

Evaluation of P16/Ki67 (CINtecPlus) and L1-capsid compared with HPV-genotyping in cervical cytology in women ≥ 35 years old focusing on patients with atypical squamous cells of undetermined significance

MAHMOUD ABBAS¹, ISMAIL ERDURAN², JAN DE JONGE³ and OLAF BETTENDORF³

¹Department of Pathology, Gerhard-Domagk Institute for Pathology;

²Department of Pathology, Medical Hospital, University Muenster, D-48149 Muenster;

³Department of Pathology, Institute for Pathology and Cytology (IPN), D-48465 Schuettorf, Germany

Received March 25, 2022; Accepted May 6, 2022

DOI: 10.3892/ol.2022.13362

Abstract. Cervical cancer is the third most common cancer in women worldwide. Conventional cytological examination as a screening method with Papanicolaou has been established to reduce the incidence of dysplasia and cervical cancer for years. In addition to the conventional screening, the introduction of immunocytochemical examinations, including CINtecPlus and L1-capsid, has been demonstrated to have a positive impact on screening results. In addition to morphological screening methods, human papillomavirus (HPV)-testing has also been demonstrated to possess an enormous potential in the cervical screening process. Additionally, different screening models ranging from conventional cytological screening to primary HPV-testing do exist in different countries. At the beginning of the year 2020, a combination of cytological screening and HPV-testing was introduced in Germany for women ≥ 35 years. The aim of the present study was to evaluate the role of morphological screening, including immunocytochemistry, and to compare it with HPV-genotyping. Immunocytochemistry was added to confirm the diagnosis but needs established infrastructure and well-trained personnel. Furthermore, there was a need to establish the HPV-screening method. In the Institute for Pathology and Cytology (Schuettorf, Leer, Germany), 146,800 samples of women (>35 years old) were examined between January 2020 and January 2021. The present study retrospectively analyzed 146,800 samples. Each sample was examined using a conventional cytological technique and HPV-high risk-Test (HPV-HR-Test) with Viper-BD.

Immunocytochemistry with CINtecPlus and L1-capsid was added in some cases. A total of 555 cases were cytological diagnosed as atypical squamous cells of undetermined significance (ASC-US; IIP). After performing immunocytochemistry, 79% of cases were suspected to be positive and 1.48% of cases were definitely positive. The HPV-HR-Test was positive in 26.4% of cases. Among cases of ASC-US and HPV-HR-negativity, 33.7% were suspicious of immunocytochemical positivity and 0.5% were definitely positive. Among patients with HPV-16-negativity, 13.6% were patients with highly squamous intraepithelial lesion (HSIL) and 22.7% were patients with low-grade squamous intraepithelial lesion (LSIL) and HSIL. Among patients with HPV-18-negativity, 14.3% were patients with HSIL and 19.5% were patients with LSIL and HSIL. There were 107 cases in this group of cases with negativity of both HPV-16 and HPV-18. After performing the colposcopy and biopsy, there were 6.5% with cervical intra-epithelial neoplasia (CIN) I, 8.4% with CIN II and 5.6% with CIN III. In conclusion, there is still a need for conventional cytological examination and maybe the addition of immunocytochemistry to confirm the diagnosis and to exclude dysplasia of cervical epithelium. The HPV-HR-Test is not enough as a screening method and may be misleading.

Introduction

Cervical cancer is the third most common cancer in women with around 0.5 million cases worldwide (1). There is an annual increase of 0.6% in new cases (2). Approximately 76% of recent cases occur in low-resources nations, with numbers increasing in high-income countries (3). In Germany, there is an incidence of 2.2% of all new cases of cervical cancer in women. It belongs to the less common malignancies in Germany. There is a marked decline in the incidence, because of cytological screening with Papanicolaou of 80% (4). It is also recognized that cervical cancer is a rare long-term outcome of persistent infection of the lower genital tract by one of about 15 high-risk HPV types. In Germany, there was the use of Papanicolaou classification

Correspondence to: Dr Mahmoud Abbas, Department of Pathology, Gerhard-Domagk Institute for Pathology, University Muenster, Domagkstrasse 17, D-48149 Muenster, Germany
E-mail: mahmoud.abbas@ukmuenster.de

Key words: CINtecPlus, L1-capsid, cervical cytology, atypical squamous cells of undetermined significance

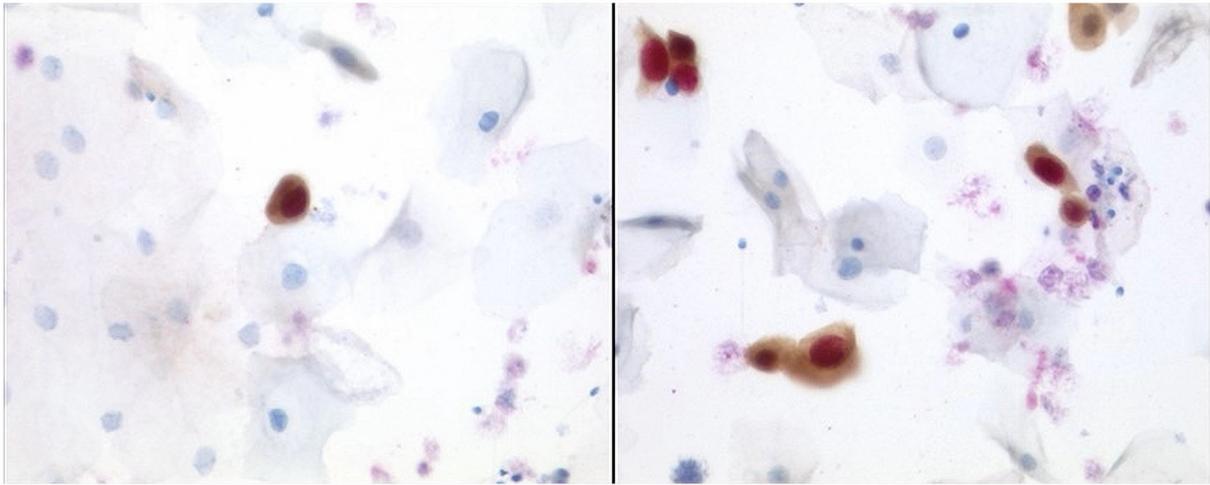


Figure 1. Two representative images with atypical squamous cells of undetermined significance after performing CINtecPlus (P16/Ki67). The nucleus is indicated by red signal (Ki-67) and the cytoplasm is indicated by brown signal (p16). Magnification, x40.

system in conventional cytology until 1975. After that there was the muenchener classifications II, which has been used until 2014 and then muenchener classification III. In the last classification, there was more details and subgroups than the muenchener classification II, like IIP (abnormal squamous cells). Group IIP (ASC-US) was for a long time under controversial discussions. It may be due to the irritation of cells with inflammation or mechanical irritation, which leads to metaplasia but it does not mean that it is dysplasia. With the beginning of the new screening program for women in Germany for early detection of cervical cancer, there are new controversial discussions about the importance of this group and about the importance of conventional cytological smears. The screening program based on the following: all women under the age of 30 years old should only be examined using conventional cytological examination. Women between the age of 30 and 34 years old should be examined with the conventional cytological examination and if the sample showed abnormal suspicious cells, then this sample should be processed for HPV-Test for high-risk subtypes after 6 months from the cytological examination. Women at or above 35 years old should be examined every 3 years with both conventional cytological examination and HPV-HR-Test. Some researchers think that it is enough to protect women from cervix carcinoma by examining women only with subtyping the HPV high-risk without doing the conventional cytological examination or immunocytochemistry. Our work is focusing on the group IIP (ASC-US) in women at or above 35 years old, to evaluate the importance of conventional cytological examination with the optional use of immunocytochemistry (CINtecPlus and L1-capsid) to evaluate the abnormal cells and grading the dysplasia, if present, in comparison with the HPV-HR-Test.

Materials and methods

Data collection. In the Institute for Pathology and Cytology-Schuettorf-Leer-Germany were at the beginning of 2020 until the beginning of year 2021 approximately 146.800 samples of women above 35 years old. These samples

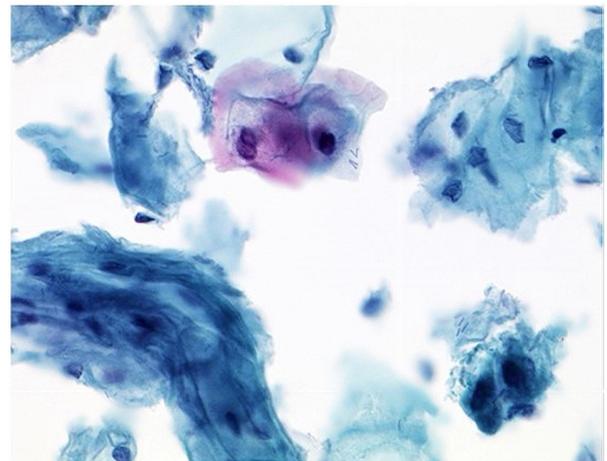


Figure 2. Conventional cytological stain in a case with atypical squamous cells of undetermined significance (IIP). There were signs of human papillomavirus changes, including cells with slight enlargement of the nucleus and hyperchromasia. Magnification, x20.

have been processed for both the cytological examination with Surepath-liquid based-technique (BD) in parallel with processing for HPV-HR-Test (BD Viper), according to advices and protocol of BD-manufacture.

Data analysis and examination. Among these samples, there were 555 cases, which have been subgrouped as IIP (Synonym for ASC-US in Germany) from certified Cytological technical assistants (CTA) and certified Cytopathologists (JJ, BT and MA). In about 135 cases (24.3%), there was a need to perform immunocytochemistry (IC) to confirm or to exclude the dysplasia. 112936 women (77%) have no HPV-vaccine, 296 women (0.2%) have had vaccine and only one woman in this age group with IIP (ASC-US) has had HPV-Vaccine.

Immunocytochemistry. Immunocytochemistry has been applied in Dako-Automat with manual kit from Roche, CINtecPlus cytology kit under advices and protocol of the manufacturer. Immunocytochemical staining of L1-capsid

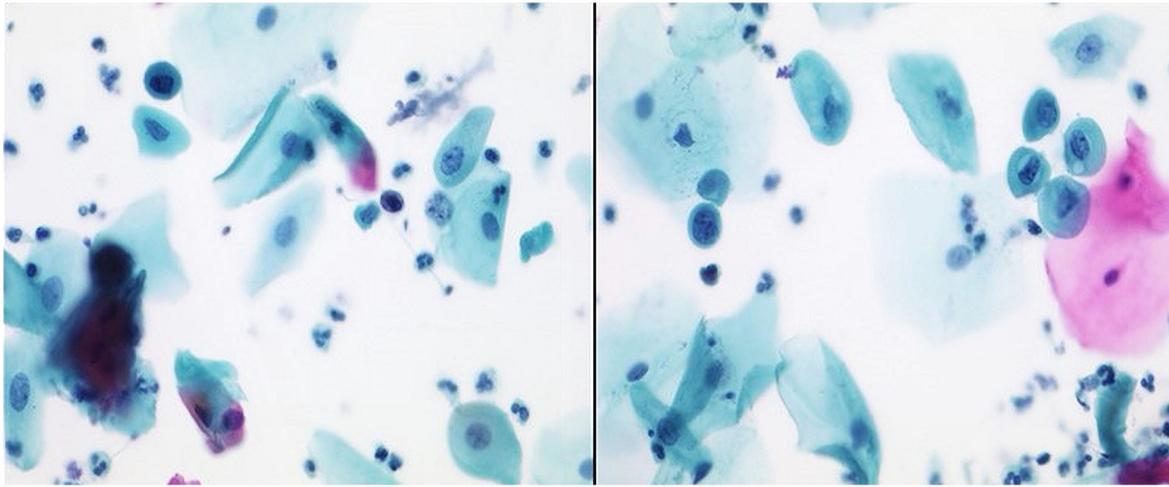


Figure 3. Two representative images of conventional cytological stain with inflammatory cells in the background. Numerous cells with metaplastic changes, which appear similar to dysplastic cells. Immunocytochemistry is essential in these cases to exclude IID2 or IVa-p. Magnification, x20.

antibody was performed following manufactures instruction (Virofem, dilution 1:10). The results of immunocytochemistry have been subdivided into negative, suspect of positivity, positive and technically not suitable to be judged and negative. If there is any weakness of the immunocytochemical reaction such as weak red signal of L1-capsid or weakness of brown signal in the cytoplasm of CINtecPlus, we have to call it suspect of positivity. Surely positive results are when the dysplastic cells clearly react to L1-capsid or to CINtecPlus or to both without element of suspicious. All immunocytochemical results were confirmed from both the CTA and the Cytopathologist.

HPV-Test for high-risk subtypes (HPV-HR-Test). The results of HPV-HR-Test were subdivided into negative, positive and technically not suitable to be judged.

Statistical analysis. Significant results will be considered if the P-Value is <0.05. Statistics were calculated with GraphPad Prism (GraphPad Software, Inc.) with non-parametric Kruskal-Wallis test and Dunn's post hoc test (Prism 5-2007). Data are presented as the mean \pm SD of three experimental repeats.

Ethics statement. The approval was granted by the ethics committee (Ethics Committee of the medical association-Hannover-Germany. An informed consent for inclusion into the study was waived, as patient records were anonymized and retrospectively analysed. The samples were anonymous with respect to measurements of data protection.

Results

Immunocytochemistry (IC). After performing the immunocytochemistry (IC) using P16/Ki67 (CINtecPlus, Fig. 1), there were cases of Group ASC-US (Iip, Figs. 2 and 3) with suspect of positivity in 79%, negative in 17% and surely positive in 1.48%. Positivity in these cases of Iip leads to sure diagnosis of IID2 or IVa-p (highly squamous intraepithelial lesion, HSIL). After performing the

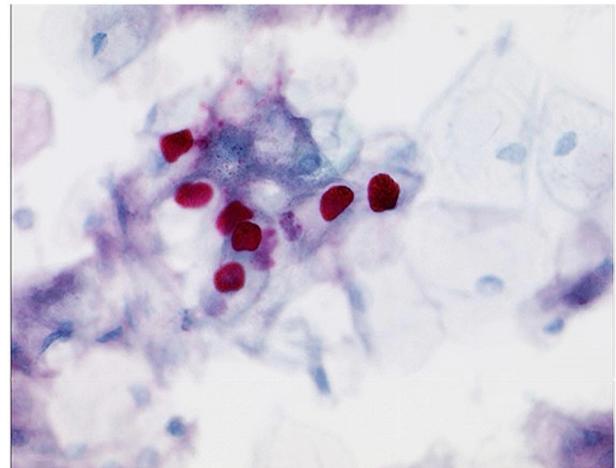


Figure 4. Atypical squamous cells of undetermined significance (Iip) after performing immunocytochemistry with L1-capsid. Nuclear positivity (red signals) was observed for L1-capsid, which indicated that there were human papillomavirus changes and the diagnosis should be IID1. Magnification, x40.

immunocytochemistry (Tables I and II) using L1-capsid (Fig. 4; Table III), there were 95.3% negative cases, 0.7% suspect of positivity and 3% surely positive. Approximately 2.2% of the cases were due to a small number of cells or due to technical problems unsuitable to be judged.

HPV-Test for high-risk subtypes. All cases were parallel processed to HPV-HR-Test. 41.6% were negative for HPV-HR, 26.4% were positive and approximately 31.8% were unsuitable to be judged. As a rule of this screening, the cases with Iip and negative for HPV-HR, histological biopsy should not be done, although 33.7% of these cases were immunocytochemically evaluated as suspect of positivity and approximately 0.5% were surely positive (Table IV). In cases with Iip and positivity of HPV-HR, there were 12.9% positive for HPV-16 and 0.7% for HPV-18. In cases with HPV-16-positivity, there was sure histological diagnosis of CINII and CINIII (HSIL) in 15.7%. In cases of

Table I. Results of IC after performing CINtecPlus and L1-capsid in cases of ASC-US (IIP).

ASC-US (IIP)/IC	Suspicion of positivity, n (%)	Negative, n (%)	Positive, n (%)	Technically not analyzable, n (%)
CINtecPlus	105 (77.70)	20 (15.30)	2 (1.48)	8 (5.52)
L1-capsid	1 (0.77)	124 (95.38)	4 (3.08)	1 (0.77)

ASC-US, atypical squamous cells of undetermined significance; IC, immunocytochemistry.

Table II. Association between HPV 16 and 18 results and the results of histopathology after colposcopy.

HPV16 and 18/histopathology	Without biopsy, n (%)	Without dysplasia, n (%)	CIN I, n (%)	CIN II, n (%)	CIN III, n (%)	Clinically without dysplasia
HPV-16-positive cases (n=19/147; 12.9%)	12 (63.30)	2 (10.50)	0 (0.00)	2 (10.50)	1 (5.20)	2 (10.50)
HPV-16-negative cases (n=22/147; 14.9%)	15 (68.40)	2 (9.00)	1 (4.50)	3 (13.60)	0 (0.00)	1 (4.50)
HPV-18-positive cases (n=1/147; 0.7%)	1 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
HPV-18-negative cases (n=21/147; 14.6%)	14 (66.90)	2 (9.50)	1 (4.70)	3 (14.20)	0 (0.00)	1 (4.70)
HPV-16 and 18-negative cases (n=107/555; 19.2%)	49 (54.54)	25 (23.30)	7 (6.45)	9 (8.25)	6 (5.60)	2 (1.86)

CIN, cervical intra-epithelial neoplasia; HPV, human papillomavirus. Out of 147 HPV-HR-positive cases, 84 cases that had HPV-HR positivity are not included, because they had another type of HPV-HR positivity, other than HPV-16 or -18; therefore, 63 cases, which are only either positive or negative for HPV-16 or -18 are included. After excluding cases with other types of HPV-HR reactivity and cases that were technically not suitable, there were only 107 cases out of 555 with negativity for both HPV-16 and -18.

Table III. Association between results of L1-capsid and results of histopathology after colposcopy.

L1-capsid	Without biopsy, n (%)	Without dysplasia, n (%)	CIN I, n (%)	CIN II, n (%)	CIN III, n (%)	Clinically without dysplasia, n (%)
Suspicion of positivity	1 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Negative	112 (90.40)	5 (4.00)	0 (0.00)	2 (1.60)	2 (1.60)	3 (2.40)
Positive	2 (50.00)	0 (0.00)	1 (25.00)	1 (25.00)	0 (0.00)	0 (0.00)

CIN, cervical intra-epithelial neoplasia.

Table IV. Association between results of CINtecPlus and results of histopathology after colposcopy.

CINtecPlus	Without biopsy, n (%)	Without dysplasia, n (%)	CIN I, n (%)	CIN II, n (%)	CIN III, n (%)	Clinically without dysplasia, n (%)
Suspicion of positivity	94 (89.67)	5 (4.70)	0 (0.00)	3 (2.80)	1 (0.93)	2 (1.90)
Positive	1 (50.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (50.00)	0 (0.00)
Negative	20 (91.40)	0 (0.00)	1 (4.30)	0 (0.00)	0 (0.00)	1 (4.30)

CIN, cervical intra-epithelial neoplasia.

HPV-16-negativity, there was histological diagnosis of HSIL in 13.6%. There was interestingly approximately 18.1% of these cases (IIP and negative HPV-16) with surely histological diagnosis of CIN I (LSIL), CIN II and III (HSIL). In cases with HPV-18-negativity and IIP, there were 21 cases (14.6%).

14.2% of them with HSIL and 18.9% of them with LSIL and HSIL. There were 107 cases (19.2%) in this group of cases (ASC-US) with negativity of both HPV-16 and HPV-18. After performing the colposcopy and biopsy, there were 6.5% with CIN I, 8.4% with CIN II and 5.6% with CIN III (Table II).

Statistical analysis. There was no significant difference ($P=0.3679$) between the frequency of a HSIL diagnosis between the HPV-16-positive and HPV-16-negative cases. Statistics were calculated with GraphPad Prism with Kruskal-Wallis test (Prism 5-2007). Significant results will be considered if the P-Value is <0.05 .

Discussion

After applying the Munich nomenclature III in Germany in 2015, there were annually approximately 0.59% of patients with group IIP (ASC-US) as reported Hilal Z. and colleagues in 2015 (5). In our work, we have only 0.37% of IIP in approximately 146.800 cases. In the literature so far, there is no study with this number of cases focusing on group IIP (ASC-US). This little number of cases with group IIP in our collection may be due to the extensive training of our certified CTAs and our Cytopathologists and after adding the immunocytochemistry other research groups like Rokita *et al*, 2012 (6) and Wentzensen *et al*, 2012 (7) as well as Dupin *et al*, 2015 (8) have added the immunocytochemistry (CINtecPlus). They have reported respectively increased sensitivity to detect dysplasia in the cervix or anus of 78, 92.3 and 64%. In our collection, there is approximately 1.48% positivity for CINtecPlus, which means approximately 3.7% detection of high-grade lesions (CINII and CINIII). If we added the cases with assumed (suspect of) positivity, we will get approximately 80.5%. Up to date we do not find research groups that have investigated L1-capsid in the group IIP. In our collection, there are four (3%) definitely positive cases, which confirms the diagnosis of CIN I.

The sensitivity of HPV-subtyping to detect dysplasia was approximately 87.2% in the work of Gilani *et al*, 2014 (9), and approximately 84.1% in Pichon *et al*, 2019 (10). In our work, there is HPV-HR-positivity in 26.4% in Group IIP. We have also focused on the HPV-HR-subtypes 16 and 18 in the cases of ASC-US. 12.9% of these cases were positive for HPV-16 and 0.7% were positive for HPV-18. We have also analyzed the HPV-HR-negative cases, which were 41.6%. We have found that in 13.6% in the cases with IIP and HPV-16-negativity, histologically certain CINII and III (HSIL), and 22.9% with LSIL and HSIL. We have also found that 14.6% with HPV-18-negativity, histologically certain HSIL (CIN II) and approximately 19.5% with LSIL and HSIL. Interestingly, there was in general 19.2% of these cases with negativity for both HPV-16 and HPV-18. 14% of them were diagnosed later as HSIL [CIN II (8.4%) and CIN III (5.6%)].

In conclusion, the results of this study with this number of cases ensure the need of conventional cytological examination as well as the additive immunocytochemistry in suspicious cases of group IIP to confirm the diagnosis and to exclude the higher dysplasia. 3.7% of the cases of IIP will have high-grade dysplasia (HSIL). The sensitivity of conventional cytological examination and the added immunocytochemistry was up to 80.5%. The limitation by this examination is the need of human power (CTAs and certified cytopathologist) as well as the continuous training. 33.7% of cases with IIP and HPV-HR-negative were immunocytochemically evaluated as suspect of positivity and approximately 0.5% were surely positive. The advantage of HPV-subtyping is that machinery work

with screening too much number of cases in little time but it is not accurate in indicating the presence of dysplasia and can be misleading, especially in negative cases as other high- or low-risk subtypes of HPV not included in the HPV-HR test may be present.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MA developed the idea of the work and study design, interpreted the results and drafted the manuscript. OB collected data, interpreted the results and provided final approval. IE was involved in sample selection, collection and analysis of results. JDJ interpreted the results and was involved in analysis of figures. MA and OB confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The approval was granted by the ethics committee (Ethics Committee of the medical association, Hannover, Germany). The samples were anonymous with respect to measurements of data protection. An informed consent for inclusion into the study was waived, as patient records were anonymized and retrospectively analyzed. The samples were anonymous with respect to measurements of data protection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Arbyn M, Bergeron C, Klinkhamer P, Martin-Hirsch P, Siebers AG and Bulten J: Liquid compared with conventional cervical cytology: A systematic review and meta-analysis. *Obstet Gynecol* 111: 167-177, 2008.
- Forouzanfar MH, Foreman KJ, Delossantos AM, Lozano R, Lopez AD, Murray CJ and Naghavi M: Breast and cervical cancer in 187 countries between 1980 and 2010: A systematic analysis. *Lancet* 378: 1461-1484, 2011.
- Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 127: 2893-2917, 2010.
- Robert Koch Institute: Malignancy in Germany 2007/2008. *Epidemiology* 8, Berlin, 2012.

5. Hilal Z, Tempfer C, Schiermeier S, Reinecke J, Ruppenkamp C and Hilal Z: Progression or regression?-strengths and weaknesses of the new munich nomenclature III for cervix cytology. *Geburtshilfe Frauenheilkd* 75: 1051-1057, 2015.
6. Rokita W, Skawiński D, Zmelonek-Znamirowska A, Kedzia W, Karowicz-Bilińska A, Spaczyński R and Spaczyński M: Results of pap smears and immunocytochemical detection of the p16 and Ki67 proteins in women with cervical intraepithelial neoplasia and cervical cancer. *Ginekol Pol* 83: 822-826, 2012 (In Polish).
7. Wentzensen N, Schwartz L, Zuna RE, Smith K, Mathews C, Gold MA, Allen RA, Zhang R, Dunn ST, Walker JL and Schiffman M: Performance of p16/Ki-67 immunostaining to detect cervical cancer precursors in a colposcopy referral population. *Clin Cancer Res* 18: 4154-4162, 2012.
8. Dupin C, Siproudhis L, Henno S, Minjolle S, Arvieux C and Tattevin P: Use of human papillomavirus genotyping and biomarkers for targeted screening of anal dysplasia in human immunodeficiency virus-infected patients. *Dig Liver Dis* 47: 423-428, 2015.
9. Gilani SM, Tashjian R and Fathallah L: Cervical cytology with a diagnosis of atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H): A follow-up study with corresponding histology and significance of predicting dysplasia by human papillomavirus (HPV) DNA testing. *Arch Gynecol Obstet* 289: 645-648, 2014.
10. Pichon M, Joly M, Lebreton F, Benchaïb M, Mekki Y and Devouassoux-Shisheboran M: Evaluation of p16/Ki-67 dual staining compared with HPV genotyping in anal cytology with diagnosis of ASC-US for detection of high-grade anal intraepithelial lesions. *J Cytol* 36: 152-156, 2019.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.