REVIEW

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Review of the biotechnologies and tests used for precancerous cervical lesions diagnosis

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Abstract

This paper draws on the author's extensive experience in the clinical research focused on the implementation of the new biotechnologies able to identify precancerous cervical lesions and is intended to be a systematic approach to new achievements. The goal of this review is to provide updated information concerning the significance of each biotechnology used in clinical medicine to screen women for cervical cancer or to allow a pertinent discrimination between spontaneous remission lesions and progressive lesions. The data is arranged according to the most widely used biotechnologies and the worldwide recommendations of specialized guidelines.

Keywords: Pap test, HPV genotyping test, colposcopy, immunocytochemistry, immunohistochemistry, sensitivity.

Introduction

Advances in scientific research opened new opportunities for physicians to identify untimely cervical lesions that can turn into cervical cancer. Despite these promising circumstances, the results of various types of laboratory investigations are sometimes unsatisfactory and could lead to inappropriate decisions in the management of cervical lesions. This is the reason why a useful approach is to associate and correlate the results of different laboratory tests and then seek a unitary understanding. Despite the many achievements made in the diagnosis of precancerous cervical lesions, correct interpretation of results requires a deeper level of detail, able to justify monitoring or surgical attitude.

The purpose of this paper is to highlight the up-to-date laboratory tests recommended for an early detection of cervical dysplasia that could gradually develop into cancer. The data sources used for this paper consist in international databases (PubMed, Medscape, Scopus) and the results of the author's own research conducted in this field. Our research was funded by the Romanian National Research Grant: Partnerships in Priority Areas No. 61-44/ 2007-2010; Grant Director: Ruxandra Viorica Stănculescu.

In support of the above-stated view, the next sub-chapters include a description of the most important laboratory tests used. Therefore, we are going to mention and depict, one by one, the principal methods used by the clinicians and researchers operating in this area of major medical and social interest; we are going to focus on the following types of tests: Papanicolaou stain – Pap test; human papillomavirus (HPV) genotyping test; colposcopy; immunofluorescence expression of cyclin markers in cell culture; immunocytochemistry tests able to identify cyclin markers; immunohistochemistry tests able to identify cyclin markers and vascular endothelial growth receptors (VEGFRs).

Papanicolaou stain (Pap test) cytological diagnosis

The Papanicolaou stain – worldwide known as Pap test – is the most high-performing method used for cervical screening. Cervical cells have to be extracted from the squamocolumnar junction (SCJ) of the cervix junction. Two different methods are available for the same Papanicolaou stain, as regards the technology able to collect cervical cells. They are known as conventional type and liquid based cytology type methods.

The conventional type method employs the cellular sample fixation pursued by classical Pap staining (EA 50, Harris' Hematoxylin, Orange G and various concentrations of ethanol). The procedure is short and does not take more than 45 minutes.

Another more advanced biotechnology uses the liquid-based collection milieu. Therefore, cervical cells are pulled

together by means of a cytobrush, which is then put into a collection support liquid (*e.g.*, CytoFAST). This method provides the opportunity to preserve the physiological constitution and the morphology of any cell types for 24 months at room temperature, and also to supplementary explore (*e.g.*, HPV oncotypes, cyclin immunomarkers).

The published data show that the monolayer slides obtained from a liquid-based collection environment are more effective than the results of the conventional smear screening method. The classification of cytological results had been done until 2014 according to the *Bethesda System Criteria* as established in 2001. In 2014, these criteria underwent minor changes, such as the use of the female patient's age to pathologically consider the presence of endometrial cells in the samples. According to the latest *Bethesda System Criteria*, when endometrial cells are detected in the samples taken from a patient aged over 45 years, clinicians should consider the possibility of an endometrial abnormality and an endometrial curettage must be performed [1, 2].

On the other hand, the new *Bethesda System Criteria* do not acknowledge the necessity to create a new category for squamous lesions with LSIL (low-grade squamous intraepithelial lesion) and few cells suggestive of concurrent HSIL (high-grade squamous intraepithelial lesion). According to the latest *Bethesda System Criteria*, the cytologist must only decide whether the diagnosis is LSIL or HSIL and take into account that the latter requires to move the woman to colposcopy.

HPV genotyping test

Many methods are available to identify the high-risk (HR) and low risk (LR) genotypes of HPV infections. The right method should be chosen properly for each individual case, considering the advantages and limitations of each procedure.

HPV genotyping test can be done by using one of the next methods: nucleic acid hybridization assays, signal amplification assays and nucleic acid amplification [3].

We are going to briefly depict each group of biotechnology involved in the HPV genotyping test.

Nucleic acid hybridization assays generate high-quality information, but have some disadvantages, such as low sensitivity and the need for a large amount of purified DNA [4].

Signal amplification assays correspond to another biotechnology represented by two tests known as the Digene HPV test, which uses Hybrid Capture 2 (hc2) technology, and the Cervista HR-HPV assay [5].

Nucleic acid amplification methods imply many types of methodologies based on microarray analysis, Papillo Check, polymerase chain reaction (PCR), real-time (RT) PCR, Abbott RT-PCR, HPV genome sequencing, the Linear Array, CLART HPV, INNO-LiPA, Clinical Array HPV, microplate colorimetric hybridization assay (MCHA), HPV-mRNA detection, HPV viral load quantification and integration.

The COBAS 4800 HPV test was approved by *Food and Drug Administration* (FDA) in the USA, in 2014. It is a PCR method using the same fluorescent label for the fluorescent signal from 12 HR-HPV genotypes and simultaneously highlighting three distinct fluorescent labels of HPV16, HPV18 and β-globulin signals.

The principal methods used to uncover HPV integration are PCR, fluorescence *in situ* hybridization (FISH), and RT-PCR. It is important to know that the methodologies, which used FISH and RT-PCR, permit to assess the ratio between the levels of E2 and E6/E7 HPV oncoproteins. When the viral genome reveals a 1:1 ratio between the E2 and E6/E7 oncogenes, the clinician gains information about the HPV nucleus integration [6].

Colposcopy

Colposcopy was first introduced in Europe by Dr. Hans Hanselman (1920) and improved by Schiller (1927); 40 years later, it was introduced in USA by Dr. Sheffey and used throughout the years as an important tool to diagnose cervical abnormality. Nowadays, to provide an accurate diagnosis, colposcopy is used along with HPV test or Pap test according to the medical practice specific to the various geographic areas of the world. The methodology used to perform colposcopy comprises acid acetic test followed by Lugol test or standalone visual inspection with acid acetic (VIA) test, usually recommended in USA.

The utility of colposcopy as regards its ability to early detect a cervical dyplasia increased over time due to the implementation of the risk score, which allows to integrate the colposcopic diagnosis with the corresponding histopathological diagnosis. The criteria agreed by the *International Federation for Cervical Pathology and Colposcopy*, in 2011, require a description of the following conditions: adequate or inadequate (the reason should also be specified), squamocolumnar junction visibility and transformation zone type (TZT); colposcopy must also reveal the size and region of cervical colposcopical lesions, with or without the TZT, the "inner border sign" and the "ridge" sign. Finally, the colposcopic findings are able to be joined/assessed into a colposcopic diagnosis degree ranging from minor to ICC or miscellaneous conditions such as erosion, condyloma, polyp, cyst, endometriosis, inflammation, vaginal stenosis, congenital transformation zone [7].

Despite the large number of biotechnologies developed for use in precancerous cervical diagnosis, colposcopy continues to be a very important tool able to reveal the dysplasic changes of the cervical epithelium and estimate the borders of cervical resection [8].

Immunofluorescence expression of cyclin markers in cell culture

Immunofluorescence (IF) assay on epithelial cervical cell culture is a research biotechnology able to confirm the diagnosis obtained by other investigation methods. Specific literature reveals some research results concerning the immunoexpression of cell cycle regulators such as $p16^{INK4a}$, p21, p27, p53 and Ki67 cyclins. The technology is laborious, requires many steps, histopathology training and a more time-consuming slide preparation. The

biotechnology for IF assay is exposed to many dangerous events that could compromise the diagnosis results.

The pieces of tissue are selected from suspected lesions detected by colposcopic images; the size of the cervix biopsy tissue for one piece must be at least 2 mm in diameter and the sample must be taken from each quarter portion of the cervix. The biopsy specimens must be immediately sent to laboratory in a transport medium (2% Modified Eagle's Medium Dulbecco with antibiotic), in thermally insulated bags. The primary cell culture is obtained by cultivating small fragments of tissue known as explants. To reach a greater number of cells, laboratory workers must achieve a stable and durable cell culture. Therefore, the methodology uses the keratinocytes restriction milieu. To accurately establish the epithelial origin of cell cultures, the researcher must pay attention to cytokeratin expression, which is considered an epithelial cell marker. The anti-pan-cytokeratin antibodies enable the recognition of highly conserved sequences present in all types of cytokeratins and therefore are able to identify the epithelial nature of CulCel. After the epithelial cells culture has been obtained, the next step consists in the incubation with monoclonal primary antibodies derived from rabbit, both for cyclin inhibitor proteins ($p16^{INK4a}$, p21, p27), and for anti-p53 and anti-Ki67. The technique used is indirect immunofluorescence (DakoCytomation, Glostrup, Denmark) with primary antibodies (Santa Cruz Biotechnology California, USA) on 1:50 dilution. Anti-rabbit secondary antibody produced in goat was used in combination with Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen, USA). 4',6-Diamidino-2-phenylindole (DAPI) dye (Sigma Chemical, St. Louis, MO, USA) was used to highlight the nuclei.

The examination of cell cultures was performed by using the Nikon TE300 microscope equipped with Nikon D1X image acquisition system, 40×, 60× and 100× Plan Apo Nikon objectives. The IF expression of Ki67 and $p16^{INK4a}$ cyclins in nucleus and cytoplasm was performed on 500 cells for each cell culture. In order to quantify the percentages of IF expression on epithelial cell cultures, Stănculescu *et al.* (2013) introduced, by similarity, a score resembling the well-known score used by Eleutério *et al.* (2007) in immunohistochemistry diagnosis [9].

Therefore, the immunofluorescence score for $p16^{INK4a}$, p21, p27, p53 cyclin inhibitors and Ki67 proliferative marker is described as the percentage of immunofluorescence expression ranging from <1% to 100%; therefore, it is possible to create a score from 1 to 5. Stănculescu *et al.* (2013) applied this working methodology in their research, which allowed them to achieve enhanced diagnosis accuracy.

Immunofluorescence on cell epithelial cervix culture could be used as a research biotechnology able to reveal the intensity of cyclins expression, which allows physicians to perform a more thorough analysis that enables clinical connection. We conclude that these biotechnologies, albeit requiring many abilities, can supply valuable immunofluorescence expression, able to increase the diagnosis accuracy. The following figures endorse the aforementioned observations (Figures 1–3).

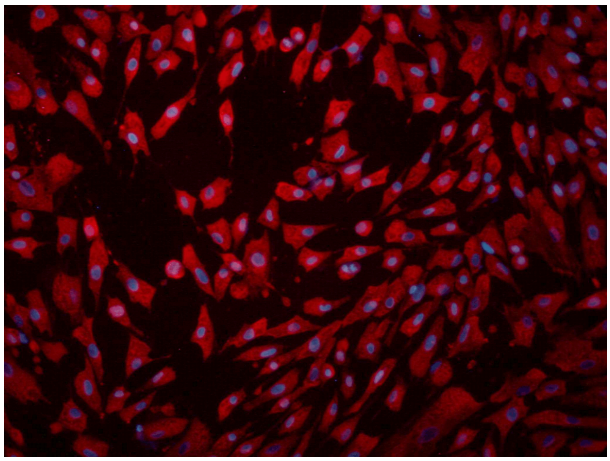

Figure 1 – ***Immunofluorescence: cytokeratin in cell cervix culture, medium cervical dysplasia; red – pan-cytokeratin; blue – nuclei, ×200 (Romanian National Research Grant: Partnerships in Priority Areas No. 61-44/2007-2010; Grant Director: Ruxandra Viorica Stănculescu. Collection of Vasilica Bauşic, MD, PhD).***

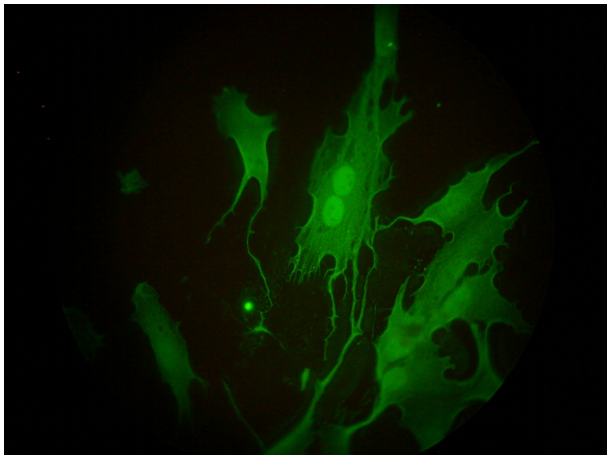

Figure 2 – ***Immunofluorescence: expression of cyclin D1 in cell culture, cervix cancer, ×600 (Romanian National Research Grant: Partnerships in Priority Areas No. 61-44/2007-2010; Grant Director: Ruxandra Viorica Stănculescu. Collection of Vasilica Bauşic, MD, PhD).***

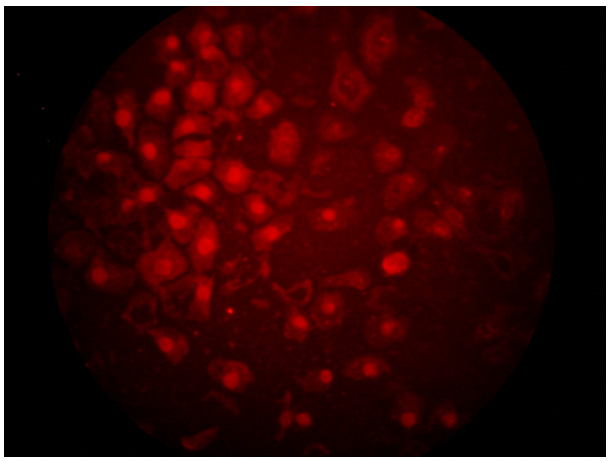

Figure 3 – ***Immunofluorescence: E6 oncoprotein in cervical cell culture, medium cervical dysplasia, ×200 (Romanian National Research Grant: Partnerships in Priority Areas No. 61-44/2007-2010; Grant Director: Ruxandra Viorica Stănculescu. Collection of Vasilica Bauşic, MD, PhD).***

Immunocytochemistry tests able to identify cyclin markers

Two main tests are available in clinical practice to distinguish between ASC-US (atypical squamous cells of undetermined significance) and ASC-H (atypical squamous cells, cannot exclude high-grade intraepithelial lesion) abnormal cytology results or between LSIL and HSIL results. These tests are performed using methodologies able to only recognize $p16^{INK4a}$ cyclin-dependent kinase inhibitor or this inhibitor combined with Ki67 proliferation marker – a test known as dual test. The immunochemistry for $p16^{INK4a}$ cyclin-dependent kinase inhibitor is recognized by CINtec $p16^{INK4a}$ ready-to-use cytology kit (clone E6H4) or by CINtec PLUS assay, both factory-made by MTM Laboratories AG (Heidelberg, Germany). While the $p16^{INK4a}$ immunocytological expression is retrieved both in nucleus and cytoplasm by a brown color, the immunostaining of Ki67 is present within the nucleus of the cells only and reveals a red color [9] (Figure 4). The dual test allows for the identification of two immunomarkers in the same cell.

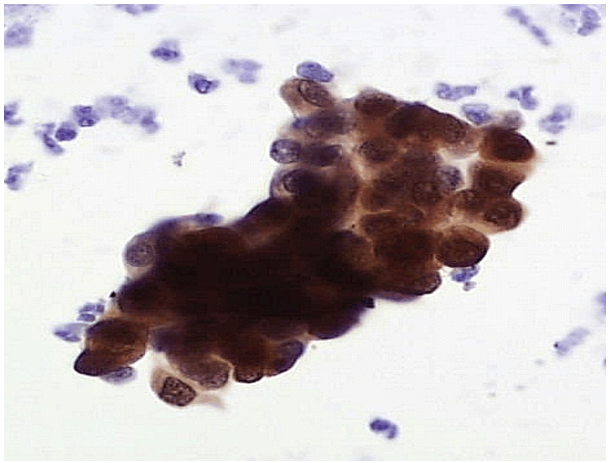

Figure 4 – ***Immunocytochemistry: $p16^{INK4a}$ cyclin in HSIL cervical lesion, ×400 (Romanian National Research Grant: Partnerships in Priority Areas No. 61-44/2007-2010; Grant Director: Ruxandra Viorica Stănculescu. Collection of Teodora Camelia Vlădescu, MD, PhD).***

Wentzensen *et al.* highlighted the key areas of interest in one of his published papers. According to him, the cytologist should focus on the following criteria: nucleus size, increased nucleus/cytoplasmic ratio, irregular nuclear shape, granular or hyperchromatic chromatin and cytoplasmic staining intensity [10].

In order to assess $p16^{INK4a}$ cyclin-dependent kinase inhibitor, the cytologists agreed to categorize samples as $p16^{INK4a}$ negative and $p16^{INK4a}$ positive. In practice, a sample is assessed as $p16^{INK4a}$ positive when the cytologist describes at least two positive characteristics from the above-mentioned criteria.

For enhanced diagnosis accuracy, Wentzensen *et al.* proposed a change-based score system, that incorporates the changes in the cell morphology in the absence of nuclear abnormalities, the presence of a single nuclear abnormality within the cells, the presence of at least two nuclear alterations within the same cell. Along with these criteria, the sample interpretation is correlated to the scoring system, which assigns points from 0 to 3 in line with the observation criteria. The next image is very suggestive because the $p16^{INK4a}$ intensity is strongly immunoexpressed both in cytoplasm and the nuclei; the last sample is larger in size, with irregular borders and chromatin texture changes (Figure 4). The handling of the score system allows cytologists to formulate a more precise cytological diagnosis able to assess the intensity of the immunocytological expression staining. A positive dual test shows that cervical dysplasia has the potential to develop into cancer. Schmidt *et al.* stress that the test must be assessed only when the CINtec Plus staining test is positive within the nucleus of atypical cells. Therefore, Schmidt *et al.* underlines the necessity to consider the dual test only in a morphologically atypical cell [11].

The assessment of the immunocytological samples in terms of $p16^{INK4a}$ or by $p16^{INK4a}$/Ki67 dual test, influenced by the cytologists' experience or by the use of a different reading platform, increases diagnosis accuracy.

Immunohistochemistry tests able to identify cyclin markers and vascular endothelial growth factor receptors

Before the progress of immunocytological tests, many pathologists only performed immunohistochemical expression on cells from biopsy specimens, especially to identify Ki67 immunohistochemistry marker as evidence of proliferative cervical lesion. Later, the immunohistochemistry test was performed by using indirect three-step ABC-Hsu technique, as modified by Bussolati & Gugliotta (A-20): sc-152 clone, source Santa-Cruz, 1:50 dilution (Figure 5).

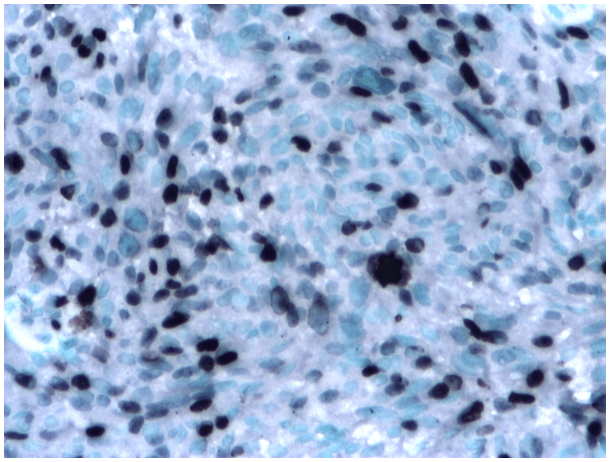

Figure 5 – ***Immunohistochemistry: Ki67 in tumor cells nuclei; 25–30% positivity; non-keratinizing squamous cell carcinoma of cervix, ×200 (Romanian National Research Grant: Partnerships in Priority Areas No. 61-44/2007-2010; Grant Director: Ruxandra Viorica Stănculescu. Collection of Florina Vasilescu, MD, PhD).***

However, immunohistochemistry tests have recently gained more importance as regards the treatment management by using anti-angiogenic factors. This statement is endorsed by the researchers who demonstrated that the intensity of the VEGFRs, the cervical lesion severity and the oncological treatment efficiency are all correlated to one another. Data suggest that for a better interpretation

of the immunohistochemistry expression of VEGFR2, a score system should be used in order to assess, by percentage points, the intensity of positive cells reactivity, as follows: 0–10% for very low reactivity (VLR), 10–25% for low reactivity (LR), 25–50% for moderate reactivity (MR), and >50% for intense reactivity (IR).

Correlating the results of the research conducted in this area: benefits and limitations

Taking into consideration the many years dedicated to investigations in this area, coupled with the large studies conducted across the world to determine the benefits and limitations of the biotechnologies involved in the diagnosis of precancerous cervical lesions by using immunomarkers, researchers elaborated conclusions able to provide a better and clearer view on the possibility of screening women for early detection of precancerous cervical pathology.

The conclusions focus on the benefits and limitations of such biotechnologies that contribute to an accurate diagnosis in cervical pathology. To reduce the incidence of cervical cancer worldwide, health policies must take into account the major discrepancies existing across countries in terms of women's access to cervical screening programs. To find solutions to this health problem, we must know which are the most affordable biotechnologies and what choices are best for women located in a specific geographic community where financial resources are scarce.

Researchers have determined that Pap test provides higher specificity and has lower sensitivity than the HPV test, when these tests are used as a screening tool [12].

Pap test is used both for opportunistic cervical cancer screening and within national screening programs. The Pap test result is influenced by the need to comply with the cell collection requirements; sometimes, it is also very important to train the cytologist for a correct cytological interpretation of the sample which must be read by more than one cytologist in order to ensure better diagnosis accuracy. Under such circumstances, a high-performance diagnosis becomes more expensive. Cytological diagnosis is not influenced by the cervical cell collection method [13]. In Europe, Pap test is considered the best solution in terms of cost/benefit ratio.

The limitations of the Pap test are correlated with its inability to distinguish between cases with ASC-US lesions and LSIL lesions, which are able to spontaneously go into remission or develop into cervical cancer.

Harald zur Hausen *et al.* demonstrated that cervical cancer is induced by HPV integration into the nucleus of the host cell. The advantage of HPV DNA testing is that, unlike Pap test it provides higher sensitivity in identifying cervical cancer. This fact backs the idea that it is better to screen women for cervical cancer by using HPV test. In clinical practice, it is not enough to demonstrate the presence of HPV, because HPV testing alone is not able to distinguish between transient and transforming HPV infection. Clarification comes by detecting E6 and E7 oncoproteins of HR-HPV genotypes that mainly encompass 16 and 18 HPV genotypes besides other oncogenotypes.

New research activities must be conducted to investigate the potential aggressiveness of HR-HPV oncogenes. One of the global perspectives must focus on the classification of HR-HPV sequence variations, particularly 16 and 18 genes, from women living in various geographic areas worldwide. The HR-HPV genotype variants have different oncogenic potential and can eventually lead to cervical cancer. A solution to early detect the most aggressive variants of HR-HPV genotypes is to identify the cases that may rapidly develop into cancer [14].

The management of cervical cancer incidence could be improved by drawing up a structural map indicating the worldwide distribution of HPV genotypes in cervical cytology specimens. This map could be a useful tool in appropriately recommending HPV vaccines to female populations in each geographic area. An argument in favor of this idea is the research conducted by Anton *et al.* (2011) in different geographic areas of Romania. The authors statistically proved that the high prevalence of HPV16, HPV18, HPV31 and HPV51 fully justifies the use of the HPV vaccines that are currently available [15].

A new approach was backed by the published conclusion of ATHENA study, in 2014. The study demonstrated that 57% of the women aged 25–29 years, whose cytological diagnosis was negative for intraepithelial cervical lesion (NIEL), had shown a histopathological diagnosis suggesting high-grade cervical intraepithelial neoplasia (CIN3+). These statistical observations are a strong argument in favor of recommending HPV test for cervical cancer primary screening [16, 17]. Nowadays, COBAS HPV technology has turned out to be a good solution able to mainly identify 16 and 18 HPV types together with other 12 HPV genotypes.

An HPV primary screening test, which can be used in conjunction with Pap test, was approved by FDA in USA, in 2014. Therefore, this new management criterion allows a screening interval extension from one year to 2–3 years when women are found to be HPV DNA positive with negative $p16^{INK4a}$ invasive cervical cancer (ICC).

As regards the advantage of the $p16^{INK4a}$ ICC and Ki67 dual test, statistical studies showed that a positive dual test could mean a high-grade cervical intraepithelial neoplasia as established by the histopathology exam [18, 19]. The dual test is able to reveal that the nucleus of the abnormal cell is affected by a transforming HR-HPV genotype infection.

A large number of immunohistochemical tests, including $p16^{INK4a}$, Ki67 and VEGFR, can provide a better assessment of histopathological diagnosis. As regards the significance of VEGFR2 immunohistochemistry expression on the biopsy samples, physicians must take into account that cytokines are able to stimulate angiogenic activity by increasing vascular permeability and therefore, by acting as mitogenic endothelial cells. Based on this observation, authors such as Nagy *et al.* (2011) and Stănculescu *et al.* (2015) focused their research on the possibility of identifying cases, which could have a different evolution consisting in spontaneous remission or progression to cervical cancer, and of managing the therapy response. Therefore, immunohistochemistry expression of VEGF receptors has the potential to suggest, by using a scoring system, a histological diagnosis such as CIN1, CIN2,

CIN3 or CIS (cancer *in situ*), cervical cancer (CC) [20]. A strong immunohistochemistry expression of VEGFR2 bias advocates the lack of metastasis but without any statistical proven data concerning overall survival or disease recurrence [21]. The researchers' results revealed that there is a tight correlation between LSIL cytology, HR-HPV and HSIL HP (histopathological) diagnosis. The data collected from published research showed that immunohistochemistry markers such as $p16^{INK4a}$ and VEGFR2 are closely correlated, as regards their intensity expression, with the gradual proliferative evolution of a cervical lesion. Negative or low positive $p16^{INK4a}$ ICC test results in HR-HPV positive cases are associated with LSIL HP diagnosis, which, in our view, clinically supports case supervision rather than immediate surgical intervention, an attitude validated by practice. By contrast, highly ICC $p16^{INK4a}$ positive nuclei in HR-HPV positive cases are associated with HSIL HP diagnosis and are also correlated with highly immunoexpression of VEGF receptors.

Many studies, including ALTS (ASCUS-LSIL Triage Study), ATHENA study, PALM, Compass Trial study from Australia, tackled the comparison between the sensitivity and specificity of $p16^{INK4a}$ immunocytomarker staining, alone or dual ($p16^{INK4a}$ and Ki67), *versus* HPV test, in order to unveil whether any of these tests is statistically more powerful to detect high-grade HP lesions ≥CIN2+. This issue has received great attention over the past 10 years and meta-analyses have been conducted by well-known researchers, including Holladay *et al.* (2006) [22], Wentzensen *et al.* (2007) [23], Denton *et al.* (2010) [24], Bergeron *et al.* (2010) [25], Samarawardana *et al.* (2010) [26], Izaaks *et al.* (2011) [27], Roelens *et al.* (2012) [28] and others. The brief conclusion of all these studies is that $p16^{INK4a}$ ICC is more accurate than HR-HPV test as concerns the triage of ASC-US cytology samples. As regards LSIL samples, $p16^{INK4a}$ proved to be more specific, but less sensitive than HR-HPV in the detection of HP lesions ≥CIN2+. The above-mentioned researches agreed that $p16^{INK4a}$ ICC positive test raises Pap test sensitivity and has higher specificity than HR-HPV test when it comes to identifying the lesions that could develop into high-grade HP lesions [22–28]. In line with these results, the novel $p16^{INK4a}$/Ki67 dual test proved to be a powerful supplemental biomarker useful in the triage of ASC-US and LSIL cytologies, closely related to the presence of HR-HPV within the nuclei of the cells where cellular cell cycle disruption is in progress [29–31].

All these achievements in the field of early cervical cancer diagnosis resulted in the incorporation of specific biomarkers in guidelines. The recommendations included in guidelines vary across countries depending on each national screening program. There are several possibilities to screen women for cervical cancer. First of all, it is worth mentioning that the United States' and Australia's national programs have included HPV vaccination, while screening is available to all women, vaccinated or not. Both American and Australian Guidelines introduced HPV testing in the national primary screening program. While the American Guidelines recommend the use of HPV test for screening and Pap test for co-testing in HPV-positive cases, the Australian Guidelines only recommend the standalone HPV test. In Europe, the Guidelines' recommendation for national cervical screening programs only sets out the use of Pap test as the primary test to screen women for cervical cancer. Worldwide, where no national screening programs are in place, biomarkers are recognized as able to perform the triage between abnormal cervical cytologies such as ASC-US or LSIL, by using $p16^{INK4a}$ ICC test or $p16^{INK4a}$/Ki67 dual test. Another biomarker useful in the triage between transient and proliferative HPV infection is E6/E7 mRNA oncoproteins. E6/E7 mRNA identification within the cell nuclei in ASC-US or LSIL cytologies is considered strong evidence of the presence of HPV transforming infection.

The progress made in relation to the national screening programs' results prompted changes as regards the screening interval. A one-year interval between tests was recommended when the screening for cervical cancer was firstly introduced. Today, considering the foremost research results endorsed by extensive statistical data, the *World Health Organization* (WHO) developed in 2013 the *WHO Guidelines for Screening and Treatment of Precancerous Lesions for Cervical Cancer Prevention*, which include changes as regards the screening interval and the age of women who need this assessment. The main recommendation is to enroll all women aged 30 to 49 years. The new conception highlights that such criteria are more important than the screening of individual women by using multiple screening tests [32, 33].

The Guidelines' recommendation is to use HPV test and VIA screening. The Guidelines indicate that screening should be repeated in three to five years following a negative VIA result and in five years when the HPV test result is negative. Therefore, a negative result in the HPV test makes it possible to extend the interval between screening rounds. The Guidelines advise that Pap test should be performed by trained cytologists within national screening programs, and be repeated with rescreening within three years [34].

Following the ATENA study results, the Guidelines recommend that the time period between two screening rounds is one to two or three years when the HPV DNA test result is positive and the $p16^{INK4a}$ ICC test is negative.

Successful screening programs need funds and proper management in line with national resources. Financial assistance from donors is welcome in least developed countries [35].

Conclusions

The *Guidelines for Screening and Treatment of Precancerous Lesions for Cervical Cancer Prevention* (2014) set out that the screening programs consisting in HPV test or Pap test must be performed both for unvaccinated and vaccinated women. Reported data highlight that when the HR-HPV test is positive, a co-testing with a Pap test increases diagnosis accuracy. The most advisable option to triage ASC-US and LSIL cervical lesions that can develop into cancer is the use of immunomarkers such as $p16^{INK4a}$, $p16^{INK4a}$/Ki67, E6/E7 mRNA. The interval between two screening rounds can be longer for women with a negative test, as follows; five years for women with negative HPV results and three years for women with negative Pap test results. Women who test positive for HR-HPV must be directed to colposcopy. Immuno-

cytomarkers and immunohistochemistry markers are also involved in the management of cervical dysplasia follow-up program, with VEGFR2 immunohistochemistry marker particularly able to show the oncological treatment response. In the future, new biotechnologies and extensive research on HR-HPV genes sequence variations might be an opportunity to early identify cases of HPV infection that could rapidly develop into cancer.

Conflict of interests

The authors declare that they have no conflict of interests.

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Author contribution

Ruxandra Viorica Stănculescu and Elvira Brătilă equally contributed to this article.

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