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Chapter 4

Triaging HPV-positive women with normal cytology by p16/Ki-67 dual-stained cytology testing: Baseline and longitudinal data

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Abstract

Primary human papillomavirus (HPV)-based screening results in a 2–5% lower specificity for cervical intraepithelial neoplasia Grade 2 or worse (CIN2+) compared to Pap cytology. To identify HPV-positive women with CIN2+, we retrospectively evaluated the cross-sectional and longitudinal performance of p16/Ki-67 dual-stained cytology in HPV-positive women with normal cytology participating in population-based cervical screening. Conventional Pap cytology specimens of 847 of these women derived from the VUSA-Screen study were dual-stained for p16/Ki-67. Cross-sectional clinical performance in detecting CIN3 or worse (CIN3+), and CIN2+ was compared to that of baseline HPV genotyping. Moreover, 5-year cumulative incidence risks (CIR) for CIN3+ (CIN2+) were determined. The sensitivity of p16/Ki-67 dual-stained cytology for CIN3+ (CIN2+) was 73.3% (68.8%) with a specificity of 70.0% (72.8%). HPV16/18 genotyping showed a sensitivity for CIN3+ (CIN2+) of 46.7% (43.8%), with a specificity of 78.3% (79.4%). The 5-year CIR for CIN3+ in HPV-positive women with normal cytology was 6.9%. Testing these women with p16/Ki-67 dual-stained cytology resulted in a significantly lower CIN3+ 5-year CIR of 3.3% ($p = 0.017$) in case of a negative test result. A negative HPV16/18 genotyping test result also led to a lower 5-year CIN3+ CIR of 3.6%. p16/ Ki-67 dual-stained cytology detects more than 70% of underlying CIN3+ lesions in HPV-positive women with normal cytology at baseline and is therefore suitable for triaging these women to colposcopy. Furthermore, the CIN3+ 5-year CIR of 3.3% after a negative dual-stain result is significantly lower compared to the 5-year CIR of 6.9% in women without p16/Ki-67 dualstained cytology triage.

Introduction

Persistent infections with carcinogenic human papillomavirus (HPV) genotypes represent the main causative event in the multistep process of cervical carcinogenesis. Recent efforts to improve cervical screening have focused on introducing HPV testing as a adjunct to Pap cytology testing, or as a primary screening tool. Unlike cytology, HPV testing is objective and has consistently been shown to be more sensitive for the detection of cervical intraepithelial neoplasia Grade 2 (Grade 3) or worse (CIN2+/3+) compared to cytology-based testing (for CIN3+ 94% vs. 65%).^{1,2} Based on this evidence, HPV testing will replace cytology as the primary cervical cancer screening method in The Netherlands in 2016.³ However, the main limitation of primary HPV testing is a 2–5% lower specificity for high-grade CIN compared to cytology.⁴ Most HPV-infections are transient and regress spontaneously. Therefore, to limit the number of unnecessary follow-up procedures, a triage of women tested positive for HPV is necessary.⁵ Currently, cytology is the preferred triage test.^{6,7} Women who test negative for both tests are at very low risk for developing CIN3+, while HPV-positive women with abnormal Pap cytology are at sufficient risk for underlying disease to be directly referred to colposcopy. However, the optimal management for HPV-positive women with normal cytology is still under debate. Several triage strategies for HPV-positive women have been studied. With repeat cytology testing after 6 or 12 months, the 2-year CIN3+ risk decreases to <1%, which is acceptable for dismissal to the next screening round after 3–5 years).^{5,8} However, repeat testing after initial HPV test positives, cytology triage test negative at baseline incurs a risk of loss to follow-up. To overcome the disadvantage of repeat cytology testing, more objective, alternative triage markers that allow direct triage of HPV-positive women with normal cytology would be helpful. Recently, p16/Ki-67 dual-stained cytology has emerged as an interesting candidate of such a biomarker.^{9,10} The simultaneous detection of the overexpression of the p16-protein, which under normal physiological conditions induces cellcycle arrest in the course of cellular differentiation, and the expression of the proliferation marker Ki-67 within the same cervical epithelial cell points to HPV-induced deregulation of the cell cycle. This may be utilized as an indicator for the presence of CIN2+/3+ lesions.^{9,11} The aim of this study was to evaluate both the cross-sectional and longitudinal performance characteristics of p16/ Ki-67 dual-stained cytology in HPV-positive women with normal cytology. The dual-staining was performed on the original, destained conventional Pap cytology specimens of a subset of women participating in the VUSA-Screen study, a population-based cohort, longitudinal screening study conducted in The Netherlands.^{6,8,12} The cross-sectional results for p16/Ki-67 dual-stained cytology were compared to the results for HPV16/18 genotyping. Furthermore, we examined the follow-up results of the p16/Ki-67 dual-staining for the presence or absence of CIN3+ and CIN2+ lesions for a median period of 5 years.

Material and Methods

Study population

The present study was conducted as a post hoc-study within in the VUSA-Screen study, a cohort study within the setting of The Netherlands population-based cervical cancer screening programme (2003–2005). The aim of VUSA-Screen had been to evaluate the effectiveness of HPV testing using the Digene Hybrid Capture 2 (HC2) assay (Qiagen, Venlo, The Netherlands) in a population-based screening cohort. The design of the study has been previously described in detail.¹² Briefly, HPV-positive women with normal cytology (n=1,021) that were selected for this substudy had been retested using cytology and HPV testing after 12 and 24 months. Women with abnormal cytology, regardless of their HPV status, at 12 months or with abnormal cytology and/or a positive HPV test result at 24 months of follow-up were referred to colposcopy.

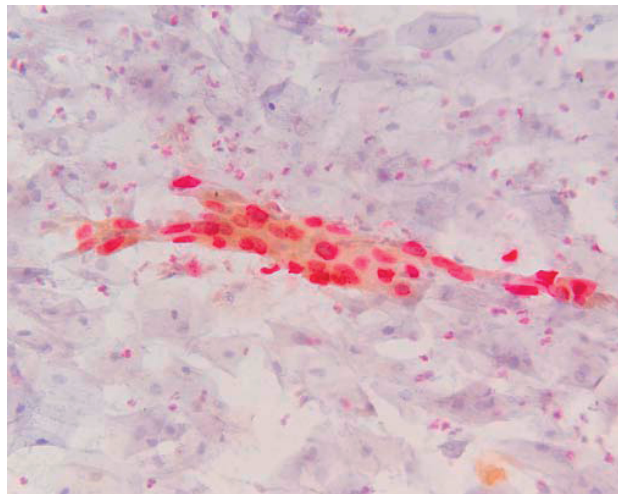
Procedures

In the VUSA-Screen study, a conventional cytological smear using a cervical brush (Cervex-Brush®, Rovers Medical Devices B.V., Oss, The Netherlands) had been collected from all participating women. After conventional preparation of the smear on a glass slide, the brush was placed in a vial containing 1 mL UCM (Universal Collection Medium; Qiagen Corporation, Gaithersburg, Maryland, USA) for HPV testing. Slides were used for Pap staining, and cytologic findings were classified according to the CISOE-A classification. Reading of cytologic slides was done by in a routine cytology screening laboratory by cytotechnicians and cytopathologists according to the Quality Assurance guidelines of the Dutch Pathological society. This classification is easily translatable into either the British or the Bethesda 2001 classification.^{13,14} All UCM collection vials were tested with the HC2 highrisk HPV DNA test in an automated format on a rapid capture system according to the manufacturer's instructions (Qiagen). This test uses a cocktail of probes to detect 13 high-risk HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). Samples with HC2 outcome of 1 RLU/ CO were considered HPV-positive. For HPV genotyping, all HC2 positive cases were subsequently tested with hrHPV GP5+/6+ -PCR and enzyme immunoassay (EIA) readout. Positive samples were further characterized by reverse line blot hybridization.¹⁵ In this study, HC2 positive women were divided into the two groups of women with versus without HPV16/18 positive test results. Colposcopic assessment was performed if women showed an abnormal cytology test result at the 12 or 24 months follow-up visits, and/or when the HPV test was positive at 24 months. Biopsies were taken from all suspect areas, according to standard procedures in The Netherlands.¹⁶ Histological examination of these biopsies was done at local pathology laboratories and specimens were classified as normal or "no CIN", CIN Grades 1, 2 or 3, or as invasive cancer according to international criteria.¹⁷ In this study, all archived conventional cytology slides of the

1,021 HPV-positive women with normal cytology participating in the VUSA-Screen study were selected. We retrieved 847 of these slides for retrospective p16/Ki-67 dual-staining. For the p16/Ki-67 dual-staining, a commercial kit specifically designed for the simultaneous detection of p16 and Ki-67 in cervical cytology preparations was used (CINtecV R PLUS, Roche mtm laboratories AG, Mannheim, Germany). Slides were destained and subjected to p16/Ki-67 dual-staining according to the instructions of the manufacturer. The slides were analyzed and scored by an experienced cytotechnologist blinded to all other study data. Cells were considered positive when immunoreactivity for both p16 and Ki-67 was detected within the same cell (i.e., a cytoplasmic brown staining for p16, together with a nuclear red staining for Ki-67). The presence of at least one dual-stained cell was used as a cut-off to rate the sample as positive for the CINtecV R PLUS test (see Fig. 4.1). Additional assessments using alternative cut-offs were performed. In December 2012, The Netherlands' nationwide network registry and network of histological and cytological results (PALGA; Bunnik, The Netherlands)¹⁸ was accessed and reviewed with regards to follow-up data for all women participating in the VUSA-Screen study. In this timeframe of up to 9 years (median follow-up time 61 months, range 2–78 months), all women at the age of 55 years or younger at baseline should have had a follow-up visit (i.e., more than a full cycle in the Dutch population-based screening program). The results for using p16/Ki-67 dual-stain and HPV genotyping as a triage test for HPV-positive women with normal cytology were analyzed for the time points at baseline and after the entire follow-up period. For each woman, the most severe diagnosis during follow-up was used.

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Figure 4.1 Example of p16/Ki-67 dual-stained cervical cytology after de-staining of the original Pap cytology slide



Brown cytoplasmic signal for p16 overexpression and red nuclear signal for Ki-67 expression within the same cell points to cell-cycle deregulation.

Statistical analysis

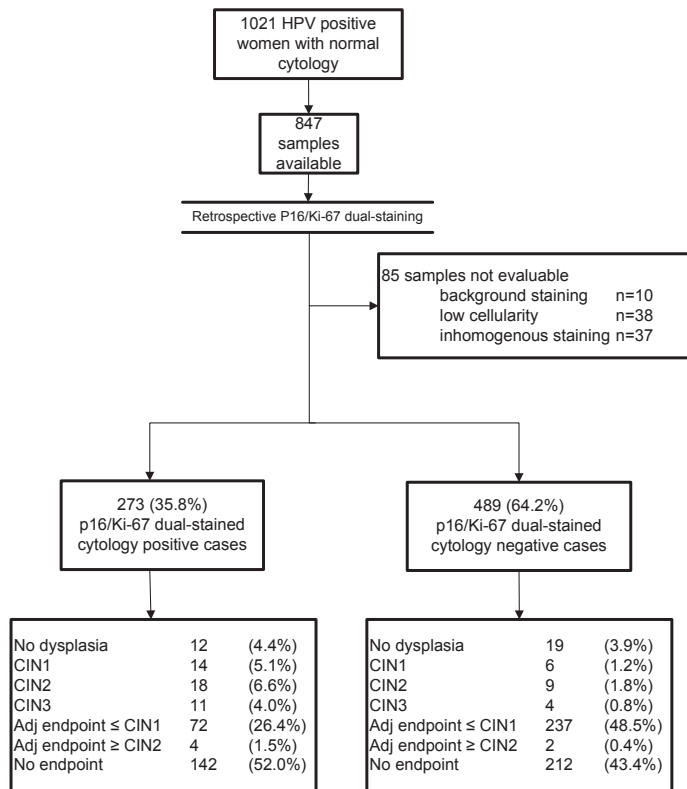
The VUSA-Screen study was designed as a historically prospective cohort study. Women reached their study endpoint if they had a histological outcome of CIN1, CIN2, CIN3, or carcinoma, or an adjusted endpoint. Adjusted endpoints for HPV-positive women with normal cytology were defined as \leq CIN1 when they had a normal cytological test result within 3 years of follow-up. Alternatively, HPV-positive women with normal cytology were considered to have CIN2+ when they had cytology test results categorized as moderate dyskaryosis or worse (\geq BMD) during their follow-up without any histological test result.^{19,20} Because of the unknown histological outcome these were only considered as CIN2+ and not as CIN3+. Women with other (combinations of) repeat test results were considered as having no adjusted endpoint and were therefore excluded from the cross-sectional analysis. We used 3 year follow-up results for the association between p16/Ki-67 positivity, HPV genotyping, and CIN3+ detection at baseline since within this time frame all women should have had their follow-up visits after a positive test result. The results were analyzed using the chi-square test. CIN2+ was used as an additional, secondary outcome because treatment of CIN2 is common practice in most Western countries. Differences in results for CIN3+ and CIN2+, between the evaluable slides and nonevaluable slides were calculated using the two-sided Fisher's Exact Testing. Estimates for sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated from cross-tabulation of test results and adjusted endpoints. Referral rates were calculated by dividing the number of women with a positive test result by the total number of women. Significant differences between tests with regard to sensitivity, specificity, and referral rates were calculated using the McNemar test. The two tests were compared with respect to PPV and NPV using the method of Leisingring et al.²¹ A p-value below 0.05 was considered significant and all test were two-sided. Exact 95% confidence intervals were calculated. These 3-year analyses were done with IBM SPSS version 20 (International Business Machines Corp., Armonk, New York). For the long-term predictive value of both the p16/Ki-67 dual staining and HPV genotyping triage testing interval censoring was used in case of a CIN2+/CIN3+ event and right censoring otherwise. The cumulative 5-year CIN2+/3+ risk and 95% confidence intervals were calculated with the interval package within the statistical software program R (version 3.0.1).²²

Results

Of the 1,021 HPV-positive women with normal cytology, a total of 847 conventional slides were available for p16/Ki-67 dual-stained cytology. Eighty-five of these 847 cases (10.0%) were excluded from the analysis because of testing failure: 37/85 (43.5%)

cases due to nonhomogenous staining, 10/85 (11.8%) cases due to background staining, and 38/85 (44.7%) cases did not show sufficient cellular material. A total of 762 cases were included in the final analysis (see Fig.4.2). There were no significant differences in the proportion of CIN3+ (2.0 vs. 1.2%; p50.543) or CIN2+ (6.3 vs. 2.4%; p50.129) cases between the evaluable and nonevaluable slides.

Figure 4.2 Flowchart of the VUSA-Screen study design including p16/Ki-67 dual-stained cytology testing in HPV-positive women with normal cytology – 3 years follow-up



HPV, human papillomavirus, CIN, cervical intraepithelial neoplasia (grade 1, 2 or 3); Adjusted endpoints: normal cytology within three years of follow-up equals \leq CIN1, $>$ BMD cytology test results in follow-up equals CIN2+.

Study cohort characteristics

The median age of the 762 women included in this study was 35.0 years (range 29–61 years). Five hundred ninety nine out of 762 (78.6%) women attended a follow-up visit within 3 years, and all women had a follow-up visit within 5 years. The median follow-up time was 61 months (range 2–78 months). For this analysis, 93/762 (12.2%) cases had a histological endpoint. Adjusted endpoints could be established for another 315 women. Three hundred and nine women had normal cytology within 3 years of follow-up and

were therefore considered \leq CIN1. Furthermore, six women with moderate dyskaryosis or worse within 3 years of follow-up were considered to have CIN2+. Ultimately, endpoints (both histologic and adjusted) could be established for 408 women, leaving 354 women who did not meet the criteria of an adjusted endpoint and were therefore excluded from the analysis to estimate the sensitivity and specificity. These comprised 164 women (46.3%) without a 3 year follow-up test result, 33 (9.3%) women with a borderline or mild dyskaryotic follow-up smear, 154 (43.5%) women with a HPV-positive, cytomorphologically normal follow-up smear, and finally, there were 3 women (0.8%) with nonevaluable cytology test results.

Test performance characteristics

Two-hundred-seventy-three out of 762 (35.8%) women with or without (adjusted) endpoint tested positive for p16/Ki-67 dual-stained cytology at baseline. These included 18 women with CIN2 and 11 with CIN3 diagnosed within 3 years of follow-up. In the dual-stain negative group, nine CIN2 lesions and four CIN3 lesions were confirmed within 3 years. The estimated 3-year longitudinal sensitivity of p16/Ki-67 dual-stained cytology testing was 73.3% (95% CI 44.9–92.2%) for CIN3+ and 68.8% (95% CI 53.7–81.3%) for CIN2+ (Table 4.1). The corresponding specificity values were 70.0% (95% CI 65.2–74.6%) for CIN3+ and 72.8% (95% CI 67.9– 77.3%) for CIN2+.

As the interpretation of dual-stained cells in some cases may show some remaining subjectivity, we explored various thresholds for calling the test result positive, that is, one positive dual-stained cell versus using thresholds of two or more, six or more, and 50 or more. As a result the sensitivity was substantially lowered and specificity increased (Table 4.1).

Performance of p16/Ki-67 dual-stained cytology in comparison to HPV16/18 genotyping

One-hundred-nineteen of the 273 (43.6%) women with a positive dual-stained cytology tested also positive for HPV16/18 (Table 4.2). In this group, 7 CIN3 and 19 CIN2+ lesions developed. Of the 489 women with a negative dual-staining result, 95 (19.4%) tested positive for HPV16/18. In this dual-stain negative, HPV16/18 positive group, no CIN3 or worse, and two CIN2 lesions developed during 3 years of follow-up. The sensitivity of HPV16/18 genotyping for CIN3+ (46.7%; 95% CI 21.3–73.4%) was lower than the sensitivity of p16/Ki-67 dual-stained cytology (73.3%; 95% CI 44.9–92.2%), although not reaching statistical significance ($p=0.125$). For CIN2+ the sensitivity of HPV16/18 genotyping (43.8%; 95% CI 29.5–58.8%) was significantly lower than that of dual-stained cytology (68.8%; 95% CI 53.7–81.3%; $p=0.004$). The specificity of HPV16/18 genotyping was 78.3% (95% CI 73.9– 82.3%) for CIN3+ and 79.4% (95% CI 75.0–83.5%) for CIN2+, both significantly higher compared to dual-stained cytology

Table 4.1 Clinical performance of p16/Ki-67 and HPV testing to detect CIN2+ or CIN3+ in HPV-positive women with normal cytology using the standard positivity cut-off of ≥ 1 dual-stained cell in the upper part and alternative cut-offs in the lower part

	n/N	Sensitivity	(95% CI)	n/N	Specificity	(95% CI)	PPV	(95% CI)	NPV	(95% CI)
p16/Ki-67 ≥ 1 pos										
CIN2+	33/48	68.8%	(53.7-81.3%)	262/360	72.8%	(67.9-77.3%)	25.2%	(18.0-33.5%)	94.6%	(91.2-96.9%)
CIN3+	11/15	73.3%	(44.9-92.2%)	271/387	70.0%	(65.2-74.6%)	8.7%	(4.4-15.0%)	98.5%	(96.3-99.6%)
HPV16/18										
CIN2+	21/48	43.8%	(29.5-58.8%)	286/360	79.4%	(75.0-83.5%)	22.1%	(14.2-31.8%)	91.4%	(87.7-94.3%)
CIN3+	7/15	46.7%	(21.3-73.4%)	303/387	78.3%	(73.9-82.3%)	7.7%	(3.1-15.2%)	97.4%	(95.0-98.9%)
alternative cut-offs										
p16/Ki-67 ≥ 2 pos										
CIN2+	30/48	62.5%	(47.4-76.0%)	281/360	78.1%	(73.4-82.2%)	27.5%	(19.4-36.9%)	94.0%	(90.7-96.4%)
CIN3+	11/15	73.3%	(44.9-92.2%)	293/387	75.7%	(71.1-79.9%)	10.5%	(5.3-18.0%)	98.7%	(96.6-99.6%)
p16/Ki-67 ≥ 6 pos										
CIN2+	22/48	45.8%	(31.4-60.8%)	326/360	90.6%	(87.1-93.4%)	39.3%	(26.5-53.2%)	92.6%	(89.4-95.1%)
CIN3+	10/15	66.7%	(38.4-88.2%)	344/387	88.9%	(85.3-91.8%)	18.9%	(9.4-32.0%)	98.6%	(96.7-99.5%)
p16/Ki-67 >50 pos										
CIN2+	8/48	16.7%	(7.5-30.2%)	352/360	97.8%	(95.7-99.0%)	50.0%	(24.7-75.3%)	89.8%	(86.4-92.6%)
CIN3+	2/15	13.3%	(1.7-40.5%)	376/387	97.2%	(95.0-98.6%)	15.4%	(1.9-45.4%)	96.7%	(94.4-98.2%)

Abbreviations: CIN, cervical intraepithelial neoplasia (grade 2 or higher; grade 3 or higher); HPV, human papillomavirus; CI, confidence intervals; PPV, positive predictive value; NPV, negative predictive value; n, number of cases; N, total number of cases

($p=0.005$ and $p=0.031$). Referral rates on the basis of p16/Ki-67 dual-stained cytology would have resulted in higher referral rates of these women for colposcopy (35.8% vs. 28.0%; $p<0.001$) compared with referral on the basis of HPV16/18 genotyping.

Table 4.2 Analysis of HPV16/18 genotyping versus histologic endpoint stratified by p16/Ki-67 dual-stained cytology

p16/Ki-67 dual-stained cytology	HPV 16/18	\leq CIN1*	CIN2+*	CIN3	No endpoint
Positive	Positive	29	19	7	71
	Negative	69	14	4	71
Negative	Positive	45	2	0	48
	Negative	217	13	4	164

Abbreviations: CIN, cervical intraepithelial neoplasia (grade 1 or less; grade 2 or higher; grade 3); HPV 16/18, human papillomavirus type 16 and/or 18; * \leq CIN1 comprises all women with no dysplasia ($n=31$), CIN1 ($n=20$) or an adjusted endpoint ($n=310$); CIN2+ comprises all women with a CIN2 ($n=27$), a CIN3 ($n=15$) and the 6 women with an adjusted endpoint.

Five-year follow-up data

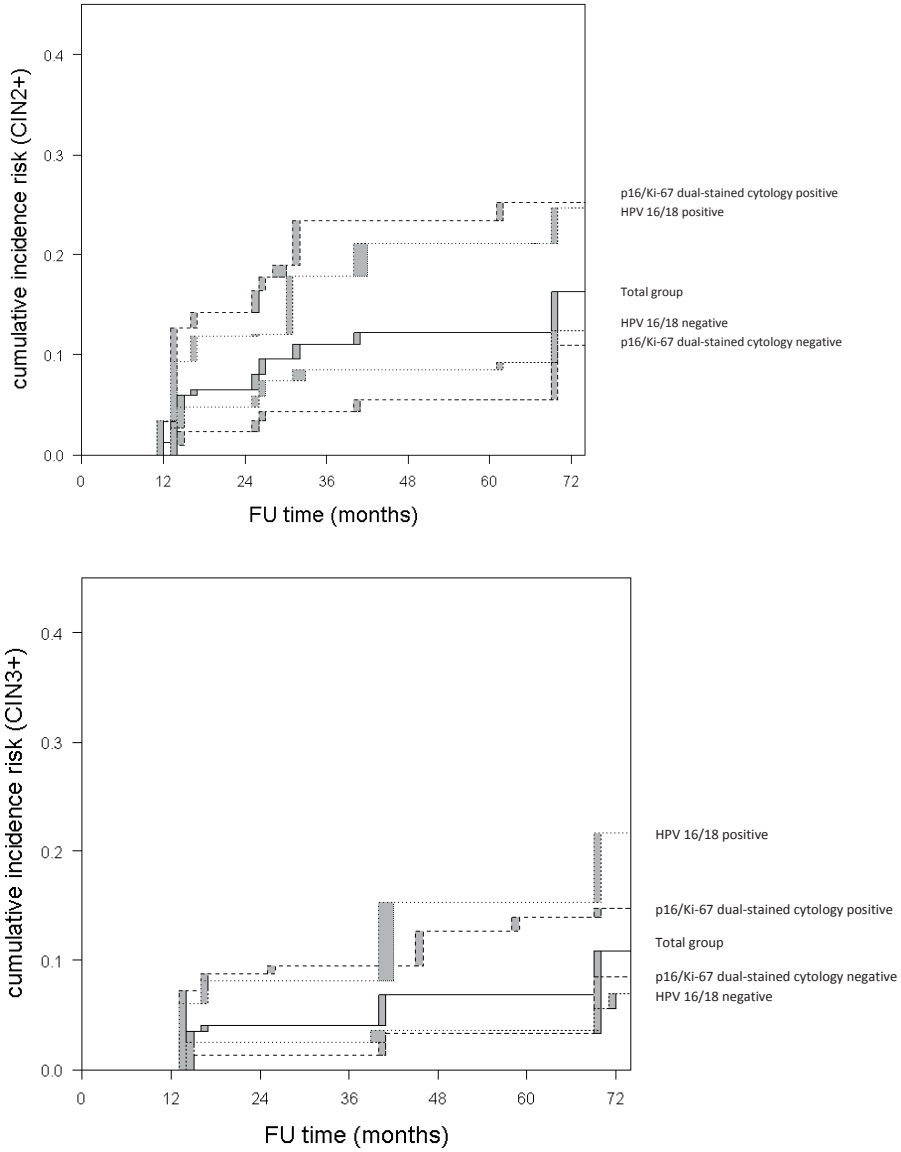
The 5-year cumulative incidence risk (CIR) estimates of the HPV-positive women with normal cytology stratified by p16/Ki-67 dual-stained cytology or HPV16/18 genotyping results are presented in Table 4.3, with corresponding cumulative incidence curves for CIN3+ and CIN2+ provided in Figure 4.3. HPV-positive women with normal cytology at baseline had a 5-year CIR of 6.9% (95% CI 2.4–8.8%) and 12.2% (95% CI 8.5–14.4%) for CIN3+ and CIN2+, respectively. In case of a negative dual-stained cytology test result, the 5-year CIR for CIN3+ (3.3%; 95% CI 0.3–4.7%) and CIN2+ (5.4%; 95% CI 2.9–7.6%) were statistically significantly lower ($p=0.017$ and $p<0.001$). There were no significant differences in 5-year CIN3+ CIR for either women with a negative dual-stain result compared to women with a negative HPV16/18 genotyping test result (3.6%, 95% CI 1.2–5.0%, $p=0.81$), nor for 5-year CIN2+ CIR (5.4%, 95% CI 2.9–7.6%, vs. 8.5%, 95% CI 5.3–11.0%, $p=0.58$).

Table 4.3 Cumulative incidence risks for CIN2+ and CIN3+ for HPV-positive women with normal cytology and different triage tests

		CIN2+		CIN3+	
		CIR	95% CI	CIR	95% CI
HPV-positive women with normal cytology		12.2%	8.5–14.4%	6.9%	2.4–8.8%
Normal repeat cytology within 12 months		7.8%	4.2–10.6%	4.5%	0.7–7.3%
p16/Ki-67 dual stain \geq 1 cell	Positive	23.4%	16.1–28.3%	13.9%	7.8–17.8%
	Negative	5.4%	2.9–7.6%	3.3%	0.3–4.7%
HPV16/18	Positive	21.1%	11.9–25.6%	15.2%	4.9–19.5%
	Negative	8.5%	5.3–11.0%	3.6%	1.2–5.0%

Abbreviations: CIN, cervical intraepithelial neoplasia (grade 2 or higher; grade 3 or higher); CIR, cumulative incidence risk; HPV, human papillomavirus; CI, confidence intervals.

Figure 4.3 Cumulative incidence risk (CIR) curves for CIN2+ and CIN3+ for HPV-positive women with normal cytology and different triage tests. Abbreviations: cervical intraepithelial neoplasia (grade 2 or higher; grade 3 or higher); FU, follow-up.



Discussion

The aim of this study was to evaluate the performance of p16/Ki-67 dual-stained cytology for the triage of HPV-positive women with normal cytology. The results show that for triaging these women, p16/Ki-67 dual-stained cytology had a 3-year sensitivity of 73.3% for CIN3+, with a specificity of 70.0% and a referral rate of 35.8%. Of the 15 CIN3 lesions, four lesions developed in the group of p16/Ki-67 dual-stained negative women, and therefore would have been missed when using dual-stained cytology for triaging HPV-positive women with normal cytology and no further follow-up of those women for 3 years. However, the vast majority of CIN3+ cases was positive for p16/Ki67 dual-staining. The 5-year cumulative incidence risk for CIN3+ in case of a negative p16/Ki-67 dual-stained test was 3.3%. Petry et al. were the first to examine the use of p16/Ki-67 dual-stained cytology in HPV-positive women with normal cytology,⁹ reporting a good performance of dual-stained cytology for detecting women at risk for developing high-grade CIN lesions. Both sensitivity and specificity of dual-stained cytology for detecting women at risk for developing high-grade CIN lesions were even higher in that study. Differences in study populations and methodology used may explain the differences between these two studies. The VUSA-Screen study was performed as a population-wide cohort analysis in The Netherlands, a country with an organized screening program and a high quality of cytology screening. In contrast, the Petry et al. study was a single institutional study in a region in Germany that underwent opportunistic cervical cancer screening before. Also, Petry et al. used liquid-based cytology (LBC) specimens for p16/Ki-67 dual staining, and differences in the performance of p16/Ki-67 dual-staining might be expected between destained, archived conventional smears and freshly prepared LBC specimens.

Worth mentioning, in our study, 36 women were excluded because they had BMD test results within three years of follow-up, but without histological follow-up data. It might be expected that approximately 5–15% of these women may harbor an underlying CIN3+.^{23,24} Indeed, two women developed a CIN3, and one of them became positive for p16/Ki-67 dual-staining within 5 years. Five others (all positive for p16/ Ki67 dual-staining) developed CIN2 within 5 years. If these women would have been referred according to protocol, we could have included them in the analysis which would have resulted in higher sensitivity estimates.

Another triage strategy for HPV-positive women with normal cytology which is widely discussed and recommended in the USA is HPV16/18 genotyping. We evaluated the genotyping results in our study and made a comparison to p16/ Ki-67 dual-staining. Although CIN3+ numbers were small, the sensitivity of p16/Ki-67 dual-stained cytology for the detection of CIN3+ tends to be higher compared to HPV16/ 18 genotyping (73.3% vs. 46.7%). Specificity for CIN3+ was significantly lower for p16/Ki-67 dual-

staining versus HPV16/18 genotyping (70.0% vs. 78.3%). The 5-year CIR for CIN3+ of HPV16/18 positive women with normal cytology was 15.2%, compared to 3.6% for HPV-positive women, but HPV genotypes 16/18 negative women.

In a recently published study a successful triage strategy was recommended based on data from the Dutch VUSAScreen study.⁸ This strategy comprises repeat cytology testing in 12 months, because of the low CIN3+ risk of 1.6% in 1 year and a low referral rate. This is especially attractive in countries with efficient, organized cytology-based screening programs. In our study 8 of the 169 women with normal repeat cytology after 12 months were shown to develop CIN3+. With a 5-year CIN3+ CIR of 4.5%, repeat cytology after 12 months is a reasonable way to triage HPV-positive women.⁵ However, in countries with opportunistic and thus less efficient cytology-based screening programs, triaging HPV-positive women with p16/Ki-67 dual-stained cytology or HPV16/18 genotyping might be beneficial, allowing the detection of women with underlying high-grade CIN at baseline and minimizing the risk of loss to follow-up.

A limitation of our study was the fact that a significant amount of the dual-stained samples were not evaluable due to various reasons. The use of conventional smears, instead of LBC, could explain the higher number of cases with low cellularity.²⁵ Also, the use of archived, Pap-stained samples initiated the fact that the samples had to be destained and restained, which is an extra step with accompanying risks. As a result of the destaining process, 38 slides were not evaluable due to low cellularity. These technical drawbacks due to the use of conventional archived slides need to be balanced by the opportunity to study the long-term predictive values of p16/Ki-67 dual-stained cytology in a population-based screening cohort with long-term follow-up data.

Furthermore, there might be a possible verification bias because not all included women had a histological endpoint. We formulated adjusted endpoints for women who had normal cytology test results within 3 years of follow-up. Even though different studies have shown that women with two subsequent negative cytology test results have a low risk for CIN3+.^{6,7} it is not certain that this did not bias our results.^{26,27}

Another limitation was the low attendance rate. The attendance at repeat testing at 12 and 24 months in the original study cohort was only 59.7% within 3 years. Consequently, the high-grade lesions were detected after a relatively longer time frame than if women would have been tested at 12 and 24 months. To overcome this limitation, we used interval censoring in case of a CIN2+/3+ lesion and right censoring otherwise for the long-term inference.

Strengths of our study were the large sample size and the longitudinal design, in which follow-up data up to 9 years were available. Another strength of this study was the setting within a population-based screening program with a low-risk population, which means that the study results can be generalized to a larger group of women. For this study, cytology reading was done in the setting of a private laboratory with a long

experience in population-based screening and undergoing all quality assurance measures as obliged for screening. For this reason, our study setting can be considered as representative for the organized screening situation in The Netherlands.

In conclusion, p16/Ki-67 dual-stained cytology performs well in triaging HPV-positive women with normal cytology. More high-grade lesions are detected at baseline and could therefore be treated earlier.

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