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# Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 Dual-stained cytology

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ABSTRACT

*Objective.* Testing for human papillomavirus (HPV) has been shown to increase the sensitivity and negative predictive value for detection of high-grade cervical intraepithelial neoplasia (CIN2+), either when used in conjunction with Pap cytology testing or alone. However, there is no satisfying clinical management algorithm for women testing Pap negative/HPV positive. We therefore evaluated the clinical utility of a novel dual biomarker-based approach (p16/Ki-67 Dual-stained cytology) for the identification of CIN2+ in women with Pap negative/HPV positive screening results, without the need to refer all women to immediate colposcopy.

*Methods.* All women aged ≥30 enrolled during 2007/2008 into a regional prospective Pap/HPV co-testing screening pilot project and tested Pap negative, but positive for HPV (n=425) were included in the analysis. p16/Ki-67 Dual-stained cytology was performed from residual cellular material available from the liquid-based cytology vial collected during the initial Pap/HPV co-testing screening visit. Results were correlated to the presence of CIN2+ confirmed during preliminary follow-up.

*Results.* p16/Ki-67 Dual-stained cytology tested positive at baseline in 108 out of 425 (25.4%) Pap negative/HPV positive cases. Sensitivity of Dual-stain testing for the detection of biopsy-confirmed CIN2+ during preliminary follow-up within the group of Pap negative/HPV positive women was 91.9% for CIN2+ (34/37 cases), and 96.4% for CIN3+ (27/28 cases). Specificity was 82.1% for CIN2+ on biopsy, and 76.9% for CIN3+, respectively.

*Conclusions.* Triaging Pap negative/HPV positive screening test results with p16/Ki-67 Dual-stained cytology may identify women with a high probability of underlying CIN2+ and may efficiently complement HPV-based screening programs to prevent cervical cancer.



## Introduction

In a recent meta-analyses of 7 large longitudinal randomised controlled trials (RCTs) in The Netherlands, Sweden, Finland, Canada, Italy, India and UK as well as of non-randomised cohort studies comparing testing for human papillomavirus (HPV) and Pap cytology as primary screening tests for secondary prevention of cervical cancer, HPV testing was more sensitive for the detection of cervical intraepithelial neoplasia (CIN) of grade 2/3 or higher (CIN2+/CIN3+) than cytology [1,2]. This improved detection rate resulted in a significant decrease of CIN3+ cases in subsequent screening rounds [3]. Besides the high sensitivity for high-grade CIN which is unaffected by age, HPV testing is associated with a very high negative predictive value. More than 99% of cervical cancers are linked to HR-HPV [4], and HPV negative women will not develop cervical cancer within the next 5–7 years because there is strong evidence that the minimum latency from initial HPV infection to cancer seems to be in the range of 8 years [5]. This is confirmed by longitudinal observation studies and screening trials [2,6,7].

Despite these advantages, there are also limitations associated with HPV DNA testing in screening for cervical cancer precursors [8,9].

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Almost all screening trials that were based on HPV DNA testing examined women who were 30 years or older [1,2]. Due to the high prevalence of transient HPV infections in younger populations, the specificity of HPV testing in younger age groups is unsatisfactorily low. Therefore, referral rates to colposcopy would be too high to make HPV testing a cost efficient, viable alternative to Pap cytology-based screening in women less than 30 years old. Besides the restriction to the older age groups, one of the unsolved problems in HPV-based screening programs is the still missing best algorithm of how to identify CIN3+ cases among women with positive HPV tests. In primary screening programs based on a combination of cytology and HPV testing, women with normal Pap cytology who tested positive for HR-HPV may carry a risk of 3–7% for underlying high-grade CIN [10–12]. In general, there are several different management options for Pap negative/HPV positive cases, including immediate referral to colposcopy, repeat Pap cytology and/or HPV testing within 6–18 months, or triaging with HPV-genotyping, HR-HPV mRNA E6/E7 testing, or the use of other biomarkers to detect underlying high-grade CIN [9].

Recently, a novel biomarker concept, which is based on the combined detection of the $p16^{INK4a}$ (p16) and Ki-67 biomarker protein expression in cervical cytology specimens has been proposed. The simultaneous detection of p16 over-expression, a cell-cycle regulatory protein that induces cell-cycle arrest under normal physiological conditions [13,14], and the expression of a proliferation marker such as Ki-67 within the same cervical epithelial cell may be used as a surrogate marker of cell-cycle deregulation mediated by transforming HPV infections. This morphology independent biomarker approach most recently has been shown to allow for an efficient triage of equivocal or mildly abnormal Pap cytology results [15].

In this study, we evaluated the performance of this novel p16/Ki-67 Dual-stained cytology concept for the triage of Pap negative/HPV positive primary cervical cancer screening test results in a large cohort of women aged 30 years or older and participating in the Wolfsburg HPV screening pilot project [16].

## Materials and methods

### *Study subjects and patient management*

In February 2006 Deutsche BKK, a German health insurance changed its primary cervical cancer screening program in the region of Wolfsburg for women aged 30 and older from annual Pap cytology to combined Pap/HPV-testing every 5 years. This first German pilot project was set up to evaluate the feasibility and acceptance of primary HPV screening under routine conditions outside of randomized controlled trials. Details about recruitment and patient management within this screening project were recently described [16].

For the nested sub-study evaluating the performance of p16/Ki-67 dual-stained cytology reported in this manuscript, women were eligible who were tested Pap cytology negative (using ThinPrep® Pap Test; Hologic, Marlborough, MA), but positive for high-risk HPV DNA. Pap negative was defined as Pap cytology results of Pap I/II following the Munich II classification, which represents the Negative for Intraepithelial Lesion or Malignancy (NILM) category of the Bethesda system for reporting cervical cytology [17]. Pap negative/HPV positive women were advised to follow an expectant management described in detail by Luyten et al. [16], starting with repeat Pap cytology testing at 6 months post study inclusion, and repeat Pap/HPV testing after 12 months, respectively. In case of anxiety or other reasons, women could be referred to immediate colposcopy. Nine women out of the total cohort were referred to colposcopy within 1–3 months after the primary screening result.

Any positive Pap cytology and/or HPV test result during the follow-up period triggered referral to central colposcopy [16].

### *Colposcopy and biopsy sampling*

Colposcopists classified the type of transformation zones according to the Barcelona nomenclature of the International Federation for Cervical Pathology and Colposcopy (IFCPC) [18]. In case of type 1 or type 2 transformation zone with visible squamous columnar junction (SCJ), colposcopy was regarded satisfactory. Any visible lesion underwent histological assessments with punch biopsies. No random punch biopsies were taken in case of normal colposcopy findings. In case of type 3 transformation zones colposcopy with not fully visible SCJ was regarded as unsatisfactory and endocervical curettage (ECC) was obligatory. Type 3 transformation zones with visible lesions underwent punch biopsies and ECC.

### *HPV DNA testing*

Samples for HPV testing were collected during the initial screening visit using the digene Cervical Sampler (Qiagen, Hilden, Germany). HPV testing was performed at a central clinical laboratory (Wolfsburg) using the HC2 High-Risk HPV DNA Test (Qiagen). The standard threshold value of 1 RLU was used to define a positive HC2 HPV test result.

### *p16/Ki-67 Dual-stained cytology*

Residual cellular material was used from the ThinPrep® vial of the initial screening visit that was previously used for Pap cytology testing. An additional slide was prepared for each case using the T2000 processor (Hologic) and subsequently subjected to p16/Ki-67 dual-staining using the CINtec® PLUS Kit (REF 9531, mtm laboratories, Heidelberg, Germany) according to the instructions of the manufacturer (details described in Ref. [15]).

All cases were evaluated by a trained cytotechnologist with respect to their minimum squamous cellularity criteria as defined in Ref. [17], and subsequently reviewed for the presence of double-immunoreactive cervical epithelial cells. Slides simultaneously showing one or more cervical epithelial cells with brown cytoplasmic p16 immunostaining and red nuclear Ki-67 immunostaining were interpreted as a positive test result for dual-stained cytology, independent from morphology interpretation. Fig. 1 shows an example of dual-stain positive cells. Cases without any double-immunoreactive cell were considered finally negative, whereas cases with dual-stain positive cell(s) were subjected to an additional pathologist (DS) review to confirm the positive test result.

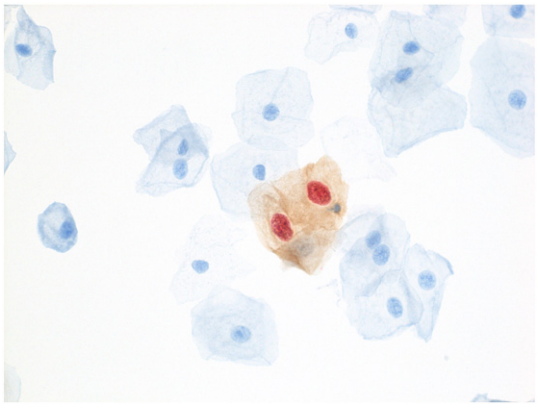

**Fig. 1.** p16/Ki-67 Dual-stained cytology example. The presence of one or more cervical epithelial cell(s) simultaneously showing p16 over-expression (brown cytoplasmic immunostain) and Ki-67 expression (red nuclear immunostain; superimposing the brown nuclear p16 stain) defines a positive test result for this combinatory biomarker test when used on cervical cytology preparations, independent from morphology interpretation.

*Histologic Gold standard*

Cervical biopsies collected during central colposcopy procedures performed at the Wolfsburg clinic were subjected to local pathologist review to establish a clinical diagnosis for patient management. For study purposes, a central pathology review was performed for all study biopsies. Majority consensus diagnoses were established on all available H&E stained cervical tissue specimen(s) by a group of independent histopathology reviewers (CB, FK, TL, JO, and SR) who were blinded to all other study results.

## Results

*Study population characteristics*

A total of 425 women aged 30 and older who were enrolled into the Wolfsburg Pap/HPV co-testing screening project during 2007/2008 and have been tested negative for Pap cytology, but positive for high-risk HPV were available for evaluation of the p16/Ki-67 dual-stained cytology testing. Median age was 41.6 years (range 30 to 87). For a total of 147 women, colposcopy follow-up results were available at the time of data analysis for this study, with biopsy results (n = 132) for the vast majority of these cases. The average number of biopsies collected for these 132 subjects was 1.6, with additional endocervical curettage specimens obtained in 58 of the cases. Mean follow-up time for women with colposcopy follow-up was 13.8 months (ranging from 1 to 27 months), starting from the date of the index screening visit. Follow-up compliance rate in the analyzed cohort was 85%. In 64 out of 425 cases there was no follow up screening visit recorded at the time when the database was frozen for the analysis presented in this report.

*Positivity rate, sensitivity and specificity estimates for p16/Ki-67 Dual-stained cytology in Pap Negative/HPV positive women*

p16/Ki-67 Dual-stained cytology testing performed from residual cellular material out of the liquid-based cytology vial collected at the time of the initial screening visit was found to be positive in 108 out of 425 (25.4%) cases (Fig. 2). Within the group of positive dual-stained cytology results, there were 34 cases of biopsy-confirmed CIN2+ during available follow-up, thereof 27 CIN3+ cases. In contrast, there were only 3 cases of biopsy-confirmed CIN2+ (i.e. 2 cases of CIN2 and 1 CIN3 case) within the Pap negative/HPV positive group that was tested negative for the presence of p16/Ki-67 dual-stained cells at baseline (Fig. 2), whereas the vast majority of cases (314 out of 317) which were tested negative for dual-stained cytology did not show biopsy-proven CIN2+ during preliminary follow-up. Thus, the estimated sensitivity of p16/Ki-67 dual-stained cytology testing for underlying CIN2+ was 34 out of 37 CIN2+ cases (91.9%; 95% CI 78.1–98.3%), and 27 out of 28 CIN3+ cases (96.4%; 95% CI 81.7–99.9%) within the cohort of Pap negative/HPV positive women during short term follow-up (Table 1).

High sensitivity rates were associated with high levels of specificity for dual-stained cytology testing. Within the group of 132 cases with biopsy results available, 51 out of 132 (38.6%) cases were positive for p16/Ki-67 dual-stained cytology. Specificity rates when limiting the analysis to those 132 cases with biopsy results only were determined at 82.1% (95% CI 72.9-89.2%) at the CIN2+ threshold (78 true negative dual-stained cytology results for 95 cases diagnosed as Negative for dysplasia or CIN1 on biopsy), and at 76.9% (95% CI 67.6–84.6%) for CIN3+ (80 true-negative cases out of 104 disease negative using the CIN3 threshold) (Table 1).

## Discussion

This is the first study to evaluate the performance of a novel dual-stained cytology concept that simultaneously detects the expression of the p16 and Ki-67 biomarkers in cervical cytology specimens, as a tool to triage women with Pap negative/HPV positive primary cervical cancer screening results to colposcopy. The results of this retrospective analysis performed on prospectively collected specimens indicate a high level of sensitivity and specificity of the p16/Ki-67 dual-stained cytology approach for identifying the subgroup of Pap negative/HPV positive women that has the highest risk for underlying high-grade precancerous disease and therefore would benefit from immediate referral to colposcopy.

HPV testing is known to significantly increase the sensitivity for the detection of CIN2+ or CIN3+ when used as an adjunctive test to Pap cytology based screening or as a primary screening method [1,2,19–22]. This was also recently confirmed by preliminary analyses

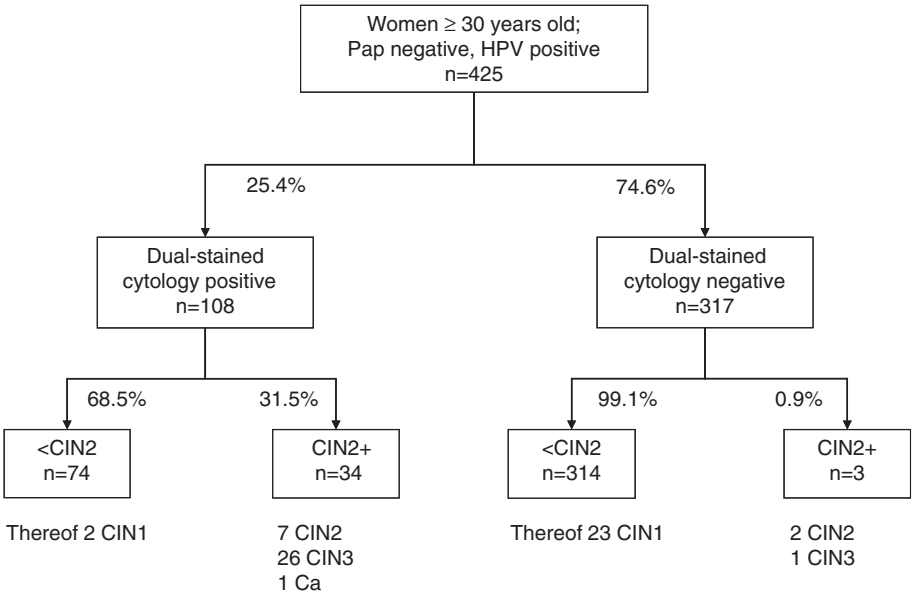


**Fig. 2.** Triage of Pap negative, HPV positive primary screening test results with p16/Ki-67 Dual-stained cytology. Flow chart summarizing the distribution of dual-stained cytology results within the group of Pap negative/HPV positive women correlated to high-grade CIN (CIN2+) detection based on preliminary available follow-up. CIN, cervical intraepithelial neoplasia; Ca, cervical carcinoma.

**Table 1**
Sensitivity and specificity of p16/Ki-67 dual-stained cytology in Pap negative/HPV positive women aged 30 years or older. Results are provided for all 132 cases with biopsy results available. HR-HPV, high-risk human papillomavirus; CIN2+ (CIN3+), cervical intraepithelial neoplasia of grade 2 (3) or higher; 95% CI, 95% confidence interval. #, number of true negatives (n) out of all disease negatives (N).

| CIN2+ | | | | CIN3+ | | | |
|---|---|---|---|---|---|---|---|
| Sensitivity | | Specificity | | Sensitivity | | Specificity | |
| n/N | % (95% CI) | n/N# | % (95% CI) | n/N | % (95% CI) | n/N# | % (95% CI) |
| 34/37 | 91.9 (78.1–98.3) | 78/95 | 82.1 (72.9–89.2) | 27/28 | 96.4 (81.7–99.9) | 80/104 | 76.9 (67.6–84.6) |

from the ongoing Wolfsburg pilot project [16]. However, without triaging HPV positive participants referral rates to colposcopy will be very high (>6% for the Wolfsburg project in women aged 30 years or older [16]). The standard follow-up algorithm for women with normal Pap cytology, but positive HPV test results which is based on repeat Pap cytology and/or HPV testing within 6–12 months after the index screening test results, proved to be unsatisfactory. In the Wolfsburg project, repeat Pap cytology testing after 6 months detected only a minority of underlying CIN3+ lesions, and 28% of all CIN3+ lesions in this project were finally diagnosed only because of HPV persistency, including 3 cases of invasive cervical cancer [16]. On the other hand, the observed spontaneous regression rate of HPV in this project was much lower than expected (46.1% regression after 12 months, vs. an originally expected 60% regression rate for HPV infection). Therefore the implemented management of repeat Pap and/or HPV testing was less efficient in reducing referrals to colposcopy than anticipated [16].

Triaging Pap negative/HPV positive women by dual-stained cytology testing in this study showed promising results. A delayed diagnosis of CIN2+ and even more importantly of CIN3+ would have been avoided in more than 90% of cases while referral rates to colposcopy could have been reduced by 75% compared to a scenario where all women with Pap negative/HPV positive test results would have been referred to immediate colposcopy. These findings are in good agreement with the results from a recent assessment of the potential use of p16 immuno-cytochemical staining in the triage of positive HPV test results in a primary screening trial in Italy which compared sensitivity levels and colposcopy referral rates of Pap cytology to those determined for primary HPV testing with subsequent triage of positive results by p16 cytology [23]. Similar to the study results presented in this manuscript for the dual-stained cytology, Carozzi and co-workers were able to show a minimal impact on the sensitivity for the combined HPV/p16 cytology triage approach versus a primary HPV screening approach without triage. However, referral rates to colposcopy that more than doubled for HPV testing over Pap cytology testing were found similar to those for Pap cytology [23]. The dual-stained cytology testing as used for the first time in this study for the triage of Pap negative/HPV positive screening results confirms the potential of this combined biomarker concept to further improve specificity over the previously reported p16 single-stained cytology approach, which still required morphology interpretation of immuno-reactive cells. A similar effect has been observed in a most recent study which assessed the sensitivity/specificity profiles of p16/Ki-67 dual-stained cytology in identifying women with underlying high-grade CIN in equivocal (atypical squamous cells of undetermined significance, ASC-US) or mildly abnormal (low-grade squamous intraepithelial lesion, LSIL) Pap cytology results and compared these results to an earlier performance assessment of p16 single-stained cytology testing performed on the same cohort [15,24,25].

Dual-stained cytology was performed from residual cellular material out of the liquid-based cytology vial collected at the initial screening visit [16]. Thus, this study evaluates the diagnostic performance of dual-stained cytology testing for the triage of HPV positives in a true reflex testing situation, whereas in the previous investigation in a comparable clinical setting p16 cytology testing was performed from a cervical sample collected during the colposcopy follow-up visit [23]. A further strength of the study is the use of fully adjudicated histologic diagnoses established on H&E-stained slides from cervical biopsy specimens obtained during colposcopy follow-up as the reference standard for study purposes.

Based on its design, a weakness of this sub-study evaluating dual-stained cytology testing is the lack of a complete disease ascertainment on all women with Pap negative/HPV positive screening test results, which may lead to an under- or overestimation of the exact performance of dual-stained cytology testing in the triage of these screening test results. This in part is an intrinsic limitation owed to the fact that the evaluations are performed on a cohort of women participating in a pilot project which includes active patient management as part of the health care service provided, versus a situation where the analyses are conducted in a true clinical study situation. Furthermore, as the collection of further follow-up data for the women with Pap negative/HPV positive results is ongoing, no assessment of the longer-term predictive value of dual-stained cytology testing can be made at this point in time.

To summarize, the results of this clinical sub-study have shown that the use of p16/Ki-67 dual-stained cytology when used as a reflex test may identify the vast majority (>90%) of underlying high-grade CIN disease in women tested Pap negative/HPV positive, while reducing the number of colposcopies towards a level of approximately 25% compared to a situation where all women aged 30 years or older would undergo colposcopy to immediately identify existing high-grade disease. Thus, dual-stained cytology testing may reduce the lead time delay for diagnosing clinically relevant cervical lesions significantly. If confirmed in further studies, dual-stained cytology appears to be a highly efficient complement of HPV-based screening programs for the triage of HPV positive participants.

**Conflict of interest statement**
KUP, DS, CB, JO and SR have been occasional clinical advisors to mtm laboratories in the past. CB and DS have been on the speaker's bureau of mtm laboratories. RR is an employee of mtm laboratories and discloses a financial interest in the company.

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