according to manufacturers' instructions.¹⁷ The isolated DNA was subjected to general primers 5+/6+ PCR–enzyme immunoassay (GP5+/6+; Diassay, The Netherlands).¹⁸ A microsphere bead-based assay (Luminex)¹⁹ was used for genotyping of the high-risk

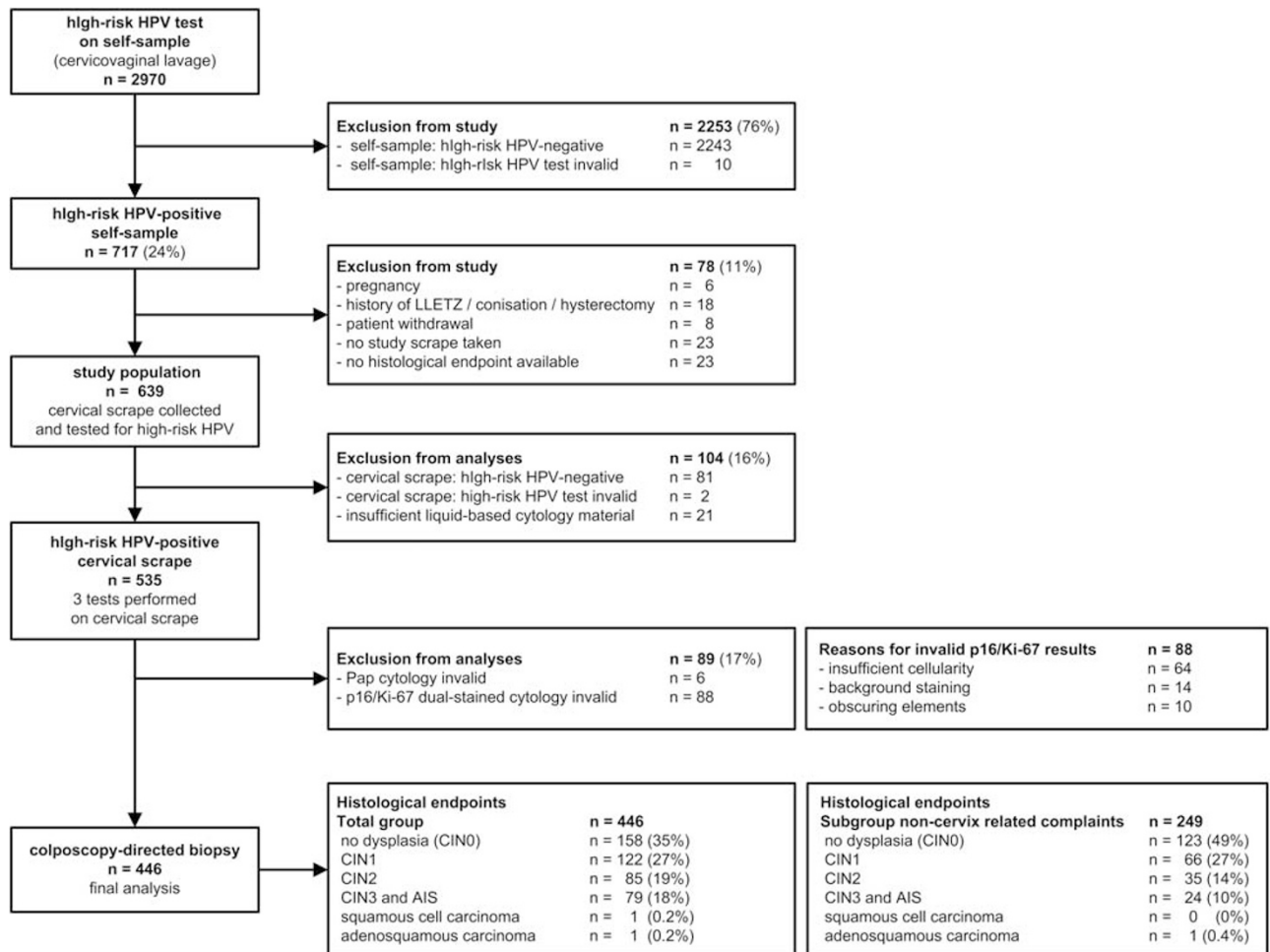


Figure 1 Composition of the study population. AIS, adenocarcinoma *in situ*; CIN, cervical intraepithelial neoplasia; HPV, high-risk human papillomavirus; LLETZ, large loop excision of the transformation zone.

HPV types 16/18/31/33/35/39/45/51/52/56/58/59/66/68.

Pap cytology

For Pap cytology testing, liquid-based cytology preparations were processed using a Thinprep 5000 processor, Pap stained, and cytologically classified according to the CISOE-A classification (reporting on Composition, the presence or absence of Inflammation, grading Squamous-, Other- or Endometrial-, and endocervical (columnar) atypia with a separate score for Adequacy) used in The Netherlands as previously described.^{16,20} CISOE-A results were translated into the Bethesda classification,²¹ in which borderline or mild dyskaryosis equals atypical squamous cells of undetermined significance (ASC-US)/ASCs, cannot exclude high-grade squamous intraepithelial lesion (ASC-H)/low-grade squamous intraepithelial lesion (LSIL), and worse than borderline or mild dyskaryosis equals high-grade squamous intraepithelial lesion. Cytotechnicians were aware of

the high-risk HPV-positive status of the cervical scrapes but not of the results of high-risk HPV genotyping.

p16/Ki-67 Dual-Stained Cytology

After HPV testing, Pap cytology testing, and the removal of aliquots for other molecular tests,¹⁶ an additional cytology slide was produced from each liquid-based cytology sample using a Thinprep 5000 processor (Hologic). For p16/Ki-67 dual-staining, a commercial kit specifically designed for simultaneous detection of p16 and Ki-67 in cervical cytology samples was used (CINtecPlus, Roche mtm Laboratories, Germany) according to the instructions of the manufacturer, as described previously.^{22,23} Slides were analyzed and scored by an experienced cytotechnologist, who was blinded to all other study data but who was aware of high-risk HPV-positive status of the cervical specimens. Samples were considered p16/Ki-67 dual-stain positive when immunoreactivity for both p16 and Ki-67 was detected within the

same cell (that is, a cytoplasmic brown staining for p16, together with a nuclear red staining for Ki-67),²² in at least one cell per slide.

Statistical Analysis

The sample size was set such that 90% power was achieved for demonstrating non-inferiority of p16/Ki-67 dual-stained cytology or HPV16/18 genotyping compared with Pap cytology using a matched-sample score test.^{24,25} A minimum of 300 high-risk HPV-positive women needed to be included at a rejection rate α of 0.05. Histologically confirmed \geq CIN3 was used as primary study endpoint. \geq CIN2 was used as secondary study endpoint, as the category of CIN2 reflects a heterogeneous disease, of which a substantial part results from productive high-risk HPV infections¹ and regresses spontaneously.^{26,27} The study endpoint was based on the histological outcome of the colposcopy-directed biopsy or, if classified worse, on the histology result of the specimen excised by LLETZ, conization, or hysterectomy. For Pap cytology, Pap cytology combined with HPV16/18 genotyping and p16/Ki-67 dual-stained cytology, sensitivity, specificity, positive predictive value, and the complemented negative predictive value (a measure of disease risk after a negative result) for the detection of \geq CIN3 and \geq CIN2 were calculated with 95% confidence intervals (95% CIs). To clarify the attribution of HPV16/18 genotyping to the combination of Pap cytology with HPV16/18 genotyping, data were also presented for HPV16/18 genotyping alone. In concordance with earlier work on this population,¹⁶ relative sensitivities (ratios of the sensitivity of one test to the sensitivity of another test) and relative specificities (ratios of the specificity of one test to the specificity of another test) were calculated with 95% CIs, to enable comparisons. If the 95% CIs of the relative sensitivity or specificity was entirely below or above 1, this difference in sensitivity or specificity was considered significant. In case such a significant difference was not found, an additional non-inferiority test was performed. Non-inferiority was defined as a relative sensitivity or specificity of at least 90% using a matched-sample score test.^{24,25} We considered three factors that might influence sensitivity and specificity of the described tests. First, the age of the participants (aged \geq 30 years vs $<$ 30 years); second, the reason of referral to the gynecologist (non-cervix-related gynecologic complaints vs a recent abnormal cytology result in cervical screening); and third, the moment that the cervical scrape was collected (during a separate visit before colposcopy vs combined with the colposcopy procedure in one visit). We used logistic regression to study the influence of these factors. After finding a factor that significantly influenced the performance of the different tests (significance: $P < 0.05$), we performed a subgroup analysis after stratification

for this factor. All statistical analyses were performed in IBM SPSS Statistics 20 and STATA 11.0.

Results

Test Positivity and Histological Endpoints

Cytology was abnormal (borderline or mild dyskariosis or worse) in 61% (272/446), p16/Ki-67 dual-stained cytology was positive in 57% (254/446) of women, and 50% (221/446) women tested positive for HPV16 and/or HPV18 (HPV16/18); 78% (348/446) of women tested positive for cytology and/or HPV16/18.

Two (0.4%) women were diagnosed with cervical carcinoma (one adenocarcinoma and one squamous cell carcinoma), 79 women (18%) had CIN3, 85 women (19%) had CIN2, 122 women (27%) had CIN1, and 158 women (35%) had no CIN.

Performance of Triage Tests in the Total Study Population

Test specifications of Pap cytology, HPV16/18 genotyping, Pap cytology combined with HPV16/18 genotyping, and p16/Ki-67 dual-stained cytology, for the detection of \geq CIN3 and \geq CIN2, are shown in Table 1. The \geq CIN3 sensitivity of p16/Ki-67 dual-stained cytology was 93.8%, which did not differ significantly from that of Pap cytology (87.7%; ratio 1.07 and 95% CI: 0.97–1.18) or Pap cytology combined with HPV16/18 genotyping (95.1%; ratio 0.99 and 95% CI: 0.91–1.07). The \geq CIN3 specificity of p16/Ki-67 dual-stained cytology (51.2%) was significantly higher than that of Pap cytology (44.9%; ratio 1.14 and 95% CI: 1.01–1.29), and that of Pap cytology combined with HPV16/18 genotyping (25.8%; ratio 1.99 and 95% CI: 1.68–2.35). The complemented negative predictive values for \geq CIN3 of p16/Ki-67 dual-stained cytology, Pap cytology, and Pap cytology combined with HPV16/18 genotyping were 2.6% (95% CI: 0.4–4.9%), 5.7% (95% CI: 2.3–9.2%) and 4.1% (95% CI: 0.2–8.0%), respectively.

Factors Potentially Influencing Test Performance

Three factors were evaluated for a potential influence on test performance: (1) age of the participants, ie, women \geq 30 years ($n=254$) or $<$ 30 years ($n=192$); (2) reason of referral, ie, because of a recent abnormal cytological scrape ($n=197$) or non-cervix-related gynecologic complaints ($n=249$); (3) moment of taking the cervical scrape, ie, at a separate visit 2–3 weeks before the colposcopy visit ($n=250$) or at the same visit as colposcopy ($n=196$).

As shown in Table 2, only \geq CIN3 specificity of Pap cytology was significantly influenced by the referral reason of the participant; \geq CIN3 specificity of Pap cytology was significantly lower in women

Table 1 Test specifications

Triage strategy	n1/N1	Sensitivity (95% CI)	n2/N2	Specificity (95% CI)	PPV (95% CI)	1-NPV (95% CI)
≥ CIN3						
Pap cytology	71/81	87.7% (80.5–94.8%)	164/365	44.9% (39.8–50.0%)	26.1% (20.9–31.3%)	5.7% (2.3–9.2%)
HPV16/18 genotyping	61/81	75.3% ^{ab} (65.9–84.7%)	205/365	56.2% ^{ab} (51.1–61.3%)	27.6% (21.7–33.5%)	8.9% (5.2–12.6%)
Pap cytology and/or HPV16/18 genotyping	77/81	95.1% ^a (90.3–99.8%)	94/365	25.8% ^a (21.3–30.2%)	22.1% (17.8–26.5%)	4.1% (0.2–8.0%)
p16/Ki-67 dual stained cytology	76/81	93.8% ^{c,d} (88.6–99.1%)	187/365	51.2% ^{ab} (46.1–56.4%)	29.9% (24.3–35.6%)	2.6% (0.4–4.9%)
≥ CIN2						
Pap cytology	144/166	86.7% (81.6–91.9%)	152/280	54.3% (48.5–60.1%)	52.9% (47.0–58.9%)	12.6% (7.7–17.6%)
HPV16/18 genotyping	101/166	60.8% ^{ab} (53.4–68.3%)	160/280	57.1% ^{e,b} (51.3–62.9%)	45.7% (39.1–52.3%)	28.9% (23.0–34.8%)
Pap cytology and/or HPV16/18 genotyping	153/166	92.2% ^a (88.1–96.3%)	85/280	30.4% ^a (25.0–35.7%)	44.0% (38.8–49.2%)	13.3% (6.5–20.0%)
p16/Ki-67 dual stained cytology	142/166	85.5% ^{c,b} (80.2–90.9%)	168/280	60.0% ^{c,b} (54.3–65.7%)	55.9% (49.8–62.0%)	12.5% (7.8–17.2%)

Abbreviations: CI, confidence interval; ≥ CIN2, cervical intraepithelial neoplasia grade 2 or worse; ≥ CIN3, cervical intraepithelial neoplasia grade 3 or worse; N1, total number of disease cases; n2, number of test negative non-disease cases; N2, total number of non-disease cases; PPV, positive predictive value; 1-NPV, complemented negative predictive value; n1, number of test positive disease cases.

Test specifications of Pap cytology, HPV16/18 genotyping, Pap cytology combined with HPV16/18 genotyping, and p16/Ki-67 dual-stained cytology for detection of ≥ CIN3 and ≥ CIN2 in high-risk HPV-positive women.

^aSignificant difference with Pap cytology.

^bSignificant difference with Pap cytology and/or HPV16/18 genotyping.

^cNon-inferior to Pap cytology.

^dNon-inferior to Pap cytology and/or HPV16/18 genotyping.

^eNo significant difference with Pap cytology; non-inferiority test not significant.

referred for abnormal cytology than in women referred for other non-cervix-related complaints. In multivariate analyses, the performance of Pap cytology was neither influenced by age nor by the sampling moment of the cervical scrape (Table 2). Both sensitivity and specificity of HPV16/18 genotyping and p16/Ki-67 dual-stained cytology were independent of the age of the participant, referral reason of the participant, and the point in time when the cervical scrape was collected (data not shown).

Subgroup Analysis of Test Performance

Given the significant influence of referral reason of the participant on the ≥ CIN3 specificity of Pap cytology, we performed additional analyses after exclusion of women who were referred for abnormal cytology ($n=197$), leaving only women who were referred for other non-cervix-related complaints ($n=249$). Histological endpoints for this subgroup are shown in Figure 1. In this subgroup, ≥ CIN3 specificity of Pap cytology (60.3%; 95% CI: 53.9–66.7%) and Pap cytology combined with HPV16/18 genotyping (33.9%; 95% CI: 27.7–40.1%) were significantly higher than in the total study population (44.9 and 25.8% respectively; Table 1). The ≥ CIN3 specificity of p16/Ki-67 dual-stained cytology (56.7%; 95% CI: 50.2–63.2%) in this subgroup was similar to its specificity in the total study population (51.2%). In this subgroup, the ≥ CIN3 specificity of p16/Ki-67 dual-stained cytology (56.7%) did not differ significantly from that of cytology (60.3%; ratio 0.94 and 95% CI: 0.83–1.07). Similar to analyses in the total study population, p16/Ki-67 dual-stained cytology had a higher ≥ CIN3 specificity compared with Pap cytology combined with HPV16/18 genotyping (56.7 vs 33.9%; ratio 1.67 and 95% CI: 1.40–1.99).

≥ CIN3 Detection by Different Tests

Table 3 shows the combinations of results for Pap cytology, HPV16/18 genotyping, and p16/Ki-67 dual-stained cytology in all women with ≥ CIN3. A majority of women with ≥ CIN3 (54/81; 67%), including one woman with an adenocarcinoma, were positive for all three tests. In another 16.0% (13/81) of ≥ CIN3 cases, including one squamous cell carcinoma, both Pap cytology and p16/Ki-67 dual-stained cytology were positive, whereas HPV16/18 genotyping was negative (the squamous cell carcinoma harbored HPV39). Among 10 Pap cytology-negative CIN3 cases (12%), 9 (11% of all ≥ CIN3 cases) tested positive for p16/Ki-67 dual-stained cytology, including 5 cases (6% of all ≥ CIN3 cases) that were also HPV16 and/or 18 positive. Among five (6% of all ≥ CIN3 cases) p16/Ki-67 dual-stained cytology-negative CIN3 cases, four (5% of all ≥ CIN3 cases) tested Pap cytology-

Table 2 Age of the participant, referral reason of the participant and sampling moment of the cervical scrape as covariates that potentially influence the performance of Pap cytology

Covariate	Inclusion criterion	Unadjusted analysis		Adjusted analysis	
		Odds ratio (95% CI)	P-values ^a	Odds ratio (95% CI)	P-values ^a
		<i>Response variable: Pap cytology</i>			
Age Women aged ≥ 30 years relative to women aged < 30 years (reference category)	\geq CIN3	1.093 (0.257–4.650)	0.904	0.791 (0.141–4.427) ^b	0.789
	< CIN3	1.096 (0.725–1.658)	0.663	0.652 (0.404–1.052) ^b	0.079
	\geq CIN2	1.342 (0.536–3.358)	0.529	0.670 (0.227–1.980) ^b	0.469
	< CIN2	0.958 (0.598–1.534)	0.858	0.654 (0.386–1.108) ^b	0.114
Reason of referral to gynecologist Women referred for abnormal cytology relative to women referred for other, non-cervix-related, gynecologic complaints (reference category)	\geq CIN3	1.587 (0.406–6.209)	0.507	1.727 (0.106–28.238) ^c	0.702
	< CIN3	5.858 (3.595–9.546)	0.000	7.450 (3.107–17.862) ^c	0.000
	\geq CIN2	3.728 (1.461–9.512)	0.006	5.839 (1.160–29.396) ^c	0.032
	< CIN2	4.630 (2.694–7.955)	0.000	5.093 (1.824–14.220) ^c	0.002
Sampling moment of cervical scrape Scrapes combined with colposcopy relative to scrapes obtained in separate visit (reference category)	\geq CIN3	1.485 (0.381–5.794)	0.569	1.041 (0.067–16.215) ^d	0.977
	< CIN3	3.889 (2.450–6.172)	0.000	0.882 (0.373–2.086) ^d	0.775
	\geq CIN2	2.480 (0.993–6.192)	0.052	0.725 (0.144–3.661) ^d	0.698
	< CIN2	3.495 (2.081–5.870)	0.000	1.024 (0.373–2.809) ^d	0.963

Abbreviations: CI, confidence interval; \geq CIN2, cervical intraepithelial neoplasia grade 2 or worse; \geq CIN3, cervical intraepithelial neoplasia grade 3 or worse.

The odds ratios represent the odds of a positive cytology (borderline or mild dyskariosis or worse) result in case of cervical disease or the absence of cervical disease in (1) women aged ≥ 30 years ($n=254$) relative to women aged < 30 years ($n=192$); (2) women referred for abnormal cytology ($n=197$) relative to women referred for non-cervix-related gynecologic complaints ($n=249$); and (3) women of whom the cervical scrape was collected in a separate visit ($n=250$) relative to those of whom it was collected in combination with colposcopy ($n=196$).

^aP-value obtained by logistic regression.

^bAdjusted for sampling method of cervical scrape and referral reason of participant.

^cAdjusted for age of participant and sampling method of cervical scrape.

^dAdjusted for age and referral reason of participant.

Table 3 Overview of Pap cytology, HPV16/18 genotyping, and p16/Ki-67 dual-stained cytology results in cervical scrapes of women with \geq CIN3 ($n=81$)

Pap cytology	HPV16/18 genotyping	p16/Ki-67 dual-stained cytology	n (% of all \geq CIN3)
+	+	+	54 ^a 66.6%
+	-	+	13 ^b 16.0%
-	+	+	5 6.2%
-	-	+	4 4.9%
+	-	-	3 3.7%
+	+	-	1 1.2%
-	+	-	1 1.2%

^aIncluding one adenosquamous carcinoma. ^bIncluding one squamous cell carcinoma.

positive and one (1% of all \geq CIN3 cases) was only detected by HPV16/18 genotyping.

Discussion

In the present study, comprising high-risk HPV-positive women of a gynecologic outpatient population, the sensitivity of p16/Ki-67 dual-stained

cytology for the detection of \geq CIN3 was similar to that of Pap cytology. In the total study population, p16/Ki-67 dual-stained cytology revealed a higher specificity for \geq CIN3 than Pap cytology. After exclusion of women who had been referred because of abnormal cytology, the \geq CIN3 specificity of p16/Ki-67 dual-stained cytology remained the same, whereas that of Pap cytology increased substantially, resulting in a similar \geq CIN3 specificity of both assays in this particular subgroup of women. p16/Ki-67 dual-stained cytology also yielded similar \geq CIN3 sensitivities to the combination of Pap cytology with HPV16/18 genotyping, at a significantly higher \geq CIN3 specificity. The complementarity of the evaluated tests for \geq CIN3 detection was limited.

In search of a tool to identify women in need of treatment among high-risk HPV-positive women, the subjective nature and related limited reproducibility of cervical cytology necessitate the exploration of more objective tests. In previous studies, p16/Ki-67 dual-stained cytology has been evaluated as a primary cervical screening test,²⁸ as a triage test for women with low-grade cervical cytology^{23,29} or for HPV-positive women with normal cytology,²² and in colposcopy referral populations.^{13,15,30} A recent large study on the performance of p16/Ki-67

dual-stained cytology as a triage marker for high-risk HPV-positive women found that p16/Ki-67 dual-stained cytology outperformed Pap cytology with regard to \geq CIN3 specificity, but had a comparable \geq CIN3 sensitivity.³¹ Our study involved a different study population and we also found a specificity advantage of p16/Ki-67 dual-stained cytology when taking into account the total study population. In our study, this specificity advantage could be attributed to the subgroup of women referred for abnormal cytology, in which a significantly lower \geq CIN3 specificity of Pap cytology, but not of p16/Ki-67 dual-stained cytology, was found.

The sensitivity of cytology in this study was relatively high. Nonetheless, in this outpatient cohort some women with CIN3 were missed by cytology. Retrospective informed cytology revision of the cytology-negative CIN3 cases showed that four CIN3 cases were initially missed by cytology (three re-classified as ASC-US and one LSIL), whereas five were again classified as cytology negative during revision. Besides the use of an outpatient population, the prior knowledge of high-risk HPV presence to the cytotechnicians and cytopathologists might be an explanation for the high sensitivity of cytology in the current study.^{32,33} By adjusting the threshold of abnormal cytology from \geq ASC-US to \geq LSIL in our study, sensitivity would decrease from 87.7 to 77.8%, with a corresponding increase in specificity from 44.9 to 72.3%. A similar finding was recently described by Ebisch *et al.* (in press).

Strengths of the present study include its sample size, the large age range of the population (18–66 years), and the presence of a histological endpoint for each participant. Moreover, our results further substantiate previous work³¹ in a different geographical region and population.

It should be kept in mind that this study was performed in an outpatient population with a relatively high \geq CIN3 prevalence, which limits the direct translatability of our results into screening settings. In addition, this study was limited by a relatively high number of cytological slides that were non-evaluable for p16/Ki-67 dual staining (16%; 88/535), mainly due to insufficient cellularity (72%; 64/88). Of note, during a retrospective informed revision, all CIN3 cases with negative p16/Ki-67 dual-stained cytology ($n=4$) were confirmed as p16/Ki-67 dual-stain negative, owing to a low cellularity. The high number of hypocellular slides might result from the secondary production of a cytological slide for p16/Ki-67 staining, after use of the liquid-based cytology samples for a previous cytology slide and several other molecular tests. Alternatively, significant lesions may have yielded hypocellular slides due to excess blood and debris clogging the filter pores. A third explanation is the fact that, for logistic reasons a proportion of liquid-based cytology samples was obtained directly before colposcopy. In these cases, cautious scraping by the physician (to avoid cervical bleeding during

colposcopy) might have led to insufficient cell numbers.³⁴ This was illustrated by the fact that the rate of non-evaluable specimens for p16/Ki-67 dual-stained cytology was 22% (56/252) among scrapes, which were obtained directly before colposcopy, compared with 11% (32/283) among scrapes that were collected in a separate visit 2–3 weeks before colposcopy.

A previous study has shown that the interpretation of p16/Ki-67 dual-stained slides is reproducible as well, if performed by non-expert staff in cervical cytology,¹⁵ implying that the use of p16/Ki-67 dual-stained cytology could improve the standardization of cervical screening in settings with a lack of cytological expertise. This more objective nature might also be of value, in particular in the triage of high-risk HPV-positive women, in which prior knowledge of high-risk HPV presence could result in a scoring 'bias' that likely will decrease specificity of Pap cytology,^{32,33} as was also observed in the present study. Yet, it should be realized that p16/Ki-67 dual-stained cytology remains microscopy-based, in contrast to other suggested molecular triage tests.

To support the clinical implementation of p16/Ki-67 dual-stained cytology, more data on the long-term \geq CIN3 risk after a negative p16/Ki-67 dual-stained cytology result are required. The currently available data are promising; in the present cross-sectional study, p16/Ki-67 dual-stained cytology tended to have a low complemented negative predictive value for \geq CIN3 compared with Pap cytology (2.6 vs 5.7%), whereas in a 2-year follow-up schedule, Wentzensen *et al*³¹ found an even lower residual \geq CIN3 risk among p16/Ki-67 dual-stain-negative women (complemented negative predictive value 0.6% and 95% CI: 0.2–2.0%).

In summary, p16/Ki-67 dual-stained cytology may serve as a more objective alternative to Pap cytology for triage of high-risk HPV-positive women.

Acknowledgments

We are grateful to the women who participated in this study. We thank the technicians and research staff of the unit of Molecular Pathology of the Department of Pathology at the VU University Medical Center, especially Marjolein Bekker-Lettink, Martijn Bogaarts, and Helma de Bruin. We also thank the cytotechnicians of the Department of Pathology at the VU University Medical Center for cytological testing and logistics, and the administrative workers and information technology team of the Department of Pathology at the VU University Medical Center for the supportive work. We thank the gynecologists, nurses, and administrative workers of the Gynecology Departments of the VU University Medical Center, UMC Utrecht Cancer Center, Onze Lieve Vrouwe Gasthuis, Reinier de Graaf Group, Sint Antonius Hospital, and Flevo Hospital, for their contribution. This study was supported by grants

from the Dutch Cancer Society (KWF VU2006-3570, KWF VU2014-7238, and KWF2009-4413) and the Seventh Framework Program of DG Research of the European Commission (CoheaHr-Health-F3-2013-603019). Roche mtm Laboratories provided p16/Ki-67 staining and evaluation of p16/Ki-67 results free of charge.

Disclosure/conflict of interest

PJFS has been on the speakers bureau of Roche, Qiagen, Abbott, Gen-Probe, and Seegene. PJFS is consultant for Crucell Holland B.V. JB has played an advisory role for Merck and Roche, has been on the speakers bureau of Qiagen, and has received a travel reimbursement from DDL Diagnostic Laboratory. TJMH, RV, and WAH have been principle investigators of a GlaxoSmithKline sponsored study. WGQV is a minority shareholder of Diassay B.V. and obtained grants from GlaxoSmithKline. DAMH has been on the speakers bureau of Hologic/Gen-Probe and serves on the scientific advisory boards of AMGEN and Pfizer. CJLMM has been on the sponsored speakers bureau of GlaxoSmithKline, Qiagen, Merck, Roche, Menarini, and Seegene, and has served on the scientific advisory board of GlaxoSmithKline, Qiagen, Merck, and Roche. CJLMM has been consultant for Qiagen and Gentical, and is a minority shareholder of Diassay B.V. Formerly, CJLMM was a minority shareholder of Delphi Biosciences. CJLMM, PJFS, and DAMH have minority stake in Self-screen B.V., a spin-off company of VU University Medical Center Amsterdam. RL, MGD, FJK, LR, WMB, GCMG, JWMS, and DKED declare no conflicts of interest.

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