JMS 2024;Vol7(3): 80-86
UDC: 618.146-006.6-076.5
doi.org/10.55302/JMS2473080m

DUAL STAINING CYTOLOGY WITH P16/KI67 FOR CERVICAL CANCER SCREENING

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Abstract

Currently, cytology, HPV testing, and combined cytology with HPV testing are the methods used for cervical cancer screening in patients. The p16/Ki67 dual staining is a biomarker with high sensitivity and specificity for CIN2+ lesions.

The introduction of such a biomarker for triage and identification of high-grade cervical lesions in patients, based on preliminary results from cytology and HPV screening, is necessary to prevent an excessive number of referrals for colposcopy and biopsy. The study was designed as a cross-sectional cohort study conducted at the University Clinic of Gynecology and Obstetrics, Skopje, over a two-year period, involving a group of 81 patients aged 21 to 65 years.

All patients underwent HPV DNA testing with typing, cytological testing (LB), and p16/Ki67 dual staining. The correlation of p16/Ki67 immunocytochemical status in patients with cytological verified squamous intraepithelial lesions and the presence of high-risk HPV was analyzed.

The analyses revealed a significant association between high-grade cervical lesions and p16/Ki67 staining status. A positive result from p16/Ki67 dual staining was significantly associated with an HSIL finding on the Pap test (p=0.01). HSIL cytological findings were significantly more common in patients with positive immunocytochemistry (36.36% vs 5.71%).

Patients positive for p16/Ki67 dual staining had more frequent positive HPV typing compared to patients with negative p16/Ki67 staining.

Keywords: p16/Ki67, immunocytochemistry, cervical cancer screening, HPV

Introduction

Cervical cancer is the fourth most common malignant tumor in women worldwide and a global public health problem, especially in countries with low to medium levels of development [1]. In 2022, 662 301 women were diagnosed with cervical cancer, and 348 874 women died from it [9].

The proven effectiveness of intervention measures, such as vaccination against the most oncogenic human papillomavirus (HPV) types and screening, makes this disease preventable [2]. In the Republic of North Macedonia, according to the WHO, the incidence of cervical cancer for 2022 was 6.7 per 100,000 women (107 cases), with mortality of 3.5 (61 deaths) [11].

Most commonly, cervical cancer is diagnosed in women aged 35 to 54 years, with an average age of 50 years. Over 20% of cases occur in women over 65 years old [4]. In 2020, the WHO launched a global initiative to eliminate cervical cancer with the goal of reducing the incidence to less than 4 cases per 100,000 women annually in every country [1,5].

The goal of the 90–70–90 initiative, which should be achieved by 2030, includes:90% of girls vaccinated by the age of 15, 70% of women screened with high-performance tests at least twice by the age of 45, 90% of women with precancerous changes or invasive cancer treated [1,5].

Cervical cancer is most often caused by persistent infection with human papillomavirus (HPV), with long-term infection by high-risk HPV types, such as HPV 16 and HPV 18, accounting for 70% of cervical cancers [6,13]. Nearly all sexually active individuals will contract HPV at some point in their life, but most infections clear up on their own within one to two years.

When the infection is persistent, it can lead to precancerous changes, which, if undetected and untreated, may develop into invasive cancer [7]. Risk factors for the progression of the infection into invasive cancer include: HPV type, immune status of the patient, presence of other sexually transmitted infections, number of childbirths, young age at first pregnancy, use of hormonal contraceptives, smoking [8].

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Persistent HPV infection can cause irreversible changes in the cervical cells, leading to the development of invasive cancer. These changes are a result of the effects of HPV genes, particularly those encoding the E6 and E7 proteins. These changes lead to deregulation of the cell cycle, creating conditions for atypical changes in cervical cells and the development of cervical cancer [16].

Typically, it takes 15–20 years for abnormal cells to progress to cancer, but in patients with weakened immune systems, such as those with untreated HIV, this process can be faster and take only 5–10 years [8].

For decades, cervical cancer screening has been based on cytological analysis of cervical cells [19]. Screening can be performed using conventional cytology (CC) or liquid-based cytology (LBC) [22]. LBC is replacing conventional cytology in many countries due to its higher efficiency and lower rate of inadequate tests (about 1% compared to 7% in CC) [23].

Many countries have started using HPV testing as the primary screening method [13]. The WHO recommends HPV DNA testing as the primary screening test, replacing cytology, for women in the general population and for women living with HIV. The WHO proposes a regular screening interval of every 5 to 10 years when using HPV DNA detection as the primary screening test for the general population of women [10].

Where HPV DNA screening has not yet been implemented, the WHO recommends regular screening every 3 years using cytology or colposcopy as the primary screening test among the general population of women and women living with HIV [11].

Cytology shows high specificity but relatively low sensitivity (30–80%) for detecting cervical cancer [15,22]. This method has low sensitivity due to the high rate of false-negative results. The sensitivity of HPV testing is high (around 90%), but specificity is low [15,19,20].

Therefore, detecting high-risk HPV is used to improve the sensitivity of cytology [15,25,26]. Due to the low specificity of HPV DNA testing, many women will be referred for colposcopy or biopsy, particularly young women under 30, as this population has the highest prevalence of HPV [27,28]. Thus, a more effective marker is needed for triaging HPV-positive patients with normal cytology and to identify women with a potentially higher (CIN2+ lesion) from cytological ASCUS/LSIL [27,28].

Most of the HPV infections are transitory and do not cause precancerous changes in the cervix, so effective methods are needed to triage patients to prevent unnecessary colposcopy and biopsy referrals. Among these, the most extensively studied are HPV genotyping, HPV methylation, and dual cytological staining with p16/Ki67 [12,29].

The p16/Ki67 dual cytological staining method is an immunocytochemical technique used to identify high-grade cervical lesions (CIN 2+). p16 is a cyclin-dependent kinase inhibitor and acts as a regulatory protein of the cell cycle, while Ki67 is a cell proliferation marker [12,15,37]. Under physiological conditions, they are not expressed simultaneously in the same cell. Co-expression of p16 and Ki67 indicates deregulation of the cell cycle and may suggest the presence of high-grade cervical lesions [15,32,34,35].

Cytology with p16/Ki-67 dual cytological staining has high sensitivity and specificity and is a useful biomarker for screening and triaging cervical cancer and its precancerous lesions. This method shows promise for reducing the number of unnecessary colposcopies and biopsies. It can be used to predict the regression or progression of CIN2 lesions (as a borderline lesion for treatment).

Compared to cytology and HPV detection, it has greater sensitivity in detecting precancerous lesions of the cervix and cervical cancer. p16/Ki-67 dual staining is recommended as a strategy for monitoring women treated for precancerous lesions [12,15].

Materials and methods

The study was designed as a cross-sectional study of a cohort conducted during the 2023-2024 period. The study included 81 participants, aged between 21 and 65 years. All participants underwent HPV DNA testing with genotyping, cytological testing (LB), and p16/Ki67 double cytological staining. Only participants with high-risk HPV confirmed during screening and/or with cytologically proven lesions were included in the analysis.

The study was performed at the University Clinic for Gynecology and Obstetrics, Skopje. All cytological results(LB) and immunocytochemical staining for p16/Ki67 were performed in the Cytology Laboratory. Participants included: patients aged from 21 to 65 years, with cytologically confirmed low- and high-grade squamous intraepithelial lesions, where HPV DNA testing revealed a high-risk HPV type, and patients with positive results for HR-HPV DNA typing but with normal cytology. Patients with low-risk HPV types, and patients who were found to have invasive cervical cancer were excluded of the study, regardless of the cytological findings.

LBC (Liquid-based Cytology) was used in this study. The sample was obtained using a brush, which was then rinsed in a liquid medium. The material was left in the medium for 15-20 minutes and then placed in a fully automated device for centrifugation and slide preparation.

The slide was removed from the device and fixed in 96% alcohol. After fixation, an automated staining process followed. Cervical smears were classified according to the Bethesda 2001 system. p16/Ki67 Double Cytological Staining: The CINtec® PLUS cytology kit is a qualitative immunocytochemical method used for detecting cells with neoplastic transformation, showing positivity for both p16 and Ki67 proteins. Signal detection occurs with two different chromogens, with 3,3'-diaminobenzidine (DAB) chromogen conversion mediated by peroxidase, and rapid red chromogen conversion mediated by alkaline phosphatase, resulting in brown and red staining of p16 and Ki-67 antigen sites, respectively.

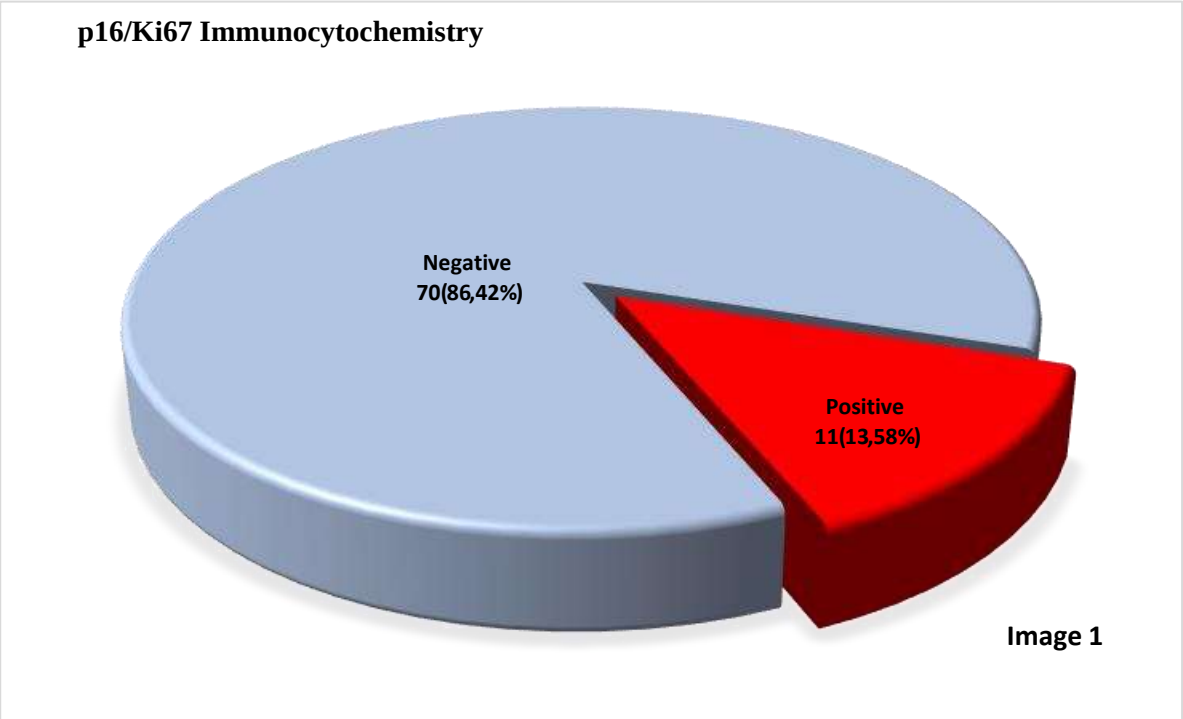
The presence of at least one cervical epithelial cell with brown-stained cytoplasm and redstained nucleus indicates a positive test result.

HPV DNA Testing/Real-time Multiplex PCR Assays for HPV: Samples for HPV typing are collected using swabs and cytobrushes. The swabs and brushes are stored in an appropriate medium - PBS (Phosphate Buffered Saline) in sterile tubes at a temperature of 2-8°C until DNA extraction. DNA extraction from the collected cervical swabs is performed using a commercial kit for urogenital samples, designed for automatic nucleic acid extraction from sexually transmitted pathogens in swabs, urine, and semen.

For the extracted DNA samples, HPV amplification is performed using real-time PCR with commercial kits. The test is a multiplex real-time PCR assay that allows simultaneous amplification, detection, and differentiation of target DNA from 19 high-risk HPV types - hrHPV (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types - lrHPV (6, 11, 40, 42, 43, 44, 54, 61, 70) along with an internal control.

Results

A total of 81 patients were included in the study, of which 11 (13.58%) were positive for p16/Ki67 double cytological staining. 70 patients (86.42%) were negative for p16/Ki67 double cytological staining.



The patients were aged between 21 and 65 years, with a mean age of 38.0±12.7 years. The average age of the patients positive for p16/Ki67 double cytological staining was insignificantly higher than that of the patients negative for this method (39.0±16.3 vs 37.9±12.2 years, p=0.79).

Table 1:

Immunocytochemistry	**Age/years**			p-level
	N	mean ± SD	min- max	
Positive	11	39.0 ± 16.3	21 – 70	t=0.27
Negative	70	37.89 ± 12.2	20 – 79	p=0.79

A positive Pap test was obtained in 60 (74.08%) patients, LSIL in 52 (64.20%), and HSIL in 8 (9.88%) patients.

Table 2: Distribution of Patients Based on Cytological Findings

LBC	**n (%)**
Negative	21 (25.92)
LSIL	52 (64.20)
HSIL	8 (9.88)

There were no differences between patients positive/negative for p16/Ki67 double cytological staining in terms of LSIL findings from the Pap test (p=1.0); LSIL was detected in 63.64% of patients with positive immunocytochemistry and 64.29% of those with negative immunocytochemistry.

Table 3:

LSIL	**Immunocytochemistry**	p-level

	N	Positive n (%)	Negative n (%)	
Undetected	29	4 (36.36)	25 (35.71)	Fisher's exact test p=1.0
Detected	52	7 (63.64)	45 (64.29)	

The positive result of p16/Ki67 double cytological staining was significantly associated with the HSIL finding from the Pap test (p=0.01). The HSIL cytological finding was significantly more frequent in patients with positive immunocytochemistry (36.36% vs 5.71%).

Table 4:

HSIL	**Immunocytochemistry**			p-level
	N	Positive n (%)	Negative n (%)	
Undetected	73	7 (63.64)	66 (94.29)	Fisher's exact test *p=0.01
Detected	8	4 (36.36)	4 (5.71)	

Patients positive for p16/Ki67 double cytological staining had a higher frequency of positive HPV typing than those with a negative p16/Ki67 immunocytochemical test.

Table 5:

HR HPV testing	**Immunocytochemistry**			p-level
	N	Positive n (%)	Negative n (%)	
Undetected	23	1 (10)	22 (37.29)	Fisher's exact test p=0.15
Detected	46	9 (90)	37 (62.71)	

Conclusion

The results obtained from this study demonstrate a significant association between the presence of high-grade cytological lesions and the positive outcome of immunocytochemical staining with p16/Ki67. Patients positive for p16/Ki67 dual cytological staining had more frequent positive HPV typing results compared to patients with negative p16/Ki67 immunocytochemical staining.

Cytology with p16/Ki67 dual cytological staining is of great importance for screening and triaging cervical cancer and its precancerous lesions due to the increased accuracy of the result when combined with current screening tests, such as cytology and HR HPV DNA testing.

Given that HR-HPV DNA detection has been established as a screening method in an increasing number of developed countries, and is also being adopted by developing countries, the challenge remains in the further management of patients who test positive, given the high sensitivity of the test and the low specificity for detecting high-grade lesions (CIN2+ lesions).

On the other hand, the management of patients in countries where cytology remains the firstline screening method for cervical cancer is a real challenge due to its low sensitivity, primarily because of the large number of false-negative results.

Thus, there is a need for a screening method that will complement existing screening programs, reducing the number of unnecessary referrals for colposcopy or biopsy.

The p16/Ki67 dual cytological staining as a screening method is expected to provide answers to the questions regarding the presence of high-grade squamous intraepithelial lesions (CIN2+ lesions)

in patients who are HR-HPV DNA positive, with a positive cytology result or dual positivity on cotesting.

The application of p16/Ki67 dual cytological staining early in the diagnostic algorithms for triaging patients with cervical precancerous lesions and cervical cancer represents a significant tool that clinicians can use to identify high-grade lesions.

At the same time, and most importantly, we expect that these results will have positive implications by improving cervical cancer screening programs and, thus, improving the health and treatment outcomes of patients in the fight against cervical cancer and its precancerous lesions in our population.

References

1. Singh D, Vignat J, Lorenzoni V, Eslahi M, Ginsburg O, Lauby-Secretan B, Arbyn M, Basu P, Bray F, Vaccarella S. Global estimates of incidence and mortality of cervical cancer in 2020: a baseline analysis of the WHO Global Cervical Cancer Elimination Initiative. Lancet Glob Health. 2023 Feb;11(2):e197-e206. doi: 10.1016/S2214-109X(22)00501-0. Epub 2022 Dec 14. PMID: 36528031; PMCID: PMC9848409.
2. Ginsburg O Bray, F Coleman MP et al. The global burden of women's cancers: a grand challenge in global health. Lancet. 2017; 389: 847-860
3. https://www.cancer.org/cancer/acs-medical-content-and-news-staff.html
4. WHO. Global strategy to accelerate the elimination of cervical cancer as a public health problem. Geneva: World Health Organization, 2020
5. Cibula D, Raspollini MR, Planchamp F, Centeno C, Chargari C, Felix A, Fischerová D, JahnnKuch D, Joly F, Kohler C, Lax S, Lorusso D, Mahantshetty U, Mathevet P, Naik R, Nout RA,
   Oaknin A, Peccatori F, Persson J, Querleu D, Bernabé SR, Schmid MP, Stepanyan A, Svintsitskyi V, Tamussino K, Zapardiel I, Lindegaard J. ESGO/ESTRO/ESP Guidelines for the management of patients with cervical cancer - Update 2023. Int J Gynecol Cancer. 2023 May 1;33(5):649-666. doi: 10.1136/ijgc-2023-004429. PMID: 37127326; PMCID: PMC10176411.
6. Key Statistics for Cervical Cancer https://www.cancer.gov/types/cervical/causes-riskprevention
7. Cervical cancer https://www.who.int/news-room/fact-sheets/detail/cervical-cancer
8. Pathak P, Pajai S, Kesharwani H. A Review on the Use of the HPV Vaccine in the Prevention of Cervical Cancer. Cureus. 2022 Sep 2;14(9):e28710. doi: 10.7759/cureus.28710. PMID: 36211088; PMCID: PMC9529156.
9. https://gco.iarc.who.int/media/globocan/factsheets/cancers/23-cervix-uteri-fact-sheet.pdf
10. WHO guideline for screening and treatment of cervical pre-cancer lesions for cervical cancer prevention, second edition (https://iris.who.int/bitstream/handle/10665/342365/9789240030824-eng.pdf?sequence=1)
11. 11.https://gco.iarc.who.int/today/en/dataviz/bars?mode=cancer&key=asr&group_populations=1&types=0&sort_by=value0&populations=807&multiple_populations=0&values_position=out&cancers_h=23&sexes=2
12. Dovnik, A.; Repše Fokter, A. The Role of p16/Ki67 Dual Staining in Cervical Cancer Screening. Curr. Issues Mol. Biol. 2023, 45, 8476–8491. https://doi.org/10.3390/cimb45100534
13. Castle, P.E.; Stoler, M.H.; Wright, T.C., Jr.; Sharma, A.; Wright, T.L.; Behrens, C.M. Performance of carcinogenic human papillomavirus (HPV) testing and HPV16 or HPV18 genotyping for cervical cancer screening of women aged 25 years and older: A subanalysis of the ATHENA study. Lancet Oncol. 2011, 12, 880–890
14. Dijkstra, M.G.; van Niekerk, D.; Rijkaart, D.C.; van Kemenade, F.J.; Heideman, D.A.; Snijders, P.J.; Meijer, C.J.; Berkhof, J. Primary hrHPV DNA testing in cervical cancer screening: How to manage screen-positive women? A POBASCAM trial substudy. Cancer Epidemiol. Biomark. Prev. 2014, 23, 55–63

15. Yu L, Fei L, Liu X, Pi X, Wang L, Chen S. Application of p16/Ki-67 dual-staining cytology in cervical cancers. J Cancer 2019; 10(12):2654-2660. doi:10.7150/jca.32743. https://www.jcancer.org/v10p2654.htm.
16. Klaes R, Woerner SM, Ridder R, Wentzensen N, Duerst M, Schneider A. et al. Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. Cancer Res. 1999;59:6132–6.
17. McLean HQ, VanWormer JJ, Chow BDW, Birchmeier B, Vickers E, DeVries E, Meyer J, Moore J, McNeil MM, Stokley S, Gee J, Belongia EA. Improving Human Papillomavirus Vaccine Use in an Integrated Health System: Impact of a Provider and Staff Intervention. J Adolesc Health. 2017 Aug;61(2):252-258. doi: 10.1016/j.jadohealth.2017.02.019. Epub 2017 Apr 24. PMID: 28462786; PMCID: PMC6749828.
18. Petrosky E, Bocchini JA Jr, Hariri S, Chesson H, Curtis CR, Saraiya M, Unger ER, Markowitz LE; Centers for Disease Control and Prevention (CDC). Use of 9-valent human papillomavirus (HPV) vaccine: updated HPV vaccination recommendations of the advisory committee on immunization practices. MMWR Morb Mortal Wkly Rep. 2015 Mar 27;64(11):300-4. PMID: 25811679; PMCID: PMC4584883.
19. Maver PJ, Poljak M. Primary HPV-based cervical cancer screening in Europe: implementation status, challenges, and future plans. Clin Microbiol Infect. 2020 May;26(5):579-583. doi: 10.1016/j.cmi.2019.09.006. Epub 2019 Sep 17. PMID: 31539637.
20. Rijkaart DC, Berkhof J, van Kemenade FJ, Coupe VM, Hesselink AT, Rozendaal L, Heideman DA, Verheijen RH, Bulk S, Verweij WM, Snijders PJ, Meijer CJ. Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. Int J Cancer. 2012 Feb 1;130(3):602-10. doi: 10.1002/ijc.26056. Epub 2011 May 5. PMID: 21400507.
21. Feldstein O, Gali-Zamir H, Schejter E, Feinberg T, Yehuda-Shnaidman E, Bornstein J, Levy T. High-risk HPV testing vs liquid-based cytology for cervical cancer screening among 25- to 30-year-old women: A historical cohort study. Acta Obstet Gynecol Scand. 2023 Feb;102(2):226-233. doi: 10.1111/aogs.14482. Epub 2022 Dec 7. PMID: 36478537; PMCID: PMC9889323.
22. Kituncharoen S, Tantbirojn P, Niruthisard S. Comparison of Unsatisfactory Rates and Detection of Abnormal Cervical Cytology Between Conventional Papanicolaou Smear and Liquid-Based Cytology (Sure Path®). Asian Pac J Cancer Prev. 2015;16(18):8491-4. doi: 10.7314/apjcp.2015.16.18.8491. PMID: 26745107.
23. Jeong H, Hong SR, Chae SW, Jin SY, Yoon HK, Lee J, Kim EK, Ha ST, Kim SN, Park EJ, Jung JJ, Sung SH, Lim SC. Comparison of Unsatisfactory Samples from Conventional Smear versus Liquid-Based Cytology in Uterine Cervical Cancer Screening Test. J Pathol Transl Med. 2017 May;51(3):314-319. doi: 10.4132/jptm.2017.03.17. Epub 2017 Apr 17. PMID: 28415159; PMCID: PMC5445207.
24. Nayar R, Wilbur DC. The Pap Test and Bethesda 2014: "The reports of my demise have been greatly exaggerated. (after a quotation from Mark Twain)". J Low Genit Tract Dis. 2015 Jul;19(3):175-84. doi: 10.1097/LGT.0000000000000115. PMID: 25943863.
25. Rijkaart DC, Berkhof J, van Kemenade FJ, Coupe VM, Hesselink AT, Rozendaal L, Heideman DA, Verheijen RH, Bulk S, Verweij WM, Snijders PJ, Meijer CJ. Evaluation of 14 triage strategies for HPV DNA-positive women in population-based cervical screening. Int J Cancer. 2012 Feb 1;130(3):602-10. doi: 10.1002/ijc.26056. Epub 2011 May 5. PMID: 21400507.
26. Katki HA, Schiffman M, Castle PE, Fetterman B, Poitras NE, Lorey T, Cheung LC, RaineBennett T, Gage JC, Kinney WK. Benchmarking CIN 3+ risk as the basis for incorporating
HPV and Pap cotesting into cervical screening and management guidelines. J Low Genit Tract Dis. 2013 Apr;17(5 Suppl 1):S28-35. doi: 10.1097/LGT.0b013e318285423c. PMID: 23519302; PMCID: PMC3616419.
27. Massad LS, Einstein MH, Huh WK, Katki HA, Kinney WK, Schiffman M, Solomon D, Wentzensen N, Lawson HW; 2012 ASCCP Consensus Guidelines Conference. 2012 updated

consensus guidelines for the management of abnormal cervical cancer screening tests and cancer precursors. Obstet Gynecol. 2013 Apr;121(4):829-846. doi: 10.1097/AOG.0b013e3182883a34. PMID: 23635684.

28. Wright TC, Stoler MH, Behrens CM, Sharma A, Zhang G, Wright TL. Primary cervical cancer screening with human papillomavirus: end of study results from the ATHENA study using HPV as the first-line screening test. Gynecol Oncol. 2015 Feb;136(2):189-97. doi: 10.1016/j.ygyno.2014.11.076. Epub 2015 Jan 8. PMID: 25579108.
29. Wentzensen N, Schwartz L, Zuna RE, Smith K, Mathews C, Gold MA, Allen RA, Zhang R, Dunn ST, Walker JL, Schiffman M. Performance of p16/Ki-67 immunostaining to detect cervical cancer precursors in a colposcopy referral population. Clin Cancer Res. 2012 Aug 1;18(15):4154-62. doi: 10.1158/1078-0432.CCR-12-0270. Epub 2012 Jun 6. PMID: 22675168; PMCID: PMC4237612.
30. Liu, J., Su, S. & Liu, Y. The value of Ki67 for the diagnosis of LSIL and the problems of p16 in the diagnosis of HSIL. Sci Rep 12, 7613 (2022). https://doi.org/10.1038/s41598-022-11584z
31. Petry KU, Schmidt D, Scherbring S, Luyten A, Reinecke-Lüthge A, Bergeron C, Kommoss F, Löning T, Ordi J, Regauer S, Ridder R. Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 Dual-stained cytology. Gynecol Oncol. 2011 Jun 1;121(3):505-9. doi: 10.1016/j.ygyno.2011.02.033. Epub 2011 Mar 21. PMID: 21420158.
32. Ikenberg H, Bergeron C, Schmidt D, Griesser H, Alameda F, Angeloni C, Bogers J, Dachez R, Denton K, Hariri J, Keller T, von Knebel Doeberitz M, Neumann HH, Puig-Tintore LM, Sideri M, Rehm S, Ridder R; PALMS Study Group. Screening for cervical cancer precursors with p16/Ki-67 dual-stained cytology: results of the PALMS study. J Natl Cancer Inst. 2013 Oct 16;105(20):1550-7. doi: 10.1093/jnci/djt235. Epub 2013 Oct 4. PMID: 24096620; PMCID: PMC3814411.
33. Yu LL, Chen W, Lei XQ, Qin Y, Wu ZN, Pan QJ, Zhang X, Chang BF, Zhang SK, Guo HQ, Qiao YL. Evaluation of p16/Ki-67 dual staining in detection of cervical precancer and cancers: a multicenter study in China. Oncotarget. 2016 Apr 19;7(16):21181-9. doi: 10.18632/oncotarget.8307. PMID: 27029033; PMCID: PMC5008277.
34. Tay TKY, Lim KL, Hilmy MH, Thike AA, Goh ST, Song LH, Hwang JSG, Mantoo S. Comparison of the sensitivity and specificity of p16/Ki-67 dual staining and HPV DNA testing of abnormal cervical cytology in the detection of histology proven cervical intraepithelial neoplasia grade 2 and above (CIN 2+). Malays J Pathol. 2017 Dec;39(3):257-265. PMID: 29279588.
35. Wentzensen N, Fetterman B, Castle PE, Schiffman M, Wood SN, Stiemerling E, Tokugawa D, Bodelon C, Poitras N, Lorey T, Kinney W. p16/Ki-67 Dual Stain Cytology for Detection of Cervical Precancer in HPV-Positive Women. J Natl Cancer Inst. 2015 Sep 15;107(12):djv257. doi: 10.1093/jnci/djv257. PMID: 26376685; PMCID: PMC4675094.