DOI: https://doi.org/10.11144/Javeriana.umed61-1.dual

p16INK4a/ki-67 Dual Immunostaining in Liquid-Based Vaginal Citology. Pilot Proof of Diagnostic Complement

Inmunotinción dual p16INK4a/ki-67 en citología vaginal en medio líquido. Prueba piloto de complemento diagnóstico

Received: 27 February 2019 | Accepted: 29 May 2019

Myriam Beatriz Puerto de Amaya
Bacteriologist, cytohistology technologist. Assistant Professor, Fundación Universitaria de Ciencias de la Salud, Bogotá, Colombia

Mercedes Olaya-C.[a]
MD, PhD. Associate Professor, Department of Pathology, School of Medicine, Pontificia Universidad Javeriana-Hospital Universitario San Ignacio, Bogotá, Colombia

Edy Marcela Corredor López
Cytohistology technologist, Fundación Universitaria de Ciencias de la Salud, Bogotá, Colombia

Carlos Humberto Pérez Moreno
MD. Director of the Hospital Universitario San José. Tenured Professor. Obstetrician/gynecologist, Fundación Universitaria de Ciencias de la Salud, Bogotá, Colombia

[a] Correspondence: olaya.m@javeriana.edu.co

Conflicts of interest: The authors declare that there are no conflicts of interest in the present manuscript.

How to cite: Puerto de Amaya MB, Olaya-C. M, Corredor López EM, Pérez Moreno CH. p16INK4a/ki-67 dual immunostaining in liquid-based vaginal citology: pilot proof of diagnostic complement. Univ. Med. 2020;61(1). https://doi.org/10.11144/Javeriana. umed61-1.dual

ABSTRACT

Objective: Apply in a pilot study tests that show the deregulation of the cell cycle and therefore the progression to cancer, as is the case of the studies with p16 and ki-67; as a complement in the study of the evolution of the lesions reported as squamous atypia (ASC-US, LSIL, and HSIL) in cytological outlines. **Methods:** An observational analytical study was carried out with liquid-based cytology, which was supplemented with immunocytochemistry, biopsy, endocervical brushing and colposcopy in positive cases. **Results:** Of the 51 Pap smears studied, 35 (68.6%) were classified as negative and 16 (31.3%) with atypia, which were classified as: ASCU-US (n=8); LSIL (n=7) and HSIL (n=1). Among those 16 positive cases, the immunocytochemical test was positive for 3 of the 7 LSIL and for the case reported as HSIL. The 8 cases reported as ASC-US were negative for biomarkers. **Conclusion:** In this study based on 51 patients, four were detected with deregulated cellular cycle by immunoreactivity to dual p16/ki67; 3 cases were classified as LSIL and one case as HSIL. Biomarkers are a useful complement in cervical cancer screening, mainly for lesions in progression to malignancy, which should be promoted in our country.

Keywords
immunocytochemistry; cervical squamous intraepithelial lesions; p16INK; papilloma.

RESUMEN

Objetivo: Introducir la prueba de desregulación del ciclo celular p16 y ki-67 como complemento en el estudio de la evolución de las lesiones

reportadas con atipia escamosa (ASC-US, LSIL y HSIL) en extendidos citológicos, dada su capacidad como marcador de progresión de lesiones precancerosas a cáncer; se compara con patrón de referencia. **Métodos:** Se ejecutó un estudio observacional analítico de corte transversal en una población a la que se le realizó citología en base líquida. A las citologías positivas se les aplicó adicionalmente inmunocitoquímica, cepillado endocervical, colposcopia y biopsia. **Resultados:** De las 51 citologías estudiadas, 35 (68,6%) se clasificaron como negativas y 16 (31,3%) con atipia: ASCU-US (n = 8), LSIL (n = 7) y HSIL (n = 1). De estos 16 casos, la prueba de inmunocitoquímica fue positiva para 3 de los 7 LSIL y para el caso identificado como HSIL. Los 8 casos reportados como ASC-US fueron negativos para biomarcadores. **Conclusión:** El presente estudio piloto realizado a 51 muestras detectó 4 de ellas con un ciclo celular desregulado, mediante inmunorreactividad a la doble tinción de p16/ki-67; 3 citologías habían mostrado LSIL y una HSIL. Los biomarcadores son un complemento útil en el tamizaje para el control de cáncer de cuello uterino, específicos para lesiones en progresión a malignidad, que se deben promover en nuestro país.

Palabras clave
inmunohistoquímica; lesiones intraepiteliales escamosas de cuello uterino; genes p16INK4; papiloma.

Introduction

Persistent infection with high-risk human papillomavirus (HR-HPV) is considered a necessary factor for the development of cervical cancer and its precursor lesions (1,2,3). At least 13 genotypes have been found associated with high risk of developing cervical cancer and have been defined as *carcinogenic* (4). Worldwide, HPV16 has been identified as the most frequent virus associated with squamous cell carcinoma and adenocarcinoma, followed by HPV18 (5). The carcinogenic mechanism involves integration of viral DNA into the cellular genome. Subsequently, there is viral DNA replication with particular importance of the expression of the E6 and E7 genes, which alters the cellular pathways of the host cell (6).

The interaction of the viral genome with fragile sites of the infected cell seems to be crucial for malignant progression. Functional inactivation of oncosuppressive genes such as p53 and pRb (retinoblastoma gene) determines the alteration of the pathways leading to the progression to cancer (7,8,9), through a proliferative state, genetic instability and formation of a malignant cell clone (9,10). The expression of E7 determines the inactivation of pRb, which increases the free form of E2F in the cell, which in turn increases the cyclin-dependent kinase inhibitor p16 (p16INK4a) and an aberrant proliferation, which can be observed by the increase in the cell proliferation marker Ki-67 (11). HR-HPV-induced molecular alterations can be studied using biomarkers used on cytology samples, as they represent a complementary tool of diagnostic and prognostic value in infected patients (7,10). These allow to differentiate the infections that are altering the cell cycle, which are the objective in the programs for early detection of cancer.

Immunocytochemistry P16INK4/Ki-67 dual staining allows recognizing neoplastic cells with altered cell cycle and uncontrolled proliferation in cytology samples of the cervix (12,13,14). Dual staining of biomarkers with immunocytochemistry is used in samples reported as atypical squamous cells of undetermined significance (ASC-US) and low-grade squamous intraepithelial lesion (LSIL) by conventional cytology or liquid-based cytology (LBC) and positive DNA HPV HR (15,16,17,18). Likewise, dual staining identifies high-grade intraepithelial lesions, with good sensitivity and specificity (96% and 83%, respectively). However, there may be inter-observer variability in these diagnoses, and the high-risk HPV test may become invalidated due to hypocellularity; in such circumstances, immunocytochemical staining helps define the diagnosis (19,20,21,22,23).

Staining is particularly useful in the differential diagnosis between non-neoplastic lesions (such as inflammatory processes or cellular changes associated with atrophy) and truly premalignant lesions (13,24,25,26,27). Cases of atrophy, tubal metaplasia, endometrial and endocervical epithelia, or squamous metaplasia (28,29,30) show a nonspecific positivity at p16 (25,31,32); while p16INK4a has been highly specific, with statistical significance in the screening of women with ASC-US, due to a relative sensitivity of 0.95 (95%; CI: 0.89-1.01) and a relative specificity of 1.82 (95%; CI: 1.57-2.12) (4).

In the present work, dual p16/Ki-67 staining was observed in cytology smears with a report of squamous atypia: ASC-US, LSIL and high-grade squamous intraepithelial lesion (HSIL), which allowed to standardize the methodology and interpret the immunocytochemistry. These results were complemented with colposcopy and biopsy in the relevant cases. In Colombia, double staining in LBC had not been used, so this study is a pioneer in the country.

Materials and methods

Type of study. Cross-sectional analytical observational study with LBC supplemented with immunocytochemistry (p16/Ki-67) in cases diagnosed as ASC-US, an atypia that does not allow ruling out high-grade lesions (ASC-H), LSIL and HSIL. The usefulness of the immunocytochemical test (p16/Ki-67) to detect patients at risk of progression to cancer was explored. Positive cytology studies were subjected to complementary immunocytochemical studies, a second review by a gynecologist, colposcopy and sampling for histological study. The study was approved by the Institutional Ethics Committee.

Population. Samples were taken from a universe of 51 women who voluntarily attended a cytology campaign at the Thirteenth Occupational Health Conference of the Hospital San José and the *Fundación Universitaria de Ciencias de la Salud (Colombia)* in 2013. Women over 18 years of age were included. Pregnant women (33) and those who had had a hysterectomy were excluded. The participants were explained the purpose of the study, after which they signed an informed consent agreeing to be part of the research.

Clinical variables: Age, parity, smoking, sexually transmitted diseases, age of sexual debut, number of sexual partners and planning methods. These variables correspond to the standardized history in the uterine cytology sampling format endorsed by the Ministry of Health and the Secretariat of Health of Bogotá. These variables are related to the population's risk of developing cervical cancer.

Liquid based cytology samples. Initially, the cervix was assessed using a colposcope and subsequently the LBC was taken (directly to the vial), using a cytobrush (Rovers®). A smear was performed for each case, using the manual equipment (BD PrepMate™ System) to process the LBC, following the supplier's instructions. The smears were processed and analyzed in the Faculty of Cytohistology of the Fundación Universitaria de Ciencias de la Salud. Papanicolau stain was used and its interpretation was based on the 2014 Bethesda criteria (34). The first reading was done by a cytohistology technologist, and the 16 cases reported with atypia (ASC-US, LSIL and HSIL) were confirmed by two pathologists. There was agreement in all cases. One of the pathologists also reviewed 10% of the negative cases, as is routinely done.

Sample for immunocytochemical staining. Residual samples interpreted as positive (ASC-US, LSIL and HSIL) were tested with vial immunocytochemistry (BD SurePath™) stored at 4 °C. Conventional immunocytochemistry was performed. The CINtec Plus Kit^{R} was used following the supplier's instructions. The kit is designed to carry out a two-step immunocytochemical staining procedure on cervical cytology preparations. Positive and negative controls were used, as indicated by the supplier. These samples were analyzed by two blinded pathologists for the morphological results of each case. The criterion for determining a sample as positive was the finding of one or more epithelial cells that showed double reactivity: both brown cytoplasm and red nucleus (10,19,23).

In the cases of positive p16/Ki-67 tests, a second colposcopy was performed by a gynecologist with experience in colposcopy. Endocervical brushing and cervical biopsy were taken as reference standards for the study, one month after the first sampling. These histological samples were read by the two pathologists.

As for women with a negative cytology result, a three-year follow-up was indicated, using the 1-3-3 scheme recommended by the Ministry of Health and Social Protection of Colombia.

Results

The universe of 51 women was included. The ages ranged from 18 to 60 years, with an average of 39.8 and a standard deviation of 10.3. Table 1 shows the demographic characteristics of patients with positive results.

Table 1

Distribution of positive cases by cytology according to variables

Case	Cytology	FP	Smoking	STD	NSP	ASD	Parity	Age
2	ASC-US	Yes	No	No	0	0	0	28
3	ASC-US	No	No	No	3	18	1	32
7	ASC-US	No	No	No	1	17	3	41
13	ASC-US	Yes	No	No	1	19	0	22
18	ASC-US	Yes	No	No	2	18	3	40
32	ASC-US	No	No	No	3	16	1	45
35	ASC-US	No	No	No	1	25	3	52
41	ASC-US	Yes	No	No	1	17	2	31
1	LSIL	No	No	No	1	19	0	25
12	LSIL	Yes	No	No	3	17	1	28
15	LSIL	Yes	No	No	5	15	2	26
38	LSIL	Yes	No	No	1	19	2	45
39	LSIL	Yes	No	HPV	2	15	2	34
44	LSIL	Yes	No	No	2	21	2	28
51	LSIL	No	No	HPV	1	18	0	46
9	HSIL	No	No	No	1	16	5	53

FP: family planning; STD: sexually transmitted diseases; NSP: number of sexual partners; ASD: age of sexual debut.

Of the 51 cytologies studied, 35 were classified as negative, and 16, with atypia, which were classified as: ASCU-US (n=8), LSIL (n=7) and HSIL (n=1) (Figure 1). The immunocytochemical test was positive for 3 of the 7 cases reported as LSIL and for the case classified as HSIL. The 8 cases reported as ASC-US by cytology were negative for biomarkers. Figures 2 and 3 show positive and negative tests.

Figure 1

Classification of the cases

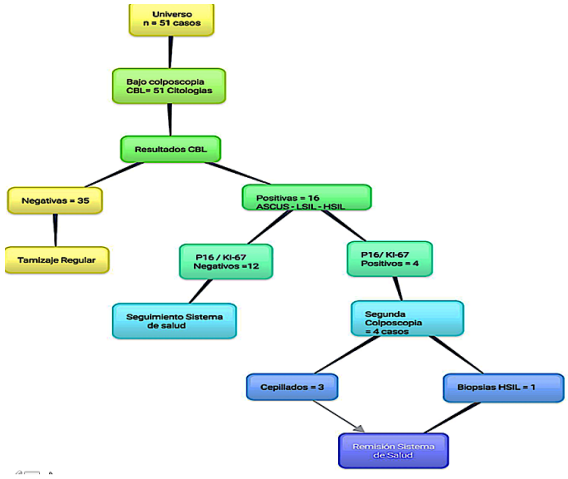


Figure 2

Positive sample

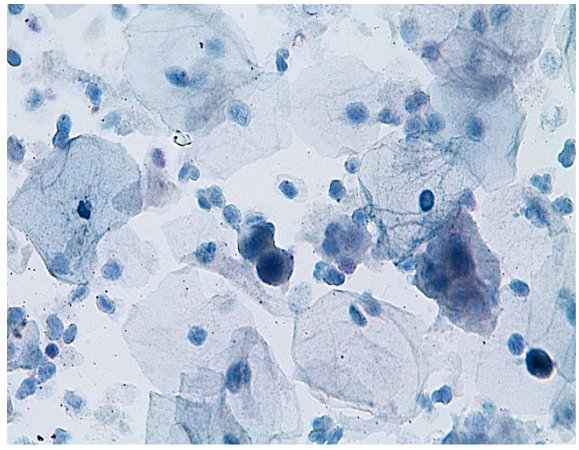
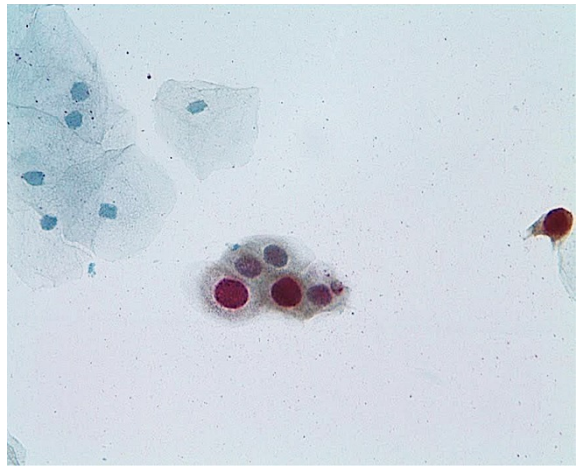


Figure 3

Negative sample



Those who had positive cytological results underwent a second colposcopic evaluation. When it was abnormal, biopsy or endocervical brushing was taken (Table 2). Brush cytology was abnormal in 3 of them (two LSIL and one HSIL), and biopsy, in 2 of the 4 patients (two cervical intraepithelial neoplasms I [CIN-I] associated with HPV). The subsequent treatment and follow-up required by the group of patients at risk was performed according to the procedure established by the health system.

Table 2

Results with complementary test

Case	Cytology	Previous cytology	Colposcopy	Biopsy	Endocervical brushing	PCR	p16/Ki67
2	ASC-US	Normal	Negative	No	No	No	Negative
3	ASC-US	Normal	Negative	No	No	No	Negative
7	ASC-US	Normal	Negative	No	No	No	Negative
13	ASC-US	Normal	Negative	No	No	No	Negative
18	ASC-US	Normal	Negative	No	No	No	Negative
32	ASC-US	Normal	Negative	No	No	No	Negative
35	ASC-US	Normal	Positive	HPV	No	No	Negative
41	ASC-US	Normal	Negative	No	No	No	Negative
1	LSIL	Normal	Negative	No	No	No	Negative
12	LSIL	LSIL	Negative	No	No	No	Negative
15	LSIL	Normal	Negative	No	LSIL	No	Positive
38	LSIL	Normal	EAB	IMD	No	No	Positive
39	LSIL	LSIL	Positive-	Inflammation	No	No	Negative
44	LSIL	Normal	Negative	No	No	No	Negative
51	LSIL	ASC-US	Positive-LEIB	LSIL-HPV	HSIL	VHP-16	Positive
9	HSIL	Normal	Negative	LSIL	HSIL	No	Positive

AWE: acetowhite epithelium (biopsy was taken); IMD: insufficient material for diagnosis.

Discussion

Worldwide, screening for early detection of cervical cancer has recently focused on the need to know if the infection is caused by a high-risk virus. More recently, studies have been to determine if the host cell genome control mechanisms are altered. Since viral infection is frequent, it is necessary to know in which cases it is a high-risk virus and when it has altered the cell regulation mechanisms, becoming a threat for cancer development.

In Colombia, the determination of high-risk HPV was introduced as a primary screening test; however, it is still not done uniformly. This should be accompanied by LBC, which has the advantage of allowing molecular tests. However, in our country this test is more than twice as expensive as conventional cytology. Problems also persist in the quality of the sampling, since they may lack a transformation zone.

Among the post-analytical problems is the fact that some patients do not pick up the result of the test, and the lack of coordination between some institutions providing health services to actively seek out patients with positive results; in addition, laboratories do not have mechanisms to inform treating physicians and patients about positive results for cervical lesions.

By tending to promote a more effective screening, in this work a first approach was made to LBC with the application of dual staining with biomarker (immunocytochemistry) for high-grade lesions. The advantage of the test is that it shows high-grade lesions that have not been identified morphologically as such, allowing early diagnosis of progression to high-risk lesions.

Multiple studies highlight the benefits of this technique. In the study by Wentzensen et al. (20), based on a pilot project (2007-2008) with 425 women who had negative Papanicolau tests but positive HPV, dual p16/Ki-67 staining was included in order to identify cases of CIN-2 that were corroborated by biopsy. The study showed a sensitivity of 91.9% to detect CIN-2 and 96.4% for CIN-3. The specificity was 82.1% for CIN-2 and 76.9% for CIN-3. The study concluded that dual-staining cytology can identify women with a high possibility of CIN-2 and can complement cervical carcinoma screening programs. In the study by Petry et al. (19), the sensitivity and specificity in the ASC-US group was 95% and 84%, and for LSIL cases, 100% and 81%, respectively. Based on the findings, the study authors concluded that the use of a biomarker allows the identification of HSIL (18,19,23,26,35,36,37). The accuracy of the p16INK4a test has been tested in cytology with a diagnosis of ASC-US and LSIL (38,39).

The advantage of this technique is that it showed that the coexpression of p16INK4a and Ki-67 only occurred in the presence of dysplasia (40). In addition, it reduces intra- and interobserver variability, which occurs in the evaluation of cervical-uterine cytologies (16) and in histopathological interpretation (15,36,41,42,43). The test is recommended to differentiate between truly dysplastic lesions.

Although the present study had a small sample controlled by conventional cytology screening, positive cases were found with dual staining (p16/Ki-67), which led to further studies with a second colposcopy and histological studies. Patients with low-grade lesions who had not been directed to subsequent studies (colposcopy: biopsy or endocervical brushing) underwent additional examinations, thanks to the result of the positive biomarker that identified cell cycle deregulation and, therefore, the potential risk of progression of the infection to cancer. Immunocytochemistry is aimed at *high-grade lesions not detected* in the morphology of the screening cytology, which would have been reported as ASC-US or LSIL.

One of the limitations of the study is that it had a closed population, probably with little exposure to risk factors. Nor was there another valuable diagnostic tool for all women, such as the high-risk HPV DNA test. More studies are necessary in the future with a more representative population in terms of risk factors and number of participants.

Specific HPV tests are very important for screening, and are an exceptional complement in cases of abnormal vaginal citologies; they will also be important for monitoring future generations to evaluate vaccine effectiveness. In cases such as the ones described, premalignant lesions were detected, not by the presence of a high-risk virus, but by evidence of an alteration in the cell cycle regulation, which draws attention to the close surveillance of these patients.

This tool allows eliminating unnecessary surveillance of infections caused by high-risk viruses that patients would eliminate without treatment, and also of high-risk cytological lesions that would return spontaneously. With this type of tests, epidemiological surveillance can be optimized, emphasizing where the true risk of progression to cancer is.

Conclusion

Globally, cervical cancer remains an enemy to women's health. Molecular tests have been complementing screening programs and helping to understand the infection and its mechanisms of progression to cancer, both from the virus and the host point of view. We have in our country this first experience in the implementation of p16/Ki-67 in LBC, which detected women whose early management changed due to the molecular result, modifying what would have been the "natural history of the illness." We expect that this experience will allow new research to expand the experience of cytologists and pathologists with these techniques, bringing these tests closer to the population at risk in our country.

Acknowledgements

To the participating patients. To Dr. Juan Carlos Bonilla for reading the samples as a pathologist.

References

1.Bosch FX, Manos MM, Muñoz N, Sherman M, Jansen AM, Peto J, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst. 1995;87(11):796-802.

2.Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol. 1999;189(1):12-9.

3.Wright JD, Rader JS, Dávila R, Powell MA, Mutch DG, Gao F, et al. Human papillomavirus triage for young women with atypical squamous cells of undetermined significance. Obstet Gynecol. 2006;107(4):822-9.

4.Tornesello ML, Buonaguro L, Giorgi-Rossi P, Buonaguro FM. Viral and cellular biomarkers in the diagnosis of cervical intraepithelial neoplasia and cancer. BioMed research international.

2013;2013:519619. https://doi.org/10.1155/2013/519619.

5.Li N, Franceschi S, Howell-Jones R, Snijders PJ, Clifford GM. Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. Int J Cancer. 2011;128(4):927. https://doi.org/10.1002/ijc.25396.

6.Hopman AH, Smedts F, Dignef W, Ummelen M, Sonke G, Mravunac M, et al. Transition of high-grade cervical intraepithelial neoplasia to micro-invasive carcinoma is characterized by integration of HPV 16/18 and numerical chromosome abnormalities. J Pathol. 2004;202(1):23-Epub 2003/12/25.

7.Dürst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig NE, Gissmann L. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene. 1987;1(3):251-6.

8.Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science. 1990;248(4951):76-9.

9.Dyson N, Howley PM, Münger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science. 1989;243(4893):934-7.

10.Toro de Méndez M, Ferrández Izquierdo A. [Detection of human papilloma virus (HPV) in liquid-based cervical samples. Correlation with protein p16INK4a expression]. Invest Clin. 2011;52(1):3-14.

11.Nakao Y, Yang X, Yokoyama M, Ferenczy A, Tang SC, Pater MM, et al. Induction of p16 during immortalization by HPV 16 and 18 and not during malignant transformation. British journal of cancer. 1997;75(10):1410-Epub 1997/01/01.

12.Shidham VB, Mehrotra R, Varsegi G, D'Amore KL, Hunt B, Narayan R. p16 immunocytochemistry on cell blocks as an adjunct to cervical cytology: Potential reflex testing on specially prepared cell blocks from residual liquid-based cytology specimens. Cytojournal. 2011;8. https://doi.org/10.4103/1742-6413.76379

13.Singh M, Mockler D, Akalin A, Burke S, Shroyer A, Shroyer KR. Immunocytochemical colocalization of P16(INK4a) and Ki-67 predicts CIN2/3 and AIS/adenocarcinoma. Cancer cytopathology. 2012;120(1):26. https://doi.org/10.1002/cncy.20188

14.Donà MG, Vocaturo A, Giuliani M, Ronchetti L, Rollo F, Pescarmona E, et al. p16/Ki-67 dual staining in cervico-vaginal cytology: correlation with histology, Human Papillomavirus detection and genotyping in women undergoing colposcopy. Gynecol Oncol. 2012;126(2):198-202. https://doi.org/10.1016/j.ygyno.2012.05.004

15.Bergeron C, Ordi J, Schmidt D, Trunk MJ, Keller T, Ridder R, et al. Conjunctive p16INK4a testing significantly increases accuracy in diagnosing high-grade cervical intraepithelial neoplasia. Am J Clin Pathol. 2010;133(3):395-406. https://doi.org/10.1309/AJCPXSVCDZ3D5MZM

16.Gustinucci D, Passamonti B, Cesarini E, Butera D, Palmieri EA, Bulletti S, et al. Role of p16(INK4a) cytology testing as an adjunct to enhance the diagnostic specificity and accuracy in human papillomavirus-positive women within an organized

cervical cancer screening program. Acta Cytol. 2012;56(5):506. https://doi.org/10.1159/000338979

17.von Knebel Doeberitz M, Reuschenbach M, Schmidt D, Bergeron C. Biomarkers for cervical cancer screening: the role of p16(INK4a) to highlight transforming HPV infections. Expert Rev Proteomics. 2012;9(2):149-63. https://doi.org/10.1586/epr.12.13

18.Ikenberg H, Bergeron C, Schmidt D, Griesser H, Alameda F, Angeloni C, et al. Screening for cervical cancer precursors with p16/Ki-67 dual-stained cytology: results of the PALMS study. J Natl Cancer Inst. 2013;105(20):1550. https://doi.org/10.1093/jnci/djt235

19.Petry KU, Schmidt D, Scherbring S, Luyten A, Reinecke-Lüthge A, Bergeron C, 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. https://doi.org/10.1016/j.ygyno.2011.02.033

20.Wentzensen N, Bergeron C, Cas F, Vinokurova S, von Knebel Doeberitz M. Triage of women with ASCUS and LSIL cytology: use of qualitative assessment of p16INK4a positive cells to identify patients with high-grade cervical intraepithelial neoplasia. Cancer. 2007;111(1):58-66.

21.Toll AD, Kelly D, Maleki Z. Utility of P16 expression and Ki-67 proliferation index in ASCUS and ASC-H pap tests. Diagn Cytopathol. 2014;42(7):576. https://doi.org/10.1002/dc.23076

22.Atkins K. p16/Ki-67 dual-stain cytology in the triage of ASCUS and LSIL papanicolaou cytology: p16/Ki-67 in cervical papanicolaou Tests. Cancer cytopathology. 2011;119(3):145. https://doi.org/10.1002/cncy.20139

23.Schmidt D, Bergeron C, Denton KJ, Ridder R, Group ECCS. 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 cytopathology. 2011;119(3):158. https://doi.org/10.1002/cncy.20140

24.Nieh S, Chen SF, Chu TY, Lai HC, Lin YS, Fu E, et al. Is p16(INK4A) expression more useful than human papillomavirus test to determine the outcome of atypical squamous cells of undetermined significance-categorized Pap smear? A comparative analysis using abnormal cervical smears with follow-up biopsies. Gynecol Oncol. 2005;97(1):35-40.

25.Duncan L, Jacob S, Hubbard E. Evaluation of p16INK4a as a diagnostic tool in the triage of Pap smears demonstrating atypical squamous cells of undetermined significance. Cancer. 2008;114(1):34. https://doi.org/10.1002/cncr.23255

26.Wentzensen N, Schwartz L, Zuna RE, Smith K, Mathews C, Gold MA, et al. Performance of p16/Ki-67 immunostaining to detect cervical cancer precursors in a colposcopy referral population. Clin Cancer Res. 2012;18(15):4154. https://doi.org/10.1158/1078-0432.CCR-12-0270

27.Tsoumpou I, Arbyn M, Kyrgiou M, Wentzensen N, Koliopoulos G, Martin-Hirsch P, et al. p16(INK4a) immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. Cancer treatment reviews. 2009;35(3):210. https://doi.org/10.1016/j.ctrv.2008.10.005

28.Walts AE, Bose S. P16/Ki-67 Immunostaining is Useful in Stratification of Atypical Metaplastic

Epithelium of the Cervix. Clin Med Pathol. 2008;1:35-42.

29.Regauer S, Reich O. CK17 and p16 expression patterns distinguish (atypical) immature squamous metaplasia from high-grade cervical intraepithelial neoplasia (CIN III). Histopathology. 2007;50(5):629-35.

30.Iaconis L, Hyjek E, Ellenson LH, Pirog EC. p16 and Ki-67 immunostaining in atypical immature squamous metaplasia of the uterine cervix: correlation with human papillomavirus detection. Arch Pathol Lab Med. 2007;131(9):1343-9.

31.Mittal K, Soslow R, McCluggage WG. Application of immunohistochemistry to gynecologic pathology. Arch Pathol Lab Med. 2008;132(3):402. https://doi.org/10.1043/1543-2165(2008)132[402:AOITGP]2.0.CO;2

32.Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, et al. Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. International journal of cancer. 2001;92(2):276-Epub 2001/04/06.

33.Trutnovsky G, Kolovetsiou-Kreiner V, Reich O. p16/Ki-67 dual-stained cytology testing may predict postpartum outcome in patients with abnormal papanicolaou cytology during pregnancy. Acta Cytol. 2014;58(3):293-6. https://doi.org/10.1159/000358817.

34.Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. JAMA. 2002;287(16):2114-9.

35.Denton KJ, Bergeron C, Klement P, Trunk MJ, Keller T, Ridder R, et al. The sensitivity and specificity of p16(INK4a) cytology vs HPV testing for detecting high-grade cervical disease in the triage of ASC-US and LSIL pap cytology results. Am J Clin Pathol. 2010;134(1):12. https://doi.org/10.1309/AJCP3CD9YKYFJDQL

36.del Pino M, Garcia S, Fusté V, Alonso I, Fusté P, Torné A, et al. Value of p16(INK4a) as a marker of progression/regression in cervical intraepithelial neoplasia grade Am J Obstet Gynecol. 2009;201(5):488.e1. https://doi.org/10.1016/j.ajog.2009.05.046

37.Yoshida T, Sano T, Kanuma T, Inoue H, Itoh T, Yazaki C, et al. Usefulness of CINtec® PLUS p16/Ki-67 double-staining in cytological screening of cervical cancer. Acta Cytol. 2011;55(5):413. https://doi.org/10.1159/000331047

38.Carozzi F, Confortini M, Dalla Palma P, Del Mistro A, Gillio-Tos A, De Marco L, et al. Use of p16-INK4A overexpression to increase the specificity of human papillomavirus testing: a nested substudy of the NTCC randomised controlled trial. The Lancet Oncology. 2008;9(10):937. https://doi.org/10.1016/S1470-2045(08)70208-0

39.Roelens J, Reuschenbach M, von Knebel Doeberitz M, Wentzensen N, Bergeron C, Arbyn M. p16INK4a immunocytochemistry versus human papillomavirus testing for triage of women with minor cytologic abnormalities: a systematic review and meta-analysis. Cancer cytopathology. 2012;120(5):294-307. https://doi.org/10.1002/cncy.21205

40.Reuschenbach M, Seiz M, von Knebel Doeberitz C, Vinokurova S, Duwe A, Ridder R, et al. Evaluation of cervical cone biopsies for coexpression of p16INK4a and Ki-67 in epithelial cells. International journal of cancer.

2012;130(2):388. https://doi.org/10.1002/ijc.26017

41.Klaes R, Benner A, Friedrich T, Ridder R, Herrington S, Jenkins D, et al. p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. Am J Surg Pathol. 2002;26(11):1389-99.

42.Galgano MT, Castle PE, Atkins KA, Brix WK, Nassau SR, Stoler MH. Using biomarkers as objective standards in the diagnosis of cervical biopsies. Am J Surg Pathol. 2010;34(8):1077. https://doi.org/10.1097/PAS.0b013e3181e8b2c4

43.Sari Aslani F, Safaei A, Pourjabali M, Momtahan M. Evaluation of Ki67, p16 and CK17 Markers in Differentiating Cervical Intraepithelial Neoplasia and Benign Lesions. Iran J Med Sci. 2013;38(1):15-21.