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Biotechnologies Involved in Differentiation of Cervical Lesions

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Abstract

The purpose of this paper is to describe the updated biotechnologies approved to be used for identifying precancerous cervical lesions in clinical practice. The paper focuses on the new biotechnologies able to detect human papillomavirus (HPV) such as nucleic acid hybridization assays, signal amplification assays, and nucleic acid amplification. Particular attention is given to the discussion regarding the differences among the biotechnologies used, such as Digene Hybrid Capture test using Hybrid Capture 2 technology and the Cervista HR-HPV assay. The scientific progress is emphasized by new markers such as cyclin p16^{INK4a}, viral oncoproteins E6 and E7, high-risk (HR) HPV genotyping, and dual test p16/Ki67. The results of the large ongoing studies conducted worldwide highlight these markers' capacity to disclose the differences between transient and transforming HPV infection and mild abnormal cytologies which could spontaneously regress or develop into cancer. Although both screening programs and opportunistic screening concerning cervical cancer are used worldwide, major geographic differences exist nowadays as regards the access to these programs. Finally, to achieve the objective of this study, the recommendations of various guidelines available across Europe, United States, and Australia, as well as the diagnosis tests accessible to women in low-resource countries are presented.

Keywords: Pap's smear, HPV genotyping test, E6/E7 mRNA, p16^{INK4a}, dual test p16^{INK4a}/Ki67

1. Introduction

According to the data reported by GLOBOCAN 2012 (IARC), cervical cancer is included among the leading causes of cancer worldwide. As regards the incidence of cervical cancer, GLOBO-

CAN report underlines that this type of cancer is the fourth most common cancer in women, and the seventh overall. The estimated number of new cases for 2012 is 528,000 [1]. For the same year, the registered number of deaths from cervical cancer was assessed at 266,000 cases. In terms of cervical cancer incidence and death by this disease, there is a major difference between the demographic regions of the world, strongly corresponding with the resource-setting level. Mortality varies 18-fold between various world regions, with rates ranging from less than 2 per 100,000 (in Western Asia, Western Europe, and Australia/New Zealand) to more than 20 per 100,000 (in Melanesia (20.6), Middle Africa (22.2), and Eastern Africa (27.6)) as stated by the GLOBOCAN 2012 report. A valuable consequence of the cervical cancer screening programs' implementation is that the incidence of cervical cancer rate dropped to half or more than half over the past 30 years, especially in countries with high levels of resources. For 2016, according to the American Cancer Society's estimates for cervical cancer in the United States, approximately 12,990 new cases of invasive cervical cancer will be diagnosed and 4120 women will die from cervical cancer. Cervical cancer is extremely rare in women younger than 20 and women over 65 years of age; only 15% of cervical cancer was reported to be identified at these ages. The improvement of collected data quality (statistics, information range) as concerns the occurrence of cervical cancer and the death by this disease is due to the implementation of national screening programs in many countries. Nowadays, more types of cervical cancer screening programs are in place, and a larger number of different biotechnologies are available across the world, which allow to identify early precancerous conditions of the cervix and therefore to obviate progression to cancer. On the other hand, it is very important that clinicians know the usable benefits and potential harms of biotechnologies able to achieve the triage of women with abnormal cytology or to identify cases with high-risk human papilloma virus in the stage of transforming infection. Due to a noteworthy scientific progress, clinicians now have many possibilities for early detection of a cervical lesion that might evolve into cervical cancer. The aim of the new biotechnology procedures is to achieve both high sensitivity and specificity in order to differentiate cervical lesions that may develop into cancer from those which spontaneously regress. Within the frame of this paper are included both the principal methods recommended by clinicians and researchers in cervical cancer field and the benefits and disadvantages of each biotechnology and marker. Our approach is designed so as to be useful especially to gynecologists with a view to a better management of the diagnosis and treatment of precancerous lesions. Hence, this paper explains what attempts should be made in the framework of each chosen biotechnology and what kind of tests increases the accuracy of an early diagnosis with regard to precancerous cervical lesions.

The data collection was performed by literature search, using PubMed, EMBASE, and the Cochrane Library (covering the 2000–2015 time frame), the main subject being detection of Human Papillomavirus Infection and cellular markers for early detection of precancerous cervical lesions. This study makes reference to the results of large studies published worldwide, such as in ATHENA, HERMES, PALMS, KPNC studies, Compass Trial, and the Newsletter on Human Papillomavirus—HPV Today (2015). This paper is structured in three sections. The first one covers different types of biotechnologies able to uncover precancerous and cancerous cervical lesions which are approved for use in medical clinical practice. The second section is dedicated to discussions about the benefits and drawbacks of each biotech-

nology reported to detect precancerous lesions. At last, attention is paid to recommendations of the overall current guidelines.

2. Biotechnologies able to detect the precancerous and cancerous cervical lesions

A strong contribution to the early detection of precancerous cervical lesions belongs to Papanicolou. The Pap's test has been in place since 1950, when George Papanicolou introduced this method as a cytological test able to detect morphological changes of abnormal cells. Generally, there are three kinds of markers for cervical cancer screening: viral markers, cellular markers, and epigenetic markers, which can identify, alone or in combination, early precancerous cervical lesions.

The laboratory tests able to reveal precancerous and cancerous cervical lesions use cytological, viral, cellular, and genetic markers such as Pap's test, HPV genotyping test, cellular markers, epigenetic markers, and other markers.

2.1. Pap's test: cytological diagnosis

Papanicolou stain—commonly known as Pap's test—is the best-performing method used for cervical screening. The cytologist analyses, via this test, the exfoliated cervical cells to detect the morphological changes characteristic to neoplastic alterations. The cervical cell samples must be taken within the squamocolumnar junction of the cervix. This area is relatively accessible, making sampling easy, but it is unable to provide information about the lesions within the endocervical canal such as adenocarcinoma precursors [2, 3]. For the same Papanicolou stain, there are two methods that differ in terms of collecting technology: one is used to collect cervical cells (being known as conventional type), and the other is liquid-based (cytology type).

The conventional type uses the fixation of cervical cells of the sample, followed by classic Pap staining (EA 50, Hematoxilina Harris, Orange G, and different concentrations of ethanol). The duration of this procedure is about 45 min.

The liquid-based collection medium biotechnology is deemed more advanced because it is more versatile. Cervical cells are collected by using a cytobrush, which is then introduced into a collection medium (e.g., Cytofast). This method gives the opportunity both to keep the physiological structure and morphology of any kind of cells for 24 months at room temperature and to perform more investigations (e.g., HPV oncotypes, cyclin immunomarkers) [4, 5]. Since 2013, Hospitex Diagnostics highlights that the monolayer slides from liquid-based collection medium are safer, faster and fully representative, in comparison with the conventional smear screening procedure. The interpretation of the cytological results is done according to the Bethesda System Criteria established in 2001. During the past 15 years, a cytological diagnosis which included medical recommendation was possible due to the Bethesda System Criteria.

2.2. Viral markers

The viral markers validated for usage in cervical cancer detection are HPV DNA and HPV genotyping, E6/E7 mRNA, and HPV proteins [6].

First of all, this paper describes, in line with its purpose, the biotechnologies which can be used to become aware of human papillomavirus infection (HPV) and identify the high-risk HPV genotypes (HR-HPV). There are several other technologies able to identify human papillomavirus infection. These methods have different benefits and drawbacks, for which reason a good option should be made to properly choose the individual test or screening program.

One of the following methods should be used to accomplish HPV detection: nucleic acid hybridization assays, signal amplification assays, and nucleic acid amplification [7, 8].

The techniques able to employ radiolabeled nucleic acid hybridization assay to identify HPV infection are Southern blotting, in situ hybridization, or dot blot hybridization.

Signal amplification assays pertain to another biotechnology consisting in two tests known as the Digene Hybrid Capture test which utilizes Hybrid Capture 2 (hc2) technology (by Qiagen) and the Cervista HR-HPV assay (by Hologic) [9].

Nucleic acid amplification methods involve many kinds of methodologies based on microarray analysis, PapilloCheck, polymerase chain reaction (PCR), real-time PCR, Abbott real-time PCR, COBAS 4800 HPV test, HPV genome sequencing, the Linear Array, CLART human papillomavirus, INNO-LiPA, clinical array HPV, Microplate colorimetric hybridization assay, HPV-mRNA detection, HPV viral load quantification and integration.

Many technologies approved in the United States and Europe, relying on large population-based studies and randomized trial, recommend using Hybrid Capture 2 test and Cervista test to spot HPV infection. Hybrid Capture 2 test was endorsed by the US Food and Drug Administration (FDA) in 2003 and is able to detect 13 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) or 5 low risk (LR)-HPV types (6, 11, 42, 43, and 44), using specific antibodies' signal amplification and chemoluminescent detection. In 2009, FDA approved the Cervista HR-HPV, which detects 14 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) by using a signal amplification method and a fluorescent signal for detecting specific nucleic acid sequences. In 2009, FDA approved another HPV DNA test, Cervista HPV 16/18, which uncovers HPV 16 and 18 oncogenotypes [10].

In April 2014, FDA approved Cobas HPV test, which is a PCR-based HPV DNA test using the same fluorescent label for the fluorescent signal from 12 HR-HPV and simultaneous recognitions with three separate fluorescent labels of HPV 16, HPV18, and beta-globulin signals.

When applied alone, the detection of HR-HPV DNA does not discriminate between the transient and transforming HPV infections. This differentiation is possible by discerning viral oncoproteins E6 and E7 mRNA and protein expression. The progression from a transient to a transforming HPV infection is identified by the high increase of E6/E7 mRNA expression [11, 12]. These oncoproteins interfere with key cellular cycles that control cell proliferation and apoptosis. E7 disrupts pRb from its binding to E2F, and E6 interferes with p53. Therefore, viral

oncoprotein E7 triggers uncontrolled cell cycling and E6 abrogates apoptosis. Two of the most widespread tests of the commercial assays designed to detect HPV E6/E7 mRNA are Pre Tect Proofer (Norchip), which detects five oncotypes of HR-HPV (16, 18, 31, 33, and 45), and APTIMA (GenoProbe) which covers 14 HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).

Another HPV assay—known as Qiagen assay (United States)—is an adoption of Digene HC2 assay. The signal amplification assay perceives 14 different HR-HPV oncotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66). The particularity of Qiagen HPV assay consists in the fact that this platform does not require electricity or water, but only needs a limited work space ($25 \times 50 \text{ cm}^2$). The result is obtained shortly (2.5 h only).

The main methods used to detect HPV integration are PCR, fluorescence in situ hybridization, and Real-Time PCR. The latter two methods allow calculating the ratio between the levels of E2 and E6/E7 HPV genes. When the HPV is integrated, the viral genome shows a 1:1 ratio between the E2 and E6/E7 genes [13].

2.3. Cellular markers

The cell markers approved by the World Health Organization (WHO) to be used in clinical practice are p16^{INK4a} and dual test p16^{INK4a}/Ki67. Another possible option arises from the ongoing research about a new clinical cell marker concerning the detection of topoisomerase 2 α , in cervical cytology slides.

2.3.1. Cellular markers: cyclin p16^{INK4a}

Uncovering of p16^{INK4a} is tightly correlated with HPV integration. In a normal cell, p16^{INK4a} blocks cyclin-dependent kinases (CDK) 4/6. Increased expression of the E6 and E7 oncogenes disrupt cell cycle regulation. In the normal cell, cell cycle progression is activated by CDK 4/6 and partially regulated by p16^{INK4a}. However, because in HPV-transformed cells, cell cycle activation is caused by E7 and not by CDK 4/6, p16^{INK4a} has no effect on the cell cycle activation. Increased expression of p16^{INK4a} in cells driven by viral oncogene-mediated cell cycle dysfunction can be distinguished through cellular immunostaining by immunocytology or immunohistology tests [14]. In brief, p16^{INK4a} is a tumor-suppressor protein and cyclin-dependent kinase (cdk) inhibitor that blocks CDK 4/6-mediated pRb phosphorylation to inhibit E2F-dependent transcription and cell cycle progression. It is obvious that the progression of dysplastic lesions to cancer is highlighted by increased expression of two viral oncogenes, E6 and E7. The last-mentioned oncoprotein, E7, inactivates retinoblastoma gene product (pRB) that inhibits transcription of the cyclin-dependent kinase inhibitor gene p16^{INK4a}. This explains why the overexpression of p16^{INK4a} is similar to the increased activity of E7, and so the overexpression of both p16^{INK4a} and E7 are markers of HPV integration in the genome of the host cell [15]. The cyclin p16^{INK4a} must be evaluated as a stand-alone test and as an adjunct to cytology or HPV testing [16, 17].

The cervical cells are collected similar to those for Pap's smear test. The liquid-based collection medium has the advantage of being able to collect more cervical cells both for Pap's smear,

immunocytochemistry tests and for HPV genotyping test. In the current activity, the clinician has the possibility to demand two tests used to identify specific immunocytomarkers, such as p16^{INK4a} alone or the dual test which evaluates, on the same cervical cell, two immunocytomarkers consisting in p16^{INK4a} and Ki67.

Over the past 10 years, the cyclin inhibitor kinase p16^{INK4a} has been identified by immunocytochemistry staining using a CINtec p16^{INK4a} ready-to-use cytological kit (clone E6H4) manufactured by mtm laboratories AG (Heidelberg, Germany). Wentzensen published the morphological characteristics necessary to evaluate the immunocytological expressions of p16^{INK4a} within the cervical cell, both for nucleus and cytoplasm. Therefore, the criteria which must be analyzed are increased nucleus size or increased nucleus/cytoplasmic ratio, irregular nuclear shape, granular or hyperchromatic chromatin with variable cellular morphology, together with the intensity of cytoplasmic staining [18].

There are many models in the published literature that allow identifying the intensity of the immunocytoexpression of p16^{INK4a}. One method used for a correct interpretation of the immunoexpression classified samples as p16^{INK4a}-negative and p16^{INK4a}-positive when positivity was observed for at least two of the aforementioned criteria. On the other hand, to obtain greater accuracy in the slides' interpretation, a scoring system was introduced, which scored the absence and the gradual intensity of the immunocytological expression staining. Therefore, the scoring system assigns 0 point for cases without p16-positive cells (p16-), 1 point for p16-positive cells with changed morphology in the absence of nuclear abnormalities (p16+), 2 points for p16-positive cells with a single nuclear abnormality (p16++), and 3 points for the presence of at least two nuclear alterations within the same cell (p16+++ [18]. In this manner, the physician can establish a correct cytodiagnosis much more accurately.

2.3.2. Cellular markers: p16^{INK4a}/Ki67 dual-stained

The CINtec PLUS assay (mtm laboratories AG, Heidelberg, Germany) is currently gaining increased credibility; it is a commercially available test which combines the immunostaining of p16^{INK4a} with the immunostaining of Ki67 within the same cervical cell [19].

This methodology allows the cytologist to see, within the same epithelial cell, the nucleus stained red for Ki67 immunoexpression, and both the nucleus and the cytoplasm stained brown for p16^{INK4a} immunoexpression. The test has predictive power to identify the progressive evolution to a higher degree of dysplasia and cancer. Positive dual staining is significant only for cells with modified morphology (atypical cells). Schmidt and other researchers [20] highlight the fact that for a better cytological diagnosis, the most important aspect is the positive dual staining within the nucleus of atypical cells. The evaluation of the p16/Ki67 dual staining could be better ascertained by an automatic reader system that obviates subjectivism.

2.3.3. Other cellular markers

Other cellular markers expressed in the S-phase of uncontrolled cellular cycle due to the activity of HPV oncoproteins in the stage of transforming infection include Topoisomerase 2 α (TOP2A) and minichromosome maintenance protein 2 (MCM2). These markers could be

noticed in SurePath cervical cytology specimens by an indirect polymer-based immunoperoxidase method (ProEx C, TriPath Oncology, Burlington, NC). The cytologist is interested in evaluating the presence of nuclear stain in epithelial cells, and the combination of nuclear staining and abnormal morphology. ProEx C is an immunocytochemical test, able to identify the possibility of proliferative process in women with low-grade cytological abnormalities [21].

There are also many other studies that recommend the immunocytochemical test ProEx C for TOP2A and MCM2 as adjunct test to conventional ASC-US cytology test.

2.4. Epigenetic markers

Nowadays there are ongoing studies about the chromosomal imbalances involved in the development of cervical cancer. Researchers pay attention to the chromosomal changes occurring early in the proliferative process. Epigenetic markers consist in DNA methylation, chromosomal abnormalities, miRNA abnormalities, and proteomics. The literature data reveal that it is about host methylation and viral methylation. Many genes are frequently methylated and remain in a silent stage during carcinogenesis, acting as negative regulators of cellular cell cycle. To identify the increased frequency of DNA, methylation of many genes (e.g., *DAPK*, *CADM1*, *TERT*, *CDH13*, *MAL*) in the early stage of carcinogenesis may hint that these could be biomarkers for early detection of cervical cancer [22]. Studies were not focused on a single gene only, and therefore, a gene panel was developed (e.g., *CADM/MAL*; *RAS β/TWST/MGMT*), targeting to improve the possibilities to attain earlier the best triage of HPV-infected cervical lesions. The analysis of viral methylation suggests that the promoter regions of oncoprotein E5 and E7 are more frequently methylated in the later stages of carcinogenesis, and as a consequence these tests allow to detect HGCIN [23].

Cervical cancer recognizes genomic instability manifested by amplification of the same regions, especially 3q/TERC or loss of other regions such as 6q and 11q. These abnormalities in the chromosomal architecture could be used in the triage of HPV-positive women with ASC-US or LSIL conventional cytology [24]. Other biotechnologies including miRNAs with increased or decreased expression, proteomics within cervical–vaginal mucus arise; their target is to discover the best predictive markers to discover, at an early stage, the risk of progress from mild to severe dysplasia and cancer.

3. Biotechnologies and test results: advantages and limitations

3.1. Pap's test: advantages and limitations

Researchers agreed that the advantage of Pap's test consists in its high specificity concerning the detection of women who do not prove to be positive for cervical lesions. On the other hand, Pap's test is limited due to its low sensitivity in identifying women with dysplastic cervical lesion prone to develop into cancer. The studies conducted on women of Europe and North America showed that the sensitivity of the cytology in detecting CIN2+ lesions is only 53%, while other studies reveal a modest sensitivity (60–70%) [25, 26].

An advantage of Pap's test consists in its use for both opportunistic cervical cancer screening and national screening programs. The clinician must know that neither the changes in the terminology of cytology by Bethesda reporting system, nor the novel techniques such as liquid-based cytology do not prove more efficient in terms of the improvement of the sensitivity and specificity of Pap's test [27, 28]. On the contrary, Hospitex Diagnostics' report (2013) highlights that the monolayer slides from liquid-based collection medium are safer, faster, and fully representative in comparison with conventional smears screening procedure. We must admit that the introduction of liquid-based cytology has decreased the number of inadequate slides and allowed to advocate a reflex testing for other viral or molecular markers [29].

A disadvantage of Pap's test is the need to be interpreted by two or more specialists trained in cytology for a more accurate cytologic diagnosis; thereby, the test implies subjectivity in the evaluation of morphology cells [30]. Also, the costs of Pap's test are not negligible by two reasons, the first is the training cost of the specialist reader and the second is the fact that screening based on cytology frequently needs repeated Pap's test during the lifetime. Initially, the Pap's test was recommended at 1-year interval, and this is why Pap testing brings substantial cost burden on the health system [31]. The method of cytological screening is able to perceive the lesions that have a high risk of progression, but this prospect is limited by the fact that when used alone, the method can distinguish neither atypical squamous cells of undetermined significance (ASC-US) lesion, nor low-grade squamous intraepithelial (L-SIL) lesions able to spontaneously go into remission against lesions able to progress. Statistical data have shown that 10–15% cases with ASC-US and LSIL cytology develop CIN3 [32, 33].

3.2. HPV test: advantages and limitations

The disadvantages of Pap's test justify why it is necessary to identify the HR-HPV types. With this aim in mind, researchers are trying to define new marks in order to get more powerful characterizations for cervical cancer prevention.

As regards the time necessary to achieve a result concerning HR-HPV assay, an advantage can be seen in Qiagen platform which needs only 2.5 h, while Digene Hybrid Capture HR-HPV testing needs about 6 h. Therefore, the former platform allows both diagnosis and treatment in a single day.

The disadvantage of HR-HPV assay is that it exposes women to overtreatment, as, when applied alone, HR-HPV assay does not reveal the difference between transient and transforming HPV infections. These features do not make it possible to differentiate women with spontaneous remission of cervical lesions, from women with progressive cervical lesions that develop into cervical cancer. Hence, this triage of women with remission or progress of cervical lesion with HPV infection requires a larger number of investigations such as detection of E6 and E7 mRNA markers whose immunoexpression is a real proof of HPV integration in host cells.

The inconveniences of the three techniques which use radiolabeled nucleic acid hybridization assays to detect HPV infection consist in relatively large amounts of purified DNA, more time-consuming procedures, and low sensitivity of the results of test [8].

The Hybrid Capture 2 system has the added advantage of detecting 13 HR-HPV types and 5 LR-HPV types [34].

Cervista assay shows a high sensitivity and specificity to HPV 16/18 genotyping and 100% sensitivity in the detection of CIN3 [35, 36].

A deep analysis of the published reviews underlines that Hybrid Capture 2 test and Cervista HR-HPV have two limitations concerning both the lack of differentiation between single HPV genotype infections and multiple concurrent HPV genotype infections, and the shortage of a test in relation to quantitative viral load [10]. It is very important for the gynecologists to obtain details about the existence of HPV infection with type 16 or 18, because these types allow for a stratification of the risk with reference to the possibility of developing cervical cancer. Thereby, the detection of HPV 16 or 18 has both the advantage of stratifying the oncologic risk of HPV infection and of providing clinicians with information which is useful in managing the precursors of cervical lesions, taking into account that in situations with persistent infections, the risk of precancerous lesions' progression to cancer is between 10 and 15% in cases with HR-HPV 16/18, and below 3% for all other combined HR types [7].

Abreu and colleagues [7] put forward a concept aiming to classify the necessity of DNA HPV testing. These authors concluded that there are six circumstances which require the test, as follows:

- a. triage of women with equivocal or low-grade cytological abnormalities
- b. follow-up of women with abnormal screening results who are negative at colposcopy/biopsy
- c. prediction of the therapeutic outcome after treatment of cervical intraepithelial neoplasia (CIN)—management of follow-up
- d. primary screening for HPV DNA testing, alone or in combination with a Pap's test, envisaging to detect cervical cancer precursors
- e. gain valuable information on the persistence of certain HPV types
- f. investigations of regional and country-based prevalence of type-specific HPV to provide the baseline values against which the global impact of HPV vaccination can be assessed in the future [7].

3.3. Cellular markers: advantages and limitations

3.3.1. Cyclin p16^{INK4a}

The p16^{INK4a} positivity shows that the cervical cells are HPV-infected but could not clearly detect a real progress to cancer. The p16^{INK4a} positivity is correlated with HPV infection but could not allow to discriminate between a HR-HPV or a LR-HPV oncotype infection. On the other hand, the p16^{INK4a} expression is independent of the HPV type, and therefore genotyping is unnecessary. In the context of disruption of cell cycle regulation, the p16^{INK4a} expression by

cycling cells is a specific marker of HPV-E7 overexpression or other events that inactivate Rb [15].

The immunoexpression of p16^{INK4a} exists both in the nucleus and cytoplasm, but its intensity is in line with the degree of cervical dysplasia. Only the p16^{INK4a} overexpression within the nucleus of the cell shows a high degree of cervical dysplasia. The presence of low p16^{INK4a} immunoexpression within the cytoplasm could not be related to cervical dysplasia's progress to cancer. Another limitation of this marker is the subjectivism in immunostaining slides' evaluation, despite the above-mentioned criteria and the scoring system proposed by Wentzensen, Samarawardana, and Denton. The evaluation of slides requires special cytologist abilities to detect the morphological abnormalities of cells and interpret the immunoexpression degree p16^{INK4a}. The clinician must take into account the possibility that sometimes the cytologist notices the physiological presence of p16^{INK4a}. In this context, it is necessary to require a morphological evaluation of p16-immunostained cells with the purpose of distinguishing HPV-transformed cells from metaplastic cells [37, 38].

Along with the aforementioned features of cyclin p16^{INK4a}, and with the benefits and drawbacks of this test, the conclusion is that it must be evaluated both as a stand-alone test and as an adjunct to cytology or HPV testing. In line with the overall opinion, cyclin p16^{INK4a} is a specific biomarker able to identify dysplastic cervical epithelia in sections of cervical biopsy samples or cervical smears [34]. The meta-analysis of reviews published shows that there is a major heterogeneity in the methods used to identify p16^{INK4a} in samples. As regards the statistical value in detecting CIN2+ lesions, cyclin p16^{INK4a} has a sensitivity between 0.59 and 0.96, and a specificity flanked by 0.41–0.96 [17].

3.3.2. Dual test p16^{INK4a}/Ki67: advantages and limitations

Dual test p16^{INK4a}/Ki67 has a better interobserver reproducibility and accuracy in cervical cancer screening compared to stand-alone p16^{INK4a}. As indicated in Kaise Permanente Northern California (KPCN) study, where cancer screening was performed based on HPV and cytology co-testing by p16/Ki67 dual staining in 2400 HPV-positive women, the recommendation to use p16/Ki67 cytologies is feasible in routine cytology laboratories with minimal time of training and easy reproducibility [39, 40].

The evaluation of p16/Ki67 dual staining could be better conducted by an automatic reader system that reduces subjectivism to the highest possible extent.

p16^{INK4a}/Ki67 dual test has a higher sensitivity than Pap's test in identifying high-grade cervical lesions associated with HR-HPV infection and the same specificity as the above-mentioned test.

3.4. Epigenetic markers: advantages and limitations

Despite some extended studies investigating the utility of epigenetic markers, such markers are not yet applied in clinical practice and guidelines do not refer to samples of these biotechnologies. However, the researchers' discoveries as regards this solution proved the utility of

epigenetic markers for the triage of HPV-positive women with mild abnormal cytologies. There seems to be an opportunity in the future to self-sample from cervical mucus or vaginal fluid, and such samples could theoretically be investigated with reference to epigenetic markers.

4. Discussion: recommendations of the large studies and worldwide guidelines

Pap's test has limited sensitivity to detect precancerous cervical lesions but has high specificity. Compared to cytological diagnosis by HPV DNA testing, it offers a higher sensitivity and a long-term reassurance as regards the minimal risk of developing cervical cancer in women who were proven to be negative in HPV-DNA testing. Hence, these women are safe as regard a progressive dysplasia and could benefit from a large interval between cytological tests, with extension to 2–3 years of the screening interval [29].

In keeping with the ASC-US/LSIL Triage Study (ALTS)—1998, HPV DNA testing is a viable strategy able to clarify especially ASC-US cytology. In fact, ALTS Study counsels clinicians to manage ASC-US cytologies using three different options: triage by HPV testing as an adjunct to cytology, immediate referral to colposcopy, and conservative management with repeating Pap's test. The triage of ASC-US cytologies has relevant significance, because approximately 10–15% of women with ASC-US cytologies proved to be CIN3 at histopathological exam. The relevance of ALTS study consists in the fact that researchers deem that HPV DNA testing could be achieved only by triage of ASC-US cytologies, while the triage of mildly abnormal cytologies is not possible due to the high number of HR-HPV belonging to this female population [32].

Numerous studies have confirmed that HPV testing demonstrates high sensitivity and lower specificity for detecting high-grade cervical intraepithelial lesions. This poor specificity is explained by the fact that most HPV infections are transient and only a lower number of cases develop transforming infections.

Due to the fact that HPV DNA testing offers higher sensitivity and Pap's testing has higher specificity, researchers have progressed in line with the necessity of developing a new marker able to corroborate high sensitivity and specificity.

Berjeron and colleagues (2010) demonstrated on 500 cases, by using H&E-stained slides, as well as p16^{INK4a}-immunostained slides analyzed by the same 12 pathologists, that the diagnosis was improved and the sensitivity increased by about 13% after adding p16^{INK4a} immunostaining. The research results recommend using p16^{INK4a} for clinical practice, because this marker was proved able to identify both CIN1 lesions associated with HR-HPV types and CIN2+ lesions [41].

The papers published in 2010 by Samarawardana and Denton have shown that p16^{INK4a} immunostaining on cytology provides significantly better specificity than HR-HPV for the triage of ASC-US and LSIL cytology cases [42, 43].

In conclusion, the diagnosis accuracy is higher when clinicians suggest one of these options: HPV DNA testing in conjunction with Pap's test or p16^{INK4a} associated with Pap's test for the triage of ASC-US cytologies.

In 2014, ATHENA study results demonstrated that COBAS HPV technology contributes to the ASC-US triage. The researchers' remarks were useful for a new approach of the cervical cancer screening. Hence, ATHENA study highlights the fact that negative intraepithelial lesions (NIEL) on cytological exam proved to be \geq CIN3 on histopathological exam. The careful data analysis of ATHENA study has shown that more than 57% of women aged 25–29 years whose cytological diagnosis is negative for intraepithelial cervical lesion proved to have a histopathological diagnosis \geq CIN3. This evidence justifies why HR-HPV testing for genotypes 16/18 is more efficient in the primary screening of the cervical cancer compared to the Papanicolaou cytology test. Cervical lesions able to progress to cancer could be prevented by the early detection of 16 and 18 HPV genotypes by COBAS HPV technology [44]. This technology is able to simultaneously identify 16 and 18 HPV genotypes (the HPV types that are the most responsible for the progress of dysplastic cervical lesions) associated with other 12 oncogenotypes.

The major contribution of COBAS HPV technology consists in the information that individualization of the 16 or 18 HPV oncoproteins underlines the necessity to change the management of an abnormal middle dysplasia from follow-up strategy with Pap's test repeated in 1 year, to other strategies involving, for example, colposcopy, biopsies.

However, HR-HPV testing alone is not able to distinguish between transient and proliferative HPV infections. In view of the gains and weaknesses proven by research as regards the usefulness of Pap's test and HR-HPV in screening women in order to discover those cervical lesions able to evolve into cancer, it is obvious that attention should also be paid to other biomarkers for increased credibility. Therefore, the progress of research led to the necessity of highlighting an immunomarker capable to increase the sensitivity of Pap's test. The cyclin p16^{INK4a}, both in cytology exam and histology exam, is able to identify the HPV-infected cells and therefore to provide a more accurate diagnosis. As a matter of fact, it is acknowledged that the positivity of p16^{INK4a} is an important feature of high-risk HPV infected cells.

Women tested HPV DNA-positive and with p16^{INK4a} ICC-negative could be safely managed with follow-up HPV DNA testing in 2 to 3 years.

Conversely, the positivity for Ki67 in the cell nucleus is a marker of nuclear proliferation. The intensity of Ki67 immunoeexpression increases more strongly in abnormal cells. The advantage of p16^{INK4a} test used alone or within a dual test (p16^{INK4a} and Ki67) is that the high intensity of p16^{INK4a} and Ki67 immunoeexpression in the nucleus warns that the cell will develop into cancer. If p16^{INK4a} is positive in the cytoplasm only, there is a transient HPV infection, and the cervical lesion is able to spontaneously regress.

As regards the significance of p16^{INK4a}/ Ki67 dual staining immunohistochemical test, Samarawardana and colleagues showed as early as 2011 that this test has increased its capacity to identify high-grade cervical lesions [45].

The novel data established that the p16^{INK4a}/Ki67 dual staining by immunocytochemical (ICC) method is a better solution to identify the high-grade cervical lesions associated with HR-HPV infection [46].

Another aspect which must be thoroughly discussed consists in the careful measurement of the sensitivity and specificity of each biotechnology used for the diagnosis of cervical lesions [43].

A meta-analysis performed by Jolien Roelens (2012) highlights that p16^{INK4a} ICC has more accuracy than the HR-HPV test concerning the triage of ASC-US cytology samples. Both tests have similar sensitivities, but p16^{INK4a} ICC has higher specificity than HR-HPV test. In LSIL samples, p16^{INK4a} was more specific, but less sensitive than HR-HPV in the detection of \geq CIN2+ [20]. Over the past 10 years, similar results have been published by well-known researchers such as Izaaks, Denton, Holladay, Wentzensen [14, 43, 48, 49].

The positive p16^{INK4a} ICC test rises Pap's test sensitivity to identify the cervical lesions prone to develop into cervical cancer. Therefore, the intensity of p16^{INK4a} immunoexpression in the nucleus is in relation to the degree of cervical lesion.

In the study conducted by Denton and Bergeron (2010), it was shown that p16^{INK4a} cytology provides significantly better specificity than HR-HPV for the triage of ASC-US and LSIL. Both HPV-testing and p16^{INK4a} immunocytomarker test have similar sensitivity percentage rates, capable to detect high-grade cervical lesions at histopathological exam. In conclusion, the specialists involved in this research field have shown that p16^{INK4a} cytology has the potential of being used as a triage of abnormal cytology LSIL [43].

Beginning with 2010, Samarawardana et al. have demonstrated by statistical analysis that the sensitivity (Se) and specificity (Sp) of p16^{INK4a} for the detection of underlying CIN \geq 2+ are 81.7% and 83.3%, respectively ($p = 0.81$). They underline that the Se and Sp of HR-HPV are lower than those of p16^{INK4a}, albeit statistically significant: 78.1% and 50.9%, respectively ($p < 0.01$) [42].

There are many research studies recommending the usage of p16^{INK4a} as a supplemental triage biomarker for ASC-US and LSIL cytologies, which have already been assigned as "high-risk" after HR-HPV detection [49, 50].

Large studies approached the comparison between the sensitivity and specificity of immunocytomarker p16^{INK4a}/Ki67 dual stain versus HPV test in order to reveal if one of these tests is statistically more powerful to detect high-grade histopathological lesions \geq CIN2+. So, PALMS study is a bulky study which has enrolled 27,349 women from five European countries. All women aged 18 and older were tested by conventional cytology and p16/Ki67 dual test, while women aged 30 or older were tested by HPV DNA. The comparison between Se of p16^{INK4a}/Ki67 dual stain cytology versus Pap's test with regard to the detection of high-grade cervical intraepithelial neoplasia (HGGIN) has shown higher values for p16^{INK4a}/Ki67 test (86.7 vs 68.5%). As regards the Sp of the tests, it was comparable (95.2 vs 95.4%). By comparing Se and Sp of HPV DNA test versus p16^{INK4a}/Ki67 dual stain, the study remarks that HPV DNA test has a higher Se to detect CIN2+, 93.3 versus 84.7%, but a low drop in specificity, 93.0 versus 96%. On the other hand, the dual test p16/Ki67 sets the idea that the test offers the same high

sensitivity and specificity to identify HGCIN (see **Table 1**). The conclusions of researchers published on August 2015 within the Specialist Forum concerning PALMS study underline that the immunoeexpression of dual-stained p16/Ki67 biomarkers is a novel approach to scrutinize efficiently for HGCIN and to achieve the same specificity conferred by Pap's test.

Detection of HGCIN	p16/Ki67		Pap's test	
	Se	Sp	Se	Sp
p16/Ki67 dual test versus Pap's test	86.7%	95.2%	68.5%	95.4%
Detection of HGCIN	p16/Ki67		HPV DNA test	
	Se	Sp	Se	Sp
p16 ^{INK4a} /Ki67 vs. HPV DNA	84.7%	96%	93.3%	93.00%

p16^{INK4a}/Ki67 = dual test CINTech Plus; Se = sensitivity; Sp = specificity; HGCIN = high-grade cervical intraepithelial neoplasia.

Table 1. Cellular markers able to detect HGCIN sensitivity and specificity—results of PALMS study.

Similar attention is given to the cervical cancer screening of vaccinated women. In this regard, the trial known as Compass Trial is being conducted today in Australia. This trial enrolled 121,000 HPV-vaccinated women who are analyzed from the point of view of cervical screening programs using HPV test versus cytology tests [52].

Methods	Unites States	Europe	Australia
HPV vaccine	NSP		NSP
HPV DNA testing	NSP primary test	GR-triage	NSP primary test
Co-test PAP	NSP		NO NSP
Pap's test	Conjunction to HPV DNA testing	NSP	
P16	GR—Triage	GR—Triage	GR—Triage
P16/Ki67	GR—Triage	GR—Triage	GR—Triage
E6/E7 mRNA	GR—Triage	GR—Triage	GR—Triage

NSP = National Screening Programme; GR—Triage = Guideline Recommendations—Triage; NO = negative.

Table 2. Cervical cancer screening programmes and guideline recommendations—update.

In line with the recent discoveries made by researches and the large studies mentioned above, the guidelines approved in the United States recommend HPV vaccination, HPV primary screening and co-testing with Pap's test, with major benefits in terms of sorting abnormally low cytologies and extending the screening interval. The ATHENA study prescribes the circumstances for applying the HPV test: triage of ASC-US in women over 21 years of age, HPV test with reflex Pap's cytology in women between 25 and 65 years, and HPV test with

HPV 16/18 genotyping for primary screening in women older than 25. As to the accepted screening interval, this is 3–5 years for women negative at both intraepithelial malign cervical lesions (NIEMML) and HPV infection, and 12 months in cases with HPV-positive test, but cytology-negative for NIEMML, followed up by classic advices when the tests are constantly changed. Positive test for HPV 16/18 genotyping push the screening directly to colposcopy due to the presence of an absolute risk for CIN3+ progression. The interval for screening is prolonged to 5 years when women are negative at co-testing. Access to this screening program is given only to women with healthcare insurance [53]. The present guideline recommendations and cervical cancer screening programs existing nowadays are summarized in **Table 2**.

In Europe, there are many differences as regards the modality of carrying out the screening of cervical cancer, because health policies and financial resources differ among Europe's demographic regions. The most commonly used national screening program is mainly Pap's test; however, it is complemented by other new markers and biotechnologies prescribed by physicians with the goal of increasing diagnosis accuracy.

The Australian National Screening Program recommends only the screening using HPV test, and encompasses ages between 25 and 74 (applicable if women were screened in the past). The program includes both vaccinated and unvaccinated women. If the HPV test is positive, the follow-up program will be in line with cervical screening pathway [52].

5. Conclusions

The detection of carcinogenetic HPV DNA, and especially of HPV 16/18 genotyping, stands for approved tests to be used as primary screening and for triage of equivocal cytology equally on vaccinated and unvaccinated women.

Recognition of E6/E7 mRNA is largely applied in adjunct to primary HPV screening to select cases with HPV integrated in the host cell. The result of E6/E7 mRNA is able to achieve the triage of equivocal or mildly abnormal cytology.

Uncovering of HPV protein by cytology and histology immunostaining underlines the accuracy of diagnosis and can be used together with primary HPV screening or for the triage of equivocal or mildly abnormal cytology.

The success that was achieved in researches across the world showed that the identification of immunocytomarkers inside the same cervical cell—dual stain p16^{INK4a} and Ki67—warns more accurately about the possibility of progression to cervical cancer.

Correctly performed and interpreted, the results of approved tests for cervical screening programs allow to obtain an extended interval screening between 2 and 5 years, with a larger number of advantages in terms of diagnosis accuracy and healthcare costs.

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