Received: 10 August 2023 | Accepted: 15 November 2023

DOI: 10.1002/jmv.29271

RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

p16/Ki67 dual stain triage versus cytology in primary human papillomavirus-based cervical cancer screening with limited genotyping

Martyna Trzeszcz[1,2] | Maciej Mazurec[1] | Robert Jach[3] | Karolina Mazurec[1] | Izabela Kotkowska-Szeps[1] | Magdalena Kania[1] | Mariola Wantuchowicz[1] | Jolanta Wasowska[1] | Monika Duczek-Polakiewicz[1] | Patrycja Rozmus[1] | Joanna Streb[4] | Agnieszka Halon[5]

[1]Corfamed Woman's Health Center, Wroclaw, Poland

[2]Division of Pathology and Clinical Cytology, University Hospital in Wroclaw, Wroclaw, Poland

[3]Division of Gynecologic Endocrinology, Jagiellonian University Medical College, Krakow, Poland

[4]Department of Oncology, Jagiellonian University Medical College, Krakow, Poland

[5]Department of Clinical and Experimental Pathology, Division of Clinical Pathology, Wroclaw Medical University, Wroclaw, Poland

Correspondence

Martyna Trzeszcz, Division of Pathology and Clinical Cytology, University Hospital in Wroclaw, Borowska 213, Wroclaw 50-556, Poland.

Email: m.trzeszcz@corfamed.pl

Abstract

The introduction of primary human papillomavirus (HPV) cervical cancer screening requires the implementation of an appropriate triage strategy that will be effective in detecting high-grade cervical disease without losing diagnostic specificity. From the 30.066 screening tests results, a total of 1086 with available high-risk human papillomavirus (HRHPV) with limited genotyping, cytology, and p16/Ki67 dual-stain were selected. Two triage strategies for primary HPV screening were analyzed retrospectively based on the study group. Performance characteristics for p16/Ki67 and cytology triage in the detection of cervical intraepithelial neoplasia grade 2 or worse (CIN2+) and grade 3 or worse (CIN3+) were calculated, detected in colposcopic biopsy. In HPV16/18-positive cases, primary HPV with p16/Ki67 triage was significantly more specific than cytology (53.1%/16.8% for CIN2+; $p < 0.0001$; 45.9%/17.0% for CIN3+; $p < 0.0001$), with yielded sensitivity (95.7%/84.8% for CIN2+; $p = 0.0955$; 100.0%/87.5% for CIN3+; $p = 0.0832$). In other HRHPV-positive cases (N16/N18), p16/Ki67 triage was also significantly higher specific (51.3%/15.3% for CIN2+; $p < 0.0001$; 44.5%/16.5% for CIN3+; $p < 0.0001$), with sensitivity (92.3%/74.4% for CIN2+; $p = 0.0522$; 90.9%/81.8% for CIN3+; $p = 0.5637$). Diagnostic predictive values were significantly higher for p16/Ki67 triage with the highest PPV in HPV16/18-positive cases for CIN2+ (45.4%; 95% confidence interval [CI]: 35.2–55.8; $p < 0.0001$) and very high NPV in all HPV-positive cases regardless of detected genotype (96.3%–100.0%). The risk (1-NPV) for CIN3+ in HRHPV16/18-positive/p16/Ki67-negative women was 0.0%. Superior diagnostic performance compared to cytology for detecting cervical cancer precursors indicates that p16/Ki67 dual-immunostain may be a highly effective tool of triage in primary HPV

screening with limited HPV 16/18 genotyping in secondary cervical cancer prevention.

KEYWORDS
cancer biomarkers, cervical cancer screening, CINtec PLUS, genotyping, high-risk HPV, p16/Ki67 dual-stain, triage

1 | INTRODUCTION

The basic paradigm of cervical cancer secondary prevention has changed, and globally primary human papillomavirus (HPV)-based screening is successively replacing cytology-based screening.[1–4] Primary HPV screening strategy is the recommended for all countries, regardless of resource settings.[5,6] This is due to higher sensitivity of HRHPV testing for the detection of cervical precancers compared to primary cytology-based screening, as well as better reproducibility and substantially lower subjectivity.[7,8] The World Health Organization (WHO) has initiated a global call to eliminate cervical cancer as a population burden, and its guidelines released in 2021 recommend using a primary HPV detection for cervical cancer screening.[6,9] However, the implementation of an effective triaging of patients with a positive high-risk human papillomavirus (HRHPV) test result remains an unresolved question and a challenge. Persistent infection of HRHPV types is the etiological factor of most cervical precancers leading to the development of cervical cancer. As it is not possible to differentiate between transient and persistent infection in women with HRHPV positive tests results, there is no doubt that primary HPV screening requires a triage test. Otherwise, too many patients would be referred for colposcopy.[10] Different triaging options have been proposed.[11,12] Triage testing should be assessed jointly with the primary screening testing, which is to ensure the safety and effectiveness of further clinical management.[11]

Dual immunocytochemical staining of cervical cytology specimens using p16 and Ki67 proteins is a morphologic-independent biomarker of a precancerous cervical lesions risk. The simultaneous immunoexpression of the p16 tumor supression protein together with the Ki67 antiproliferative marker present in one cell is an indicator of the cell cycle dysregulation that can lead to precancerous changes.[13–16] p16/Ki67 testing is characterized by high sensitivity and high specificity for the detection of CIN2+. p16/Ki67 has been proposed as secondary screening testing for minor cytological abnormalities,[15,17] for NILM HRHPV-positive cases[14,18] and for HRHPV-positive cases in primary HPV screening.[18–21] p16/Ki67 evaluation by a qualified pathologist has been approved by the Food and Drug Administration (FDA) as a triage method for HRHPV-positive N16/N18 cases in primary HPV screening and for HRHPV-positive N16/N18 NILM women who undergone cotesting.[22] The Polish Interim cervical cancer screening guidelines have recommended a wider p16/Ki67 usage during the SARS-CoV-2 pandemic.[4] Recently, it has also been studied for use in self-sampling,[23] which may open up new possibilities in the management of abnormal screening test results in non-responders in cervical cancer systems of prevention.

The scientific evidence for the use of p16/Ki67 as a triage in HRHPV-positive women undergoing primary HPV screening is limited, and after limited 16/18 genotyping there is very insufficient. Due to the need to increase the specificity resulting with the better identification of risk groups and improving of the effectiveness of cervical cancer precursors detection in primary HPV screening, we investigated whether primary HPV screening with incorporating p16/Ki67 triage of HPV-positive cases may be an alternative screening strategy for the commonly used cytologic triage. For this purpose, we conducted a retrospective analysis of cytological, virological and immunocytochemical results with histologic correlation, and assessment of the diagnostic performance of the two triage approaches for high-grade squamous intraepithelial lesion (HSIL) detection at the CIN2+ and CIN3+ thresholds (HSIL/CIN2+, HSIL/CIN3+) in primary HPV screening.

2 | MATERIAL AND METHODS

2.1 | Study design and participants

This is another study in the series, the methodology was described in detail previously.[19,24] This retrospective analysis concerns the results of patients participating in private funds-based opportunistic cervical cancer screening (August 2015–July 2020). All analyzed data come from the electronic registry one of the largest private-based outpatient gynecologic clinics in Lower Silesia in Poland, Corfamed Woman's Health Center (Center). A total of 30.066 screening tests results were analyzed, including 20.605 liquid-based cytologies, 8.331 HPV tests and 1.130 of p16/Ki67 immunostains. It was the initial study group, from which, in the first phase, patients with the performed HRHPV and LBC tests were selected (n = 8331), in the second phase with the additionally performed p16/Ki67 tests (n = 1086), and in the third phase a pre-final group with available histopathology (n = 375). In the prefinal group with colposcopic biopsy results, patients with positive HRHPV results were then selected (n = 352), and it was the final study group. Histopathological diagnoses at the HSIL/CIN2+ and HSIL/CIN3+ thresholds were clinical endpoints of the study. The final group has been retrospectively analyzed along with the diagnostic performance assessment for the primary HPV screening model, with two different triaging approaches, as follows: (1) p16/Ki67 dual-stain testing; (2) cytology. The study population was divided into three age groups (<25, 25–65, and

>65 years of age) presented in Table 1. The group of HRHPV-positive patients was divided into two subgroups depending on HRHPV type detected: 16/18 or N16/N18. The primary exclusion criteria were hysterectomy, pregnancy, history of treatment for cervical intraepithelial lesion or cancer, current cancer, missing data, or colposcopy performed outside the Center. The ethics committee approval (ID: 118.6120.36.2023).

2.2 | HRHPV detection and genotyping

Limited HPV genotyping was performed using the qualitative automated in vitro PCR Abbott RealTime High Risk HPV assay (Abbott Molecular) according to the manufacturer's instructions. The assay was designed to detect 14 HRHPV DNA types with parallel directed HPV 16 and/or 18 genotyping (HPV 16/18), and pooled phenotyping of the remaining 12 non-16 and -18 HRHPV genotypes (HRHPV N16/N18). HPV16/18-positive result was classified as detecting HPV DNA 16 or 18, or both. HRHPV N16/N18 positive was classified as detecting one or more of HRHPV N16/N18 genotypes. To amplify HPV DNA a primer combination consisting of three forward and two reverse primers targeting a conserved L1 region is used, which is also a sequence identity for the HPV classification (family *Papillomaviridae*, subfamily *Firstpapillomavirinae*, genus *Alphapapillomavirus*, species *Alphapapillomavirus 9*).[25]

2.3 | Liquid-based screening (LBS)

The cervical samples were collected from all patients once on the SurePath medium (Becton Dickinson) in a typical manner, using Cervex-Brush device (Rovers Medical Devices) according to the manufacturer's procedure, and were the basis for performing all screening tests (LBC, HRHPV, p16/Ki67 testing). Residual cytological material from all cervical samples was stored for 3 months by laboratory in conditions specified by the manufacturer.

2.4 | Liquid-based cytology

All liquid-based SurePath cytology samples were processed in the automatic PrepStain Slide system (Becton Dickinson) according to manufacturer's instructions. The cytology slides were reported by a gynecological cytopathologist according to the Bethesda 2014 system. The quality assesment and control procedures for gynecological cytopathology were based on benchmarks published by US laboratories accredited by the College of American Pathologists, and reporting rates in the study were within normal ranges reported.[24] Rescreening was amended to include all NILM HPV-positive cases. Abnormal cytology was defined as atypical squamous cells of undetermined significance (ASC-US) or worse (ASC-US+).

2.5 | p16/Ki67 dual immunostaining

Dual immunocytochemical staining of cervical samples were processed in the automatic BenchMark XT laboratory system (Ventana Medical Systems Inc.), using p16 and Ki67 proteins in CINtec PLUS detection kit (Roche, MTM AG laboratories) according to the manufacturer's protocol. A control specimen was present in each run. p16/Ki67 testing was performed from the same sample as cytology was, using residual cellular material stored in laboratory in the original SurePath vials. An immunoprofile evaluation was done by a qualified gynecological cytopathologist, who was blinded to cytology results. p16/Ki67 slides were classified as positive, negative, or unsatisfactory. A positive result was the presence of at least one cell in the slide meeting the following criteria: simultaneous red nuclear stain for Ki67 and brown cytoplasmatic stain for p16 in the same epithelial cell. In the case of the immunoexpression assessment of the cell group, the positive diagnosis was determined by a strong diffuse p16 stain and the presence of at least one cell with nuclear Ki67 staining and cytoplasmic p16 staining seen on the periphery of the cell group or sheet. If no immunostaining or single staining of p16 or Ki67 was noted within the epithelial cells, the slide was classified as negative. p16/Ki67 testing was not performed in cellular residual pellets, in which previously obtained cytology was diagnosed as inadequate.

2.6 | Colposcopy and histology

All HRHPV-positive patients with positive p16/Ki67 test result or with abnormal cytology were referred for colposcopy. The management of abnormal screening test results was based on the Polish

TABLE 1 Four-level selection of the final study group.

	<25 years, no.	25–65 years, no.	>65 years, no.
Total (HRHPV + LBC)	413	7685	233
Subtotal (HRHPV + LBC + DS)	109	961	16
Pre-final (HRHPV + LBC + DS + HP)	26	348	1
Final (HRHPV (+) + LBC + DS + HP)	24	327	1

Abbreviations: +, positive; DS, p16/Ki67 dual staining test; HP, histology; HRHPV, 14 high-risk types human papillomavirus test; LBC, liquid-based cytology.

guidelines, with the extension to American Society for Colposcopy and Cervical Pathology 2012 and 2015 guidelines for cases not covered by the Polish guidelines.[26–28] The extended colposcopic protocol used included the endocervical sampling using endocervex brushing and curettage in all cases, targeted biopsy when any abnormal colposcopic findings were found, and a random biopsy from four quadrants in the absence of any abnormal cervical lesions and visualization in the relevant quadrant the new squamocolumnar junction and major screening abnormalities present. The number of sampled biopsies ranged from 1 to 5. The International Federation of Cervical Pathology and Colposcopy 2011 nomenclature was used in colposcopic protocols. The Centre's colposcopists participate in a nationwide Colposcopy 2020 Project for i.a. colposcopy procedure standardization. The LAST 2012/WHO 2014 terminology was used for reporting histologic diagnoses and reviewed by a gynecological pathologist.

2.7 | Statistical analysis

The PQStat Software in a 1.6.0 full version (2015 PQStat Statistical Calculation Software) was used for the statistical analysis. A diagnostic value of analyzed screening approaches including primary screening test with secondary test, measured with sensitivity, specificity, positive (PPV) and negative predictive values (NPV), positive (PLR) negative (NLR) likelihood ratios, was calculated according with standard definitions. Histologic results HSIL/CIN2+ and HSIL/CIN3+ were the cut-off points. Additionally, the positivity rate with a normal approximation method used was calculated. Specificity and sensitivity for p16/Ki67 and cytology triage were compared using the McNemar's chi-square test. For comparison of PPV and NPV a method developed by Leisenring et al.[29] was applied using DTComPair package in R. Differences in diagnostic value between the analyzed triage approaches were evaluated with exact p-values, where $p < 0.05$ was considered as a significant.

3 | RESULTS

3.1 | Study participants and characteristics

The initial study group included patients with three screening tests performed: HRHPV, LBC, and p16/Ki67. Table 2 shows the number of patients with positive test results in three groups: HRHPV-positive, HRHPV-positive with p16/Ki67-positive, and HRHPV-positive with abnormal LBC. The total number of patients with three tests (HRHPV, LBC, and p16/Ki67) was 1086 of which 878 cases were HRHPV-positive, HRHPV-positive/p16/Ki67-positive were 296 cases, and HRHPV-positive with abnormal LBC were 488 cases. 45% ($n = 159/352$) of women referred for colposcopy were HPV 16/18-positive, whereas patients with one or more positive type of HRHPV N16/N18 were 55% ($n = 193/352$). Table 2 also presents the age parameters of the patients included in the study for the indicated groups: mean age with standard deviation (SD), median age, the age of the youngest and oldest patient. For the initial study group, the following parameters were achieved: mean age 36.1 (SD 10.5), median 34.

TABLE 2 Age characteristics of total and HRHPV-positive groups (with p16/Ki67-positive or LBC-positive results).

	Total, no.	HRHPV +ve, no.	HRHPV+ve DS+ve, no.	HRHPV+ve LBC+ve, no.
Total	1086	878	296	488
Mean (SD)	36.1 (10.5)	35.1 (10.0)	34.3 (8.8)	34.7 (9.7)
Median	34	33	32	33
Min, max	18, 77	18, 75	20, 70	18, 70
25–65 years	961	771	266	419
Mean (SD)	37.0 (9.1)	36.3 (8.9)	35.2 (8.2)	36.0 (8.4)
Median	35	34	33	34
Min, max	25, 65	25, 65	25, 65	25, 65
<25 years	109	98	28	63
Mean (SD)	22.4 (1.5)	22.4 (1.5)	22.9 (1.1)	22.3 (1.6)
Median	23	23	23	23
Min, max	18, 24	18, 24	20, 24	18, 24
>65 years	16	9	2	6
Mean (SD)	69.2 (3.6)	68.2 (2.9)	69.5 (0.7)	67.8 (1.5)
Median	67.5	67	69,5	67,5
Min, max	66, 77	66, 75	69, 70	66, 70

Abbreviations: +ve, positive; DS, p16/Ki67 dual staining test; HRHPV, 14 high-risk types human papillomavirus test; LBC, liquid-based cytology; LBC+ve, ASC-US+, ASC-US or worse; max, maximum; min, minimum; SD, standard deviation.

3.2 | Combining primary HPV with limited genotyping with p16/Ki67 versus cytology in age stratification

In the initial group, there were 878 of HRHPV-positive cases, of which HPV 16/18 was positive in 305 cases, including 134 positive p16/Ki67 cases, and 573 positive HRHPV N16N18 cases, including 162 p16/Ki67 positive cases (Table 3). Table 4 shows the results of cytological diagnoses for three age groups (<25, 25–65, and >65 years old) including the size of each group.

The correlation between the cytological diagnosis (NILM, ASC-US, LSIL, ASC-H, HSIL) with the HRHPV status (HPV–, HPV+) and the p16/Ki67 result (DS+, DS–) is presented in Table 5. The highest percentage of the DS+ result (from 80% to 100%) was found for HSIL and ASC-H diagnoses, regardless of the HRHPV status. The lowest for NILM, ASC-US, and LSIL diagnoses (all with negative HRHPV status): 5.5%, 13.1%, and 11.9%, respectively.

TABLE 3 Age-stratified p16/Ki67 test reporting rates with HRHPV types distribution.

Age group, Years	HPV t. 16/18+ve, no.		HRHPV t. N16/N18+ve, no.	
	DS+ve	DS−ve	DS+ve	DS−ve
<25	14	22	14	48
25–29	35	32	34	78
30–39	61	67	69	178
40–49	16	33	35	66
50–59	2	12	4	23
60–65	4	3	6	13
>65	2	2	0	5
Total	134	171	162	411

Abbreviations: +ve, positive; −ve, negative; DS, p16/Ki67 dual staining test; HPV t. 16/18, human papillomavirus types 16 and/or 18; HRHPV t. N16/N18, human papillomavirus 12 high-risk types other than types 16/18.

3.3 | Detection of HSIL/CIN2+ and HSIL/CIN3+ in colposcopic biopsy performed in p16/Ki67 versus cytology triage in women with different HRHPV positive status

In the final study group of 352 HRHPV-positive women, 35 HSIL/CIN3+ histology results were found. Table 6 presents histology results for HPV16/18-positive and HRHPV N16/N18-positive results in relation to the dual-stain results and cytological diagnoses (ASC-US+ or NILM). In the dual-stain group 24 HPV16/18-positive p16/Ki67-positive cases were found, 10 HRHPV N16/N18-positive p16/Ki67-positive cases and 1 case of HRHPV N16/N18-positive p16/Ki67-negative was noted. In cytology group were 21 HPV16/18-positive ASC-US+ and three NILM cases, nine HRHPV N16/N18-positive ASC-US+ and two NILM cases. A total of 34 cases were p16/Ki67-positive in the dual-stain group, and 30 cases in the cytology group were ASC-US+ in detection HSIL/CIN3+.

3.4 | The clinical performance for p16/Ki67 and cytology triage in HPV-positive patients

Table 7 presents the clinical performance for p16/Ki67-positive results and ASC-US+ results among HRHPV-positive women for detection HSIL/CIN2+ and HSIL/CIN3+. The highest sensitivity of 100.0% (95% confidence interval [CI]: 85.8–NA) was demonstrated for HPV 16/18-positive p16/Ki67-positive cases to detect HSIL/CIN3+, and the lowest was 74.4% (95% CI: 57.9–87.0) for HRHPV N16/N18-positive ASC-US+ cases to detect HSIL/CIN2+. The highest specificity of 53.1% (95% CI: 43.5–62.6) was demonstrated for HPV 16/18-positive p16/Ki67-positive cases to detect HSIL/CIN2+, and the lowest 14.3% (95% CI: 9.2–20.8), as was the sensitivity, for HRHPV N16/N18-positive ASC-US+ cases to detect HSIL/CIN2+. The results of a complete comparison of two triaging strategies of HRHPV-positive women are shown in Table 8. Triage of HRHPV-positive patients with p16/Ki67 was statistically significantly more specific in all HRHPV-positivity compared to cytology, including HPV 16/18-positive (53.1% vs. 16.8%, $p < 0.0001$ for HSIL/CIN2+; 45.9% vs. 17.0%; $p < 0.0001$ for HSIL/CIN3+), and HRHPV N16/N18 patients (51.3% vs. 14.3%, $p < 0.0001$ for HSIL/CIN2+; 44.5% vs. 16.5%, $p < 0.0001$ for HSIL/CIN3+). Also, PPV was

TABLE 4 Age-stratified LBC reporting rates in the study group with relevant HRHPV and p16/Ki67 results.

	HPV t. 16/18+ve, no.		HRHPV t. N16/N18+ve, no.	
Age group <25 years				
LBC result	DS+ve	DS−ve	DS+ve	DS−ve
NILM	2	5	9	19
ASC-US	5	10	2	6
LSIL	5	7	3	22
ASC-H	2	0	0	0
AGC	0	0	0	0
HSIL	0	0	0	1
Total	14	22	14	48
Age group 25–65 years				
LBC result	DS+ve	DS−ve	DS+ve	DS−ve
NILM	25	70	40	217
ASC-US	27	51	44	58
LSIL	29	24	50	80
ASC-H	11	2	9	1
AGC	3	0	0	0
HSIL	23	0	5	2
Total	118	147	148	358
Age group >65 years				
LBC result	DS+ve	DS−ve	DS+ve	DS−ve
NILM	0	1	0	2
ASC-US	0	0	0	3
LSIL	2	1	0	0
ASC-H	0	0	0	0
AGC	0	0	0	0
HSIL	0	0	0	0
Total	2	2	0	5

Abbreviations: +ve, positive; −ve, negative; AGC, atypical glandular cells; ASC-H, atypical squamous cells-cannot excluded HSIL; ASC-US, atypical squamous cells of undetermined significance; DS, p16/Ki67 dual staining test; HPV t. 16/18, human papillomavirus types 16 and/or 18; HRHPV t. N16/N18, human papillomavirus 12 high-risk types other than types 16/18; HSIL, high-grade squamous intraepithelial lesion; LBC, liquid-based cytology; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy.

TABLE 5 p16/Ki67 dual staining reporting rates with referring combinations of LBC and HRHPV results.

LBC/HPV result	Total, no.	DS+ve, no. (%)[a]	DS−ve, no. (%)[a]
NILM/HPV−ve	55	3 (5.5)	52 (94.6)
NILM/HPV+ve	390	76 (19.5)	314 (80.5)
ASC-US/HPV−ve	99	13 (13.1)	86 (86.9)
ASC-US/HPV+ve	206	78 (37.9)	128 (62.1)
LSIL/HPV−ve	42	5 (11.9)	37 (88.1)
LSIL/HPV+ve	223	89 (39.9)	134 (60.1)
ASC-H/HPV−ve	5	4 (80.0)	1 (20.0)
ASC-H/HPV+ve	25	22 (88.0)	3 (12.0)
HSIL/HPV−ve	2	2 (100.0)	0 (0.0)
HSIL/HPV+ve	31	28 (90.3)	3 (9.7)
AGC/HPV−ve	5	2 (40.0)	3 (60.0)
AGC/HPV+ve	3	3 (100.0)	0 (0.0)
Total	1086 (100.0)	325 (29.9)	761 (70.1)

Abbreviations: +ve, positive; −ve, negative; ASC-H, atypical squamous cells-cannot excluded HSIL; AGC, atypical glandular cells; ASC-US, atypical squamous cells of undetermined significance; DS, p16/Ki67 dual staining test; HPV, HRHPV14, 14 high-risk types human papillomavirus test; HSIL, high-grade squamous intraepithelial lesion; LBC, liquid-based cytology; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy.

[a]% of total results for the LBC/HPV results in the first column.

TABLE 6 HRHPV status with p16/Ki67 or cytology results by histologic diagnoses in the final study group.

	Histology result, no. (%)[a]				
	Negative	LSIL/CIN1	HSIL/CIN2+	HSIL/CIN3+	Total
HPV t. 16/18+ve DS results					
Positive	28 (28.9)	25 (25.8)	20 (20.6)	24 (24.7)	97 (100.0)
Negative	37 (59.9)	23 (37.1)	2 (3.2)	0 (0.0)	62 (100.0)
Total	65 (40.9)	48 (30.2)	22 (13.8)	24 (15.1)	159 (100.0)
HRHPV t. N16/N18+ve DS results					
Positive	34 (30.6)	41 (36.9)	26 (23.4)	10 (9.0)	111 (100.0)
Negative	52 (63.4)	27 (32.9)	2 (2.4)	1 (1.2)	82 (100.0)
Total	86 (44.6)	68 (35.2)	28 (14.5)	11 (5.7)	193 (100.0)
HPV t. 16/18 + LBC results					
ASC-US+	48 (36.1)	46 (34.6)	18 (13.5)	21 (15.8)	133 (100.0)
NILM	17 (65.4)	2 (7.7)	4 (15.4)	3 (11.5)	26 (100.0)
Total	65 (40.9)	48 (30.2)	22 (13.8)	24 (15.1)	159 (100.0)
HRHPV t. N16/N18+ve LBC results					
ASC-US+	70 (43.5)	62 (38.5)	20 (12.4)	9 (5.6)	161 (100.0)
NILM	16 (50.0)	6 (18.8)	8 (25.0)	2 (6.3)	32 (100.0)
Total	86 (44.6)	68 (35.2)	28 (14.5)	11 (5.7)	193 (100.0)

Abbreviations: +ve, positive; ASC-US+, ASC-US or worse; DS, p16/Ki67 dual staining test; HPV t. 16/18, human papillomavirus types 16 and/or 18; HRHPV t. N16/N18, human papillomavirus 12 high-risk types other than types 16 18; HSIL/CIN2, histologic high-grade squamous intraepithelial lesion with a quantification of cervical intraepithelial neoplasia in grade 2 or worse; HSIL/CIN3+, histologic high-grade squamous intraepithelial lesion with a quantification of cervical intraepithelial neoplasia in grade 3 or worse; LBC, liquid-based cytology; LSIL/CIN1, histologic low-grade squamous intraepithelial lesion with a quantification of cervical intraepithelial neoplasia in grade 1; NILM, negative for intraepithelial lesion or malignancy.

[a]% of total results for the tests results in the first column.

statistically significantly higher in triage approach with p16/Ki67 incorporation when compared to cytology, simirarly in all HPV-positivity, in HPV 16/18 (45.4% vs. 29.3%, $p < 0.0001$ for HSIL/CIN2+; 24.7% vs. 15.8%, $p < 0.0001$ for HSIL/CIN3+) and in HRHPV N16/N18 cases (32.4% vs. 18.0%, $p < 0.0001$ for HSIL/CIN2+; 9.0% vs. 5.6%, $p = 0.0332$ for HSIL/CIN3+). Triage approach with p16/Ki67 test compared to cytology was statistically significantly higher in NPV for HPV 16/18-positive cases (96.8% vs. 73.1%, $p = 0.0129$) and for women with positive HRHPV N16/N18 status (96.3% vs. 68.8%, $p = 0.0027$), however, the significance was demonstrated for HSIL/CIN2+. Despite large differences between levels of sensitivity obtained, no statistical significancy for that parameter was noted compared to p16/Ki67 and cytology triage in HPV 16/18-postitve group for HSIL/CIN2+ (95.7% vs. 84.8%; $p = 0.0955$) and HSIL/CIN3+ tresholds (100.0% vs. 87.5%; $p = 0.0832$), respectively. Similarly, for HRHPV N16/N18-positive women, for HSIL/CIN2+ (92.3% vs. 74.4%; $p = 0.0522$) and HSIL/CIN3+ (90.9% vs. 81.8%; $p = 0.5637$), respectively, the statistical significance was not found.

4 | DISCUSSION

This is one of the few large studies evaluating diagnostic performance of p16/Ki67 dual immunostaining as a triage of HRHPV-positive patients in primary HPV screening with limited genotyping for HPV16/18. Triage with p16/Ki67 showed statistically significantly higher specificity than cytology at both analyzed thresholds, HSIL/CIN2+ (51.3%–53.1% vs. 14.3%–16.8%; $p < 0.0001$) and HSIL/CIN3+ (44.5%–45.9% vs. 16.5%–17.0%; $p < 0.0001$), regardless of the HRHPV type detected. In clinical practice, it would result in a reduction in number of colposcopy referrals in primary HPV-positive women much greater than cytology triage. Sensitivity of p16/Ki67 triage had higher levels than cytology, but in both HPV-positive groups no statistical significance was found, including HPV 16/18 for HSIL/CIN2+ (95.7% vs. 84.8% respectively; $p = 0.0955$), for HSIL/CIN3+ (100% vs. 87.5%; $p = 0.0832$), in HRHPV N16/N18-positive group, for HSIL/CIN2+ (92.3% vs. 74.4%; $p = 0.0522$) and for HSIL/CIN3+ treshold (90.9% vs. 81.8%; $p = 0.5637$), as well. Positive predictive value levels were statistically significantly higher for p16/Ki67 triage than for cytology, regardless of HRHPV type detected

TABLE 7 Clinical performance of dual immunostaining versus cytology triage in HPV 16/18-positive and HRHPV N16/N18-positive women to detect HSIL/CIN2+ and HSIL/CIN3+.

	Histology results			
	HSIL/CIN2+ DS+ve	HSIL/CIN3+	HSIL/CIN2+ ASC-US+	HSIL/CIN3+
Parameter	HPV t. 16/18+ve			
Sensitivity % (95% confidence interval [CI])	95.7 (85.2, 99.5)	100.0 (85.8, NA)	84.8 (71.1, 93.7)	87.5 (67.6, 97.3)
Specificity % (95% CI)	53.1 (43.5, 62.6)	45.9 (37.3, 54.7)	16.8 (10.4, 25.0)	17.0 (11.1, 24.5)
Prevalence % (95% CI)	28.9 (22.0, 36.6)	15.1 (9.9, 21.6)	28.9 (22.0, 36.6)	15.1 (9.9, 21.6)
PPV % (95% CI)	45.4 (35.2, 55.8)	24.7 (16.5, 34.5)	29.3 (21.8, 37.8)	15.8 (10.0, 23.1)
NPV % (95% CI)	96.8 (88.8, 99.6)	100.0 (94.2, NA)	73.1 (52.2, 88.4)	88.5 (69.9, 97.6)
1—NPV % (95% CI)	3.2 (0.4, 11.2)	0.0 (NA, 5.8)	26.9 (11.6, 47.8)	11.5 (2.4, 30.1)
PLR (95% CI)	2.04 (1.66, 2.51)	1.85 (1.58, 2.16)	1.02 (0.88, 1.18)	1.06 (0.89, 1.25)
NLR (95% CI)	0.08 (0.02, 0.32)	0 (NA, NA)	0.91 (0.41, 2.01)	0.74 (0.24, 2.25)
Positivity rate %	61.0		83.6	
	HPV t. N16/N18+ve			
Sensitivity % (95% CI)	92.3 (79.1, 98.4)	90.9 (58.7, 99.8)	74.4 (57.9, 87.0)	81.8 (48.2, 97.7)
Specificity % (95% CI)	51.3 (43.1, 59.4)	44.5 (37.2, 52.0)	14.3 (9.2, 20.8)	16.5 (11.4, 22.7)
Prevalence % (95% CI)	20.2 (14.8, 26.6)	5.7 (2.9, 10.0)	20.2 (14.8, 26.6)	5.7 (2.9, 10.0)
PPV % (95% CI)	32.4 (23.9, 42.0)	9.0 (4.4, 15.9)	18.0 (12.4, 24.8)	5.6 (2.6, 10.4)
NPV % (95% CI)	96.3 (89.7, 99.2)	98.8 (93.4, 99.9)	68.8 (50.0, 83.9)	93.8 (79.2, 99.2)
1—NPV % (95% CI)	3.7 (0.8, 10.3)	1.2 (0.1, 6.6)	31.2 (16.1, 50.0)	6.2 (0.8, 20.8)
PLR (95% CI)	1.90 (1.57, 2.28)	1.64 (1.31, 2.06)	0.87 (0.71, 1.06)	0.98 (0.74, 1.30)
NLR (95% CI)	0.15 (0.05, 0.45)	0.20 (0.03, 1.33)	1.80 (0.71, 1.06)	1.10 (0.30, 4.03)
Positivity rate %	57.5		83.4	

Abbreviations: +ve, positive; ASC-US+, ASC-US or worse; DS, p16/Ki67 dual staining test; HPV t. 16/18, human papillomavirus types 16 and 18; HRHPV t. N16/N18, human papillomavirus 12 high-risk types other than types 16/18; HSIL/CIN2+, histologic high-grade squamous intraepithelial lesion with a quantification of cervical intraepithelial neoplasia in grade 2 or worse; HSIL/CIN3+, histologic high-grade squamous intraepithelial lesion with a quantification of cervical intraepithelial neoplasia in grade 3 or worse; NA, not available; NLR, negative likelihood ratio; NPV, negative predictive value; PLR, positive likelihood ratio; PPV, positive predictive value.

(32.4%–45.4% vs. 18.0%–29.3% for HSIL/CIN2+, $p < 0.0001$); 9.0%–24.7% versus 5.6%–15.8% for HSIL/CIN3+, $p < 0.001$–0.0332). Negative predictive values of triaging with p16/Ki67 were very high (even 98.8%–100.0% for HSIL/CIN3+) suggesting high safety of HPV-positive patients with negative p16/Ki67, however statistical significance between immunostain triage strategy and cytology was found only for HSIL/CIN2+, but in both HPV groups, HRHPV 16/18-positive (96.8% vs. 73.1%; $p = 0.0129$, respectively) and HRHPV N16/N18-positive (96.3% vs. 68.8%; $p = 0.0027$, respectively). The values obtained in the study of diagnostic likelihood ratios, both positive and negative, indicate a greater advantage of the number of true diagnostic results over false ones when using p16/Ki67 triage (PLR: 1.90–2.04 for HSIL/CIN2+ and 1.64–1.85 for HSIL/CIN3+; NLR: 0.08–0.15 for HSIL/CIN2+ and 0.00–0.20 for HSIL/CIN3+) compared to cytology (PLR: 0.87–1.02 for HSIL/CIN2+ and 0.98–1.06 for HSIL/CIN3+; NLR: 0.91–1.80 for HSIL/CIN2+ and 0.74–1.10 for HSIL/CIN3+). Whilst the positivity rate of p16/Ki67 in HPV-positive women was significantly lower than cytology, regardless of the detected HRHPV group or genotype, that is, HPV 16/18 versus HRHPV N16/N18 (57.5%–61.0% vs. 83.4%–83.6%, respectively), which may benefit in referring fewer patients to colposcopy when p16/Ki67 is used.

We observed a very high consistency with data published by the FDA.[22] In that approval document for p16/Ki67 premarket usage, very similar results were obtained for most of diagnostic performance parameters in triaging of HPV-positive women with p16/Ki67, including very similar sensitivity and specificity levels, almost identical positive and predictive values, and positive likelihood ratio levels. The reason for such high similarity may be related to similar values of tests positivity rates for p16/Ki67 between FDA approval document (67.3% for HPV 16; 52.2% for HPV18; 45.8% for HRHPV N16/N18) and our analysis (61.0% for HPV 16/18; 57.5% for HRHPV

TABLE 8 Comparison of clinical performance of dual immunostaining versus cytology triage to detect HSIL/CIN2+ and HSIL/CIN3+ in HRHPV-positive women.

	Histology results					
	HSIL/CIN2+			HSIL/CIN3+		
	DS+ve	ASC-US+	*p*-Value	DS+ve	ASC-US+	*p*-Value
Parameter	HPV t. 16/18+ve					
Sensitivity % (95% confidence interval [CI])	95.7 (85.2, 99.5)	84.8 (71.1, 93.7)	0.0955	100.0 (85.8, NA)	87.5 (67.6, 97.3)	0.0832
Specificity % (95% CI)	53.1 (43.5, 62.6)	16.8 (10.4, 25.0)	<0.0001	45.9 (37.3, 54.7)	17.0 (11.1, 24.5)	<0.0001
PPV % (95% CI)	45.4 (35.2, 55.8)	29.3 (21.8, 37.8)	<0.0001	24.7 (16.5, 34.5)	15.8 (10.0, 23.1)	<0.0001
NPV % (95% CI)	96.8 (88.8, 99.6)	73.1 (52.2, 88.4)	0.0129	100.0 (94.2, NA)	88.5 (69.9, 97.6)	0.0744
	HPV t. N16/N18+ve					
Sensitivity % (95% CI)	92.3 (79.1, 98.4)	74.4 (57.9, 87.0)	0.0522	90.9 (58.7, 99.8)	81.8 (48.2, 97.7)	0.5637
Specificity % (95% CI)	51.3 (43.1, 59.4)	14.3 (9.2, 20.8)	<0.0001	44.5 (37.2, 52.0)	16.5 (11.4, 22.7)	<0.0001
PPV % (95% CI)	32.4 (23.9, 42.0)	18.0 (12.4, 24.8)	<0.0001	9.0 (4.4, 15.9)	5.6 (2.6, 10.4)	0.0332
NPV % (95% CI)	96.3 (89.7, 99.2)	68.8 (50.0, 83.9)	0.0027	98.8 (93.4, 99.9)	93.8 (79.2, 99.2)	0.263

Abbreviations: +ve, positive; ASC-US+, ASC-US or worse; DS, p16/Ki67 dual staining test; HPV t. 16/18, human papillomavirus types 16 and 18; HRHPV t. N16/N18, human papillomavirus 12 high-risk types other than types 16/18; HSIL/CIN2+, histologic high-grade squamous intraepithelial lesion with a quantification of cervical intraepithelial neoplasia in grade 2 or worse; HSIL/CIN3+, histologic high-grade squamous intraepithelial lesion with a quantification of cervical intraepithelial neoplasia in grade 3 or worse; NA, not available; NPV, negative predictive value; PPV, positive predictive value.

N16/N18). FDA approval document reported from approximately 10% to 20% higher sensitivity rates (depending on the taken CIN2+ or CIN3+ threshold and HRHPV positivity type) for p16/Ki67 than for cytology triage, similar to our results. The sensitivity of p16/Ki67 immunotesting triage higher than almost 20% compared to cytology as in our investigation (92.3%–95.7% vs. 74.4%–84.8% for CIN2+; 90.9%–100.0% vs. 81.8%–87.5% for CIN3+) were also noted by Wright et al.[20] and by Giorgi Rossi et al.[12] Our study results revealed a relatively low specifitity of triage with cytology (14.3%–16.8% for CIN2+; 16.5%–17% for CIN3+) compared with levels reported, 44.6% for CIN2+ and 42.9% for CIN3+ by Wentzensen et al.,[30] 76.6% for CIN2+ by Giorgi Rossi et al.,[12] or 75.0% for CIN3+ reported by Wright et al.[20] It must be noted that the specificity may have been understimated since the proportion of true negative cytology results in HPV-positive women with NILM cytology was relatively small due to limited indications for colposcopy in that group (especially for N16/N18-positive cases) and concurrently large number of false positive cytology rates in our population. Whilst it should be rememebered a good quality of a gynecological cyto-pathology in the study.[24]

Our analysis is one of the few large studies evaluating p16/Ki67 diagnostic performance as a triage in HRHPV-positive patients in primary HPV screening setting with limited genotyping for HPV16/18. The highest sensitivity rates of immunostaining triage was noted in women with HPV 16/18-positivity for HSIL/CIN3+ (100.0%) and for HSIL/CIN2+ (95.7%), compared to sensitivity estimates obtained in women with 12 other HRHPV types positivity (92.3% for HSIL/CIN2+; 90.9% for HSIL/CIN3+). For cytology triage the sensitivity in HPV 16/18-positive group is up to 10% higher than in HRHPV N16/N18-positive group (84.8% vs. 74.4% for HSIL/CIN2+; 87.5% vs. 81.8% for HSIL/CIN3+, respectively), regardless of the cut-off point taken. Positive predictive values ofp16/Ki67 triage in the group HPV 16/18-positive demonstrated levels up to 10-15% higher than for HRHPV N16/N18-positivity (45.4% vs. 32.4% for HSIL/CIN2+; 24.7% vs. 9.0% for HSIL/CIN3+, respectively), regardless of the cut-off point. Similar differences in PPV levels between HRHPV-positivity in p16/Ki67 triage have been reported by others for CIN2+ and CIN3+.[20,22] Our results highlight the higher effectiveness of p16/Ki67 immunotesting incorporated as a single triage tool in detecting cervical precancers than strategies where cytology is used in HPV-positive women who undergone primary HPV screening, regardless of the detected HRHPV type. Though, the highest detection efficiency was observed for p16/Ki67 triage in HPV 16/18-positive women. Moreover, a negative p16/Ki67 test result was associated with a high safety of women with a positive HRHPV and with the lowest of cervical precancers risk in the most oncogenic types of HRHPV, 16 and 18 (1-NPV: 0.00%–3.2%).

Based on high or very high negative predictive value levels, not lower than 96%, obtained in both cut-off points taken, our data confirm high safety of HRHPV-positive patients with a negative p16/Ki67 test performed as a triage test. Similar estimates were obtained by Wright et al.,[20] Wentzensen et al.[30] and Ebisch et al.,[31] with the rates ranging from 93.0% to 99.0%. Measured by the 1-NPV level, the immediate risk values of the cervical precancer in HRHPV-positive women with negative p16/Ki67 test result were observed similar or slightly lower to those revealed by the others.[22] In our study, the immediate risk for CIN2+ in women with positive HRHPV N16/N18 and negative p16/Ki67 test results was 3.7% (vs. 3.6%[22]), and in HPV 16/18 positive women it was 3.2% (vs. 4.7%–6.2%[22]). The immediate risk for CIN3+ in women with positive HPV 16/18

status and negative p16/Ki67 test result was noted at the level of 0.0% in our study, while in the other study the lowest risk for CIN3+ (0.8%) was observed in HRHPV N16/N18-positive patients.[22] Moreover, very low levels of the negative likelihood ratios obtained in the study (0.08–0.15 for HSIL/CIN2+; 0.00–0.20 for HSIL/CIN3+), that were even two to four-fold lower compared to the others: (0.14–0.31 for CIN2+; 0.15–0.27 for CIN3+),[22] (0.393 for CIN2+; 0.339 for CIN3+),[20] confirm that p16/Ki67 triage strategy can demonstrate a very low proportion of negative false tests results.

Costs were not explicitly considered in the study. Polish private-based opportunistic cervical cancer screening varies substantially in pricing of screening tests, including p16/Ki67 biomarker, with an extremal diversity (more than the extent of 100%) at all levels of care providers. Pricing for a single p16/Ki67 test ranges between of 250–350 PLN ($60-84). An assessment of pricing estimates offered by laboratories revealed that the final quote is dependent on a laboratory and number of performed tests by a counterparty, with the large variations between 200 and 300 PLN ($48–72). A comparable assessment of a providers' offers revealed full pricing with estimates at the level of 150–200 PLN ($36–48) for a single test depending on number of performed tests. A higher diversity was noted for a gynecologic cytology pricing (PLN50–150/$12–36) and molecular HPV testing (PLN120–300/$29–72) for a single test applied, with differences depended on the testing is performed in the same vial or in the vial derived from the new sampling. Presented pricing levels in dollars were calculated from Polish zloty based on widely available exchanges rates valid on the date of November 07, 2023. A reasonable assessment of cost-effectiveness of p16/Ki67 triaging incorporation was disturbed also by a fluctuation of prices of all screening tests analyzed during our study was conducted. Several additional changes in pricing were associated with inflationary impacts: the average index of consumer inflation in Poland covering with our study duration was, as follows: 8.6% in 12/2021, 12/2022 16.6% in 12/2022, and w 08/2023 10.1% in 08/2023.[32] Having of stiff regular pricing would have a crucial impact on an assessment of cost-effectiveness of introducing p16/Ki67 biomarker triage making this reliable and comparable. As it was in a study of Killeen et al., where Hawaii Medicare reimbursement schedules were available for performed gynecologic and pathologic procedures.[33] In turn, no management guidelines for p16/Ki67 use in a public-based screening has been adressed, which was equivalent with lack of pricing and reimbursement schedules for this testing and associated gynecological and pathological procedures by the National Health Fund in Poland. In recently published paper of Harper et al. on cost-effectiveness of p16/Ki67 following cotesting with high-risk HPV genotyping, invasive cervical cancer death and costs related to this diagnosis were decreasing, despite increasing costs of screening tests during lifetime.[34] We would like to point it out, that our large population-based study demonstrated a superior diagnostic performance of p16/Ki67 triage for detecting cervical cancer precursors in a primary HPV-based cervical cancer screening, which is less expensive option than cotesting analyzed in the referred paper. Significantly increased detection rate for HSIL/CIN2+ and HSIL/CIN3+ combined with decreased number of colposcopies needed to detect of these lesions translate into very good effectiveness of the secondary prevention systems with the biomarker incorporation.

This study has several strengths: (1) one of the few large studies evaluating p16/Ki67 diagnostic performance as a triage in HPV-positive patients in primary HPV screening with limited genotyping; (2) one of the first such comprehensive analyses including data from private funds-based opportunistic screening in the Central European population with correlation of virological-cytological-immunocytochemical results along with histology; (3) a well-organized system of management with abnormal screening tests results, which determines good disease ascertainment at all stages of screening and further diagnostics; (4) performing all screening tests from one cervical sampling with a triage testing performed shortly after the visit (which meant short-term storage of residual cervical samples); (5) p16/Ki67 was evaluated by a qualified gynecological cytopathologist; (6) a strict adherence to extended colposcopy protocol used; (7) short interval between abnormal screening tests results and referral to colposcopy allowed immediate histologic correlation (interval not exceeding 3 months). Limitations: (1) this is a retrospective study; (2) loss of patients at various study stages; (3) data in this study comes from a real-life practice, that is not a clinical trial, which further increases heterogeneity and affects the proportions of individual studied subgroups.

In conclusion, our study showed that the application of p16/Ki67 dual-staining as a triage strategy in women with positive HRHPV test results with limited genotyping has superior diagnostic performance for detecting cervical cancer precursors compared to cytology triage in primary HPV-based cervical cancer screening. Significantly higher specificity of dual-stain triage indicates that this strategy might be associated with a substantial reduce the number of colposcopies, both in HPV 16/18-positive women, as well as in women positive for 12 other HRHPV genotypes. The diagnostic approach with the p16/Ki67 dual-staining implementation into screenings algorithms may be particularly valuable in the secondary cervical cancer prevention.

AUTHOR CONTRIBUTIONS

Conceptualization: Maciej Mazurec and Martyna Trzeszcz. *Collecting of data*: Karolina Mazurec and Patrycja Rozmus. *Data curation*: Karolina Mazurec and Maciej Mazurec. *Analysis of data*: Maciej Mazurec, Martyna Trzeszcz, and Karolina Mazurec. *Interpretation of data*: Martyna Trzeszcz and Maciej Mazurec. *Investigation*: Izabela Kotkowska-Szeps, Maciej Mazurec, Magdalena Kania, Mariola Wantuchowicz, Jolanta Wasowska, Monika Duczek-Polakiewicz, and Martyna Trzeszcz. *Writing draft*: Martyna Trzeszcz and Maciej Mazurec. *Draft editing*: Martyna Trzeszcz, Karolina Mazurec, and Maciej Mazurec. *Supervision and review*: Robert Jach. *Review*: Agnieszka Halon and Joanna Streb. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST STATEMENT

Robert Jach has given a lecture sponsored by the Roche company. The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the Jagiellonian University (opinion ID 118.6120.36.2023). The study was conducted in accordance with the Declaration of Helsinki.

ORCID

Martyna Trzeszcz http://orcid.org/0000-0002-9971-1093
Maciej Mazurec http://orcid.org/0000-0003-2152-157X
Robert Jach http://orcid.org/0000-0001-5512-763X
Joanna Streb http://orcid.org/0000-0003-2147-7177
Agnieszka Halon http://orcid.org/0000-0003-4240-7899

REFERENCES

1. Maver PJ, Poljak M. Primary HPV-based cervical cancer screening in Europe: implementation status, challenges, and future plans. *Clin Microbiol Infect*. 2020;26(5):579-583. doi:10.1016/j.cmi.2019.09.006
2. Medical Services Advisory Committee. *MSAC Outcomes. Application No. 1276—Renewal of the National Cervical Screening Program*. Australian Government Department of Health; 2014. http://www.msac.gov.au/internet/msac/publishing.nsf/Content/FD36D6990FFAA639CA25799200058940/$File/1276%20-%20Final%20MSAC%20PSD%20-%20NCSP%20Renewal.pdf
3. Perkins RB, Guido RS, Castle PE, et al. 2019 ASCCP risk-based management consensus guidelines for abnormal cervical cancer screening tests and cancer precursors. *J Low Genit Tract Dis*. 2020;24(2):102-131. doi:10.1097/LGT.0000000000000525
4. Jach R, Mazurec M, Trzeszcz M, Zimmer M, Kedzia W, Wolski H. Cervical cancer screening in Poland in current SARS-CoV-2 pandemic: interim guidelines of the Polish Society of Gynecologists and Obstetricians and the Polish Society of Colposcopy and Cervical Pathophysiology—a summary January 2021. *Ginekol Pol*. 2021;92(2):165-173. doi:10.5603/GP.2021.0043
5. Jeronimo J, Castle PE, Temin S, et al. Secondary prevention of cervical cancer: ASCO resource-stratified clinical practice guideline. *J Glob Oncol*. 2017;3(5):635-657. doi:10.1200/JGO.2016.006577
6. World Health Organization. *Cervical Cancer Elimination Initiative*. 2022. https://www.who.int/initiatives/cervical-cancer-elimination-initiative
7. Ronco G, Giorgi-Rossi P, Carozzi F, et al. Human papillomavirus testing and liquid-based cytology in primary screening of women younger than 35 years: results at recruitment for a randomised controlled trial. *Lancet Oncol*. 2006;7(7):547-555. doi:10.1016/S1470-2045(06)70731-8
8. Ronco G, Dillner J, Elfström KM, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet*. 2014;383(9916):524-532. doi:10.1016/S0140-6736(13)62218-7
9. WHO Guideline for Screening and Treatment of Cervical Pre-cancer Lesions for Cervical Cancer Prevention. *Licence: CC BY-NC-SA 3.0 IGO*. 2nd edition. World Health Organization; 2021.
10. Ronco G, Arbyn M, Meijer CJLM, et al. Screening for cervical cancer with primary testing for human papillomavirus. In: Anttila A, Arbyn A, De Vuyst H, Dillner J, Dillner L, Franceschi S, Patnick J, Ronco G, Segnan N, Suonio E, Törnberg S, Karsa L, eds. *European Guidelines for Quality Assurance in Cervical Cancer Screening*. Office for Official Publications of the European Union; 2015:1-68.
11. Wentzensen N, Schiffman M, Palmer T, Arbyn M. Triage of HPV positive women in cervical cancer screening. *J Clin Virol*. 2016;76(suppl 1):S49-S55. doi:10.1016/j.jcv.2015.11.015
12. Giorgi Rossi P, Carozzi F, Ronco G, et al. p16/ki67 and E6/E7 mRNA accuracy and prognostic value in triaging HPV DNA-positive women. *J Natl Cancer Inst*. 2021;113(3):292-300. doi:10.1093/jnci/djaa105
13. Bergeron C, Ikenberg H, Sideri M, et al. Prospective evaluation of p16/Ki-67 dual-stained cytology for managing women with abnormal Papanicolaou cytology: PALMS study results. *Cancer Cytopathol*. 2015;123(6):373-381. doi:10.1002/cncy.21542
14. Petry KU, Schmidt D, Scherbring S, et al. Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 dual-stained cytology. *Gynecol Oncol*. 2011;121(3):505-509. doi:10.1016/j.ygyno.2011.02.033
15. Schmidt D, Bergeron C, Denton KJ, Ridder R. p16/ki-67 dual-stain cytology in the triage of ASCUS and LSIL papanicolaou cytology: results from the European equivocal or mildly abnormal Papanicolaou cytology study. *Cancer Cytopathol*. 2011;119(3):158-166. doi:10.1002/cncy.20140
16. Yim EK, Park JS. The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis. *Cancer Res Treat*. 2005;37(6):319-324. doi:10.4143/crt.2005.37.6.319
17. Peeters E, Wentzensen N, Bergeron C, Arbyn M. Meta-analysis of the accuracy of p16 or p16/Ki-67 immunocytochemistry versus HPV testing for the detection of CIN2+/CIN3+ in triage of women with minor abnormal cytology. *Cancer Cytopathol*. 2019;127(3):169-180. doi:10.1002/cncy.22103
18. Uijterwaal MH, Polman NJ, Witte BI, et al. Triaging HPV-positive women with normal cytology by p16/Ki-67 dual-stained cytology testing: baseline and longitudinal data. *Int J Cancer*. 2015;136(10):2361-2368. doi:10.1002/ijc.29290
19. Trzeszcz M, Mazurec M, Jach R, et al. Is primary HPV with secondary p16/Ki67 dual-stain an alternative HSIL-risk detection strategy in cervical cancer screening for women under 30 years? *Diagnostics*. 2021;11(11):2012. doi:10.3390/diagnostics11112012
20. Wright Jr. TC, Behrens CM, Ranger-Moore J, et al. Triaging HPV-positive women with p16/Ki-67 dual-stained cytology: results from a sub-study nested into the ATHENA trial. *Gynecol Oncol*. 2017;144(1):51-56. doi:10.1016/j.ygyno.2016.10.031
21. Luttmer R, Dijkstra MG, Snijders PJF, et al. p16/Ki-67 dual-stained cytology for detecting cervical (pre)cancer in a HPV-positive gynecologic outpatient population. *Mod Pathol*. 2016;29(8):870-878. doi:10.1038/modpathol.2016.80
22. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpma/pma.cfm?id=P190024. Accessed April 1, 2020.
23. Lorenzi NPC, Termini L, Ferreira-Filho ES, et al. A positive HPV test with positive p16/Ki-67 double staining in self-sampled vaginal material is an accurate tool to detect women at risk for cervical cancer. *Cancer Cytopathol*. 2022;130(1):41-54. doi:10.1002/cncy.22498
24. Trzeszcz M, Mazurec M, Jach R, et al. Liquid-based screening tests results: HPV, liquid-based cytology, and P16/Ki67 dual-staining in private-based opportunistic cervical cancer screening. *Diagnostics*. 2021;11(8):1420. doi:10.3390/diagnostics11081420
25. https://ictv.global/report/chapter/papillomaviridae/papillomaviridae Accessed February 27, 2023.
26. National Cervical Cancer Screening Programme in Poland Coordinating Centre; Polish Gynaecological Society; Polish Society of Pathologists; Polish Society of Colposcopy and Uterine Cervix Pathology. Management of abnormal pap smear—consensus guidelines of the National Cervical Cancer screening programme in Poland Coordinating Centre, the Polish Gynaecological Society, the Polish Society of Pathologists, and Polish Society of Colposcopy and Uterine cervix pathology. *Ginekol Pol*. 2009;80:129-138.

27. Wytyczne Dotyczace Aplikacji Testów Molekularnych Identyfikujacych DNA HPV HR w Profilaktyce Szyjki Macicy. Stanowisko ekspertów PTG I KIDL [Guidelines for application of molecular tests identifying HR HPV DNA in the prevention of cervical cancer. Statement of experts from PGS (PTG) and NCLD (KIDL)]. *Ginekol Pol*. 2013;84:395-399.
28. Nasierowska-Guttmejer A, Kędzia W, Wojtylak S, et al. Polish recommendations regarding diagnostics and treatment of cervical squamous intraepithelial lesions according to the CAP/ASCCP guidelines. *Ginekol Pol*. 2016;87:676-682.
29. Leisenring W, Alono T, Pepe MS. Comparisons of predictive values of binary medical diagnostic tests for paired designs. *Biometrics*. 2000;56(2):345-351. doi:10.1111/j.0006-341x.2000.00345.x
30. Wentzensen N, Clarke MA, Bremer R, et al. Clinical evaluation of human papillomavirus screening with p16/Ki-67 dual stain triage in a large organized cervical cancer screening program. *JAMA Int Med*. 2019;179(7):881-888. doi:10.1001/jamainternmed.2019.0306
31. Ebisch RM, van der Horst J, Hermsen M, et al. Evaluation of p16/Ki-67 dual-stained cytology as triage test for high-risk human papillomavirus-positive women. *Mod Pathol*. 2017;30(7):1021-1031. doi:10.1038/modpathol.2017.16
32. Główny Urząd Statystyczny. https://stat.gov.pl/wykres/1.html. Accessed November 01, 2023.
33. Killeen JL, Dye T, Grace C, Hiraoka M. Improved abnormal Pap smear triage using cervical cancer biomarkers. *J Low Genit Tract Dis*. 2014;18(1):1-7. doi:10.1097/LGT.0b013e31828aeb39
34. Harper DM, Anderson RJ, Baker E, Yu TM. Cost-effectiveness of p16/Ki-67 Dual-stained cytology reflex following co-testing with hrHPV genotyping for cervical cancer screening. *Cancer Prev Res*. 2023;16(7):393-404. doi:10.1158/1940-6207.CAPR-22-0455

How to cite this article: Trzeszcz M, Mazurec M, Jach R, et al. p16/Ki67 dual stain triage versus cytology in primary human papillomavirus-based cervical cancer screening with limited genotyping. *J Med Virol*. 2023;95:e29271. doi:10.1002/jmv.29271