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UPDATING THE SIGNIFICANCE OF SCREENING AND TRIAGE METHODS OF CERVICAL LESIONS – REVIEW

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ABSTRACT:
THE IDENTIFICATION OF WOMEN HAVING OR BEING ABLE TO DEVELOP CERVICAL CANCER IS A MAJOR CONCERN WORLDWIDE EXPRESSED BY ENDEAVORS TO IMPLEMENT NEW METHODS OF SCREENING AND TRIAGE FOR PRECANCEROUS CERVICAL LESIONS (PCL). THE PRESENT WORK ENCOMPASSES RESULTS OF MANY TRIALS AND OBSERVATIONAL STUDIES PUBLISHED ON INTERNATIONAL DATABASES DURING LAST 10 YEARS. THE PURPOSE OF CURRENT STUDY IS TO HIGHLIGHT THE FEATURES OF ADVANCED BIOTECHNOLOGIES, CONCERNING THEIR CAPACITY OF LEADING TO AN EARLY RIGHT DIAGNOSIS SUPPORTING, TO COMPARE THE EFICACITY OF THESE TESTS SUCH SENSITIVITY AS SPECIFICITY, TO TRIAGE BETWEEN REGRESSIVE AND PROGRESSIVE PCL, TO HIGHLIGHT THE DIFFERENCE CONCERNING SCREENING METHODS ADOPTED BY THE NATIONAL PROGRAMMES WORLDWIDE. THE RESULTS SHOWED THAT PAP TEST IS AN EXPENSIVE METHOD, WITH HIGH LEVEL OF SUBJECTIVE ASSESSMENT AND WITHOUT POSSIBILITY OF DISCRIMINATION BETWEEN DIFERENT KINDS OF CYTOLOGIES. HUMAN PAPILLOMAVIRUS (HPV) GENOTYPE ALONE DOESN'T ALLOW TO DISTINGUISH TRANSIENT VERSUS PROGRESSIVE HPV INFECTION. IMMUNOSTAINING CYCLINE P16INK4A AND THE MORE PERFORMANT TEST, DUAL TEST P16INK4A/ KI 67 ALLOW THE TRIAGE OF CASES ABLE TO DEVELOP CANCER USED ALONE OR AS CO-TEST TO PRIMARY HPV SCREENING. PRIMARY CERVICAL SCREENING WITH HPV GENOTYPE ASSOCIATE WITH ONE OF CO-CIYTOLOGIC TEST IMPROVE THE ACCURACY OF AN EARLY DIAGNOSIS.

KEY WORDS: CERVICAL CANCER, PRECANCEROUS CERVICAL LESIONS, BIOTECHNOLOGIES, PAP TEST, HPV GENOTYPE, P16INK4A, P16INK4A/ KI 67

INTRODUCTION

Despite the sustained efforts to identify precancerous cervical lesions and to stop cervical neoplastic progression, the cervical cancer incidence attains high rate, being the fourth most common cancer diagnosed in women. Several screening tests for cervical cancer have been agreed over the years and the recording of the results formed a very large scientific database. This huge amount of information obtained following multiple randomized trials conducted in the last 30 years made possible the comparison between the test methods results in order to identify one method or an association of methods able to achieve the highest percentage of sensitivity in early identification of precancerous cervical lesions. Modern biotechnologies offer a variety of screening and triage tests for cervical lesions. In the current situation, it is necessary to have a better knowledge of the benefits and limitations provided by biotechnologies approved as an integral part of cervical cancer national screening programs. The identification of women who can develop or have cervical cancer is a major global concern materialized through sustained efforts to implement various cervical cancer screening methods (CC) and triage of precancerous cervical lesions (PCL). This study seeks to summarize the particular characteristics of advanced biotechnologies and their ability to establish correctly an early diagnosis able to enact adequate therapeutic approaches.

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DIAGNOSTIC AND SCREENING BIOTECHNOLOGIES IN CERVICAL CANCER - REVIEW

Our study aims to analyze the results of several trials and observational studies published in the international databases (PubMed, Medscape, Scopus) over the past 10 years. The adopted criteria consist in identification of implemented biotechnologies, comparing the effectiveness of the results of these determinations in terms of sensitivity in CC detection and PCL triage, specifying the duration of obtaining the test result, mentioning the screening interval recommend. The study also included criteria such as presenting opportunities for accessibility of women around the globe to test methods and the balance between cost and benefits of the screening and diagnosis assumed methods.

IDENTIFICATION OF DIAGNOSTIC AND SCREENING BIOTECHNOLOGIES IN CERVICAL CANCER

Babeş Papanicolaou cytological testing – PAP test

Pap cytological test is performed both in national screening programs, and in opportunistic screening situations related too cervical cancer. Pap cytological test is characterized by high specificity and low sensitivity. The Pap cytological test sensitivity regarding the detection of intraepithelial cervical neoplasia grade 2 or more (CIN2 +) is appreciated as being limited between 53%-70% [11]. Over the past 12 years, a series of comparisons have been made on the quality of the cytological testing results: dry cytology versus liquid-medium cytology [12], [13]. The testing results using dry cytology vs cytology in liquid environment remain identical without changes in specificity and sensitivity. Hospitex Diagnosis Report (2013) emphasizes the fact that single layered blades obtained from cells collected in liquid environment (CLE) are more secure and more powerful representative in comparison with the conventional screening procedure. The limitations of cytological testing consist in high cost due to investments in training for the cytologist and due to the necessity of periodic test repeating. Initially recommended to be annually repeated, currently the cytological test is recommended to be repeated between 3-5 years in case of negative testing for HPV-HR [14]. Among other Pap cytological testing limitations, an important one is the test inability to allow differentiation of AUC-US or L-SIL lesions that spontaneously

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resolve or are able to progress towards cancer. Statistical data underline that 10-15% of the cases with ASC-US and L-SIL cytology develops CIN3 [15].

Results of the ATHENA study, published in 2014 highlight the existence of a significant percentage, of 57% women aged between 25-29 years with negative cytology for intraepithelial lesions (NIEL) that have been shown to present at histopathological examination injuries ≥ CIN3. This evidence has dramatically changed the orientation regarding the priority of screening methods in cervical cancer. Currently Pap Test has acquired a special meaning in the national programs being recommended for co-testing, alongside with primary HPV testing, which allows an increased diagnosis accuracy [16].

Testing HPV infection

Nowdays, there is a statistically acknowledged tendency as regards the need to change the cervical cytological screening method with testing for the presence of infection with human papilloma virus (HPV), increased risk (HR). Therefore, the HPV testing is required as the first screening method in cervical cancer.

HPV is a non-enveloped, double-stranded DNA virus. The viral genome consists of 3 different regions: the long control region (LCR) or the URR (upstream regulatory region) - responsible for the translation and replication-, and two ORF regions (open reading frames) -early (E1-E-7) and late (L1 and L2) respectively)-. The HPV genome proteins accomplish different roles. The E1 and E2 proteins act in the cellular cycle adjustability, the E6 and E7 proteins are transforming; the late proteins are located in the L1 and L2 regions; the E4 protein function is not yet fully known and the E5 protein interferes with the cellular immortalization.

During the integration of the HPV -DNA in the host cell, the viral genome perturbs the reading hatches (ORFS) of the E1 and/or E2 levels. The loss of the E2 function causes the loss of cellular regulation control and the development of oncoproteins E6 and E7 [17].

There are three acknowledged methods of identifying the HPV types: nucleic acid-hybridization assays, signal-amplification assays, nucleic-acid amplification. These methods are used in many tests under commercial names.

The nucleic acid-hybridization assays methodology is found in the commercial tests Southem blot, In situ hybridization, Dot blot hybridization. This method detects the HPV infection in the cervical samples and provides substantial information regarding the HPV infection types. The disadvantages of the nucleic acid-hybridization assay are low sensitivity, requiring a bigger quantity of purified DNA, and taking a longer period of time for data processing.

The signal-amplification assays method is found in the commercial tests Hybrid Capture 2 -HC2- (Digene) și Cervista. The Hybrid Capture 2 -Hc2 (Digene) test approved in the US by the Food and Drug Administration (FDA) in 2003 detects 13 HPV - HR types (16, 18, 31, 33, 35, 39,

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45, 51, 52, 56, 58, 59, 68) and 5 HPV - LR types (6, 11, 42, 43, 44). The test uses the non-radioactive amplification method of the specific antibodies signal and chemiluminescence paired with hybridising the HPV-DNA target and the RNA marked in the solution samples. The Hc2 test identifies the high risk cases of LCP infected with HPV-HR 16 and 18 in a 10-15% percentage, and other types of HR-HPV with percentage < 3% [18].

The Cervista test is approved in the US by the Food and Drug Administration (FDA) since 2009. The Cervista test detects 14 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68); it identifies the nucleic acid sequences by using the signal-amplification assay and fluorescence. The advantages of the Cervista method: 100% sensitivity in detecting CIN 3, 98% sensitivity in detecting CIN 2, high sensitivity and specificity in HPV 16 and 18, lower rates of false positive results, higher percentage of identifying the precancerous cervical lesions than the H2 test [19].

The methodology that uses the nucleic acid amplification is used in the following commercial HPV tests: Microarray analysis, PapilloCheck, PCR, PCF-RFLP, Real-Time-PCR, Abbott Real Time, Cobas 4800 HPV, Genome sequencing, CLART HPV, INNO-LIPA, The Linear Array, Clinical Arrays, Pre Tect Proofer, APTIMA. The clinical use of these tests is different.

Cobas 4880 HPV test was approved in the US by the Food and Drug Administration (FDA) since 2014. The test is used as a first choice screening method in the cervical cancer. The Cobas test uses an automatic method that uses the Real-Time PCR. The amplification and the viral detection take place in a single tube with genotyping for HPV 16, HPV 18 and other 12 types of HPV (HR 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 ,68) and with using the β-globin for controlling the process [20]. The advantages of the Cobas 4880 HPV are the short period of 4 hours testing and the accuracy of identifying the HPV HR types. The method removes subjectivity by using the automated reading. The Cobas 4880 HPV test is validated for cervical cancer screening as well as for ASC-US lesions triage [21].

The signal-amplification assays method is also found in The Linear Array HPV genotyping (Roche Molecular Diagnostics, USA). The advantages of this testing consists in identifying 36 HPV types and classifying them into risk groups (15 types of HPV HR-, 15 probably HPV HR-, 10 HPV- LR types and 9 HPV types with undetermined risk).

[18] Stănculescu, Ruxandra, Elvira Brătilă, Vasilica Baușic, Teodora Camelia Vlădescu, Florina Vasilescu, Alexandra Baușic and Costin Berceanu. 2017. "Review of the biotechnologies and test used for precancerous cervical lesions diagnosis". *Rom J Morphol Embryol*. 58(1):7-1, 2066-8279

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The same methodology is used in the Clinical Arrays HPV test (Genomic SAU, Madrid Spania) which detects 35 genotypes that are individually assigned to HR-HPV or LR-HPV. The method identifies the singular or multiple infection.

In comparison with the previous tests that only confirm the presence of the HPV infection, the HPV-mRNA test adds more information because it identifies a HPV transformed, progressive infection, that affects the cellular cycle after the intracellular HPV viral genome integration into the host DNA. This is proven by detecting the E6 and E7 proteins overexpression in HPV HR infections [22].

The commercial tests able to identify the E6/ E7 mRNA HPV –HR are the Pre Tect Proofer test (Norchip) -that allows the detection of the E6/ E7 mRNA in 5 HPV-HR types (16, 18, 31, 33, 45)-, and the APTIMA test(GenoProbe) – that has a higher availability of detecting the E6/ E7 mRNA in more HPV-HR types, that is 14 HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68).

The E6/ E7 mRNA detection is also possible by using the QIAGEN test (US), covering a range of 13 HPV-HR types (16, 18, 31,33, 35, 39, 45, 51, 52, 56, 58, 59, 66). This method is used in the areas without a water source and electricity. The devices occupy a small space (25cmx50cm). Another advantage is the short period of processing the data (just 2h 30 minutes in comparison with longer duration for Hc2).

Therefore, the testing methods used for HPV infections have benefits and limits as regards an accurate diagnosis and choosing the most adequate medical attitude. According to our study criteria, we will make a distinction between the benefits and the limits of HPV testing methods using the tests presented until now.

The conclusions of collected studies reveal that the Cervista HPV testing has high sensitivity and specificity in detecting the HPV 16/18 types. The Cervista assay has 100% sensitivity in detecting the HPV types capable of evolving into CIN3 lesions [23]. Detecting the HPV 16 and 18 allow the stratification of the HPV infection oncological risk, without a crisp distinction between HPV infections that resolve spontaneously and progressive HPV infections. The identification of the E6/E7 mRNA in HPV-HR confirms the existence of a HPV transformative infection .

The presence of a 1:1 ratio between E2 and E6/7 genes confirms the HPV integration by using the Real-Time PCR method. The precancerous lesions progression risk varies with the HPV HR type: 10-15% risk for HPV HR type 16/18 and < 3% for other HPV HR types [24].

Besides the technologies proving HPV infection, a special role is played by biotechnologies that can detect the viral load. They are used within the PCR, Fluorescence in situ hybridization and RealTime PCR tests.

Analyzing the data published in the literature leads to formulating the benefits and disadvantages of detecting the presence of an HPV infection.

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[23] Johnson, Lawrence R., Cindi R. Starkey, James Palmer, James Taylor, Spencer Stout, Stephanie Holt, and Ryan Hendren et al. 2008. "A Comparison Of Two Methods To Determine The Presence Of High-Risk HPV Cervical Infections". *American Journal Of Clinical Pathology* 130 (3): 401-408. doi:10.1309/4dxeafg2jxyf34n3

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It can be unequivocally affirmed that the HPV types inducing PCL or CC are different. Types 16 and 18 are the most commonly involved types in CC, HPV type 16 is dominant in CIN3 + lesions. Equally, it is known that the variation in PCL and CC risk is influenced by the type of HPV infection, viral load, vaginal microbiome, individual immunity and it is related to geographical areas.

It is also relevant that the viral integration is an early event that occurs before morphological changes progressing to cancer. Viral HPV DNA integration does not coincide with the immediate presence of a high grade lesion[25]. Triggering strategies according to Eurogin Roadmap 2017 take into account the fact that the detection of HPV infection does not distinguish between transient and progressive infection, HPV detection has a late prediction of the risk of precancerous lesions, a risk manifested over years. Practically, the HPV testing is able to identify women who may be at increased risk of cancer in the future without the presence of detectable cervical lesions.

The prevalence of the type of HPV infection is correlated both with the presence of PCL/CC and the presence of HPV in the healthy population [26]. The use of detecting the presence of the HPV infection and genotyping is extensive, and it offers extensive diagnostics. Currently, HPV DNA genotyping requires being the primary screening method in many national programs and is equally useful for co-testing together with the Pap test, and also for the triage of cases with equivocal cytology or low grade, surveillance of cases with abnormal cytological screening results with negative colposcopies and biopsies. Also, repeating the HPV testing is required for the prediction of the evolution after the treatment of CIN lesions. These recommendations are also joined by the need to check the persistence of HPV strains, identifying the prevalence of certain HPV strains to assess the overall impact of HPV vaccination.

Therefore, HPV genotyping is required as the primary screening method within national programs, but can be also significant in the triage methods of the progressive cervical lesions.

The benefits of primary HPV-HR screening consist in identification of cases assigned to risk groups, setup of time interval for new control, stating the diagnostic for lesions ≥ CIN2+ in a percentage significantly higher than that for Pap test. Another advantage is prolongation to 5 years for screening interval for tested cases with negative HPV-HR result.

The results of studies show that, by identifying the HPV-HR positive persistent cases with normal cytological result, the primary HPV-HR screening leads to increased number of colposcopy recommendations in order to get a presumptive colposcopic diagnostic.

Co-testing and triage of the ASCU-US and LSIL lesions

A delicate problem is the one related to the precancerous cervical lesions triage for the purpose of using methods to differentiate the lesions that spontaneously resolve from the progressive ones. This difficulty in diagnosis is exceeded by highlighting the immunocytological/immunohistological markers in cases with positive Pap Cytology for PCL. The benefits and limitations of these tests are revealed by the large studies results published

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worldwide, as well as the following studies: ATHENA, HERMES, PALMS, KPNC, Compass Trail and Newsletter on Human Papillomavirus-HPV Today 2015, Study Pilot UK 2019. The first marker approved to be introduced in clinical practice for the identification and triage of PCL is represented by p16ink4a cyclin. This is a tumoral suppression protein that intervenes in adjusting the cellular cycle. Overexpression of the p16ink4a cyclin represents an indicator for the excessive presence of E7 viral oncoprotein, as well as other oncoproteins that inactivates pRB [27]. p16ink4a cyclin is a specific marker capable of identifying the dysplastic cervical epithelium, respectively CIN2+ lesions. The sensitivity and specificity of P16ink4a cyclin varies between 0.59 and 0.96, respectively 0.41-0.96 [28]. According to the diagnostic and supervision protocol algorithm of the PCL, positive testing for P16ink4A in ASCUS and LSIL cytology requires investigation completed by colonoscopy/biopsy [29]. Testing can be performed both on cytological sample and on histological sections. The benefit of immunoexpression identification of 16 ink4a cyclin consists in the fact that testing allows positive HPV cases identification. The New Technologies for Cervical Cancer Screening (**NTCC**) Trial, which compares the results of the HPV testing association with cytological testing versus singular cytological testing as primary screening method, concluded that women at age between 35-60 have a 6 time increased risk of developing CIN3+, respectively 4,7% vs 0,8% in cases with HPV positive/p16ink4a positive (HPV+/p16+) vs HPV positive/p16ink4a negative (HPV+/16-) [30]. Besides the mentioned benefits, P16ink4a testing has limitations related to the fact that individually morphological interpretation is necessary regarding the nucleus and cytoplasm appearance inside which the p16INK4a cyclin immunoexpression appears and also completing the Wentzensen score of quantification/intensity. So, interpreting the test involves subjectivity reported at cytologist's own experience of evaluating [31]. Among other limitations is the fact that p16INK4a test cannot select with clarity a real progress towards cancer, cannot distinct between high and low transformative risk HPV infections. Many studies conclusions revealed the fact that women that have been tested HPV+/p16+ require colposcopy immediately, meanwhile in cases with women tested HPV+/p16-

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[31] Wentzensen, Nicolas, Christine Bergeron, Frederic Cas, Denise Eschenbach, Svetlana Vinokurova, and Magnus von Knebel Doeberitz. 2005. "Evaluation Of A Nuclear Score For P16ink4a-Stained Cervical Squamous Cells In Liquid-Based Cytology Samples". *Cancer* 105 (6): 461-467. doi:10.1002/cncr.21378.

second testing after 2 years is recommended[32]. P16ink4a cyclin testing limitations are exceeded by introducing in clinical practice 16INK4a/Ki 67 testing, (Dual test), statistically proven to have a better reproducibility and accuracy than P16ink4a testing.

Statistical data confirm that p16INK4a/Ki 67 testing has a greater sensitivity than Pap test in identifying cervical lesions associated with HPV-HR, while specificity remains identical to Pap test.

In the last years testing of HPV infection associated with cytological co-testing by dual test p16INK4a/Ki67 was established in clinical practice, thus enhancing the accuracy of the diagnosis. Double testing advantages consist in the fact that it allows triage of HPV positive cases, with identifying HPV positive progressive cases. Among other benefits of p16INK4a/Ki 67 double testing include automatic reading of the result, which cancels subjectivity in interpreting the results, as well as reducing the duration of cytologist training [33], [34]. Immunoexpression of the p16ink4a/Ki 67 dual test biomarker allows to identify high histological lesions of intraepithelial cervical neoplasia. Comparisons on the specificity of the p16ink4a/KI 67 test versus Pap test and HPV genotyping demonstrate that dual assay has specificity similar to PAP test but higher in relation to HPV DNA genotyping.

CONCLUSIONS

Primary screening in cervical cancer by HPV genotyping with Pap co-testing are endorsed by current biotechnologies so this becomes an adequate option, supported by statistical data.

Primary HPV screening associated with Pap co-testing increases the colposcopies number but mitigates inappropriate surgery.

The negative result of abovementioned tests justifies widening the screening time interval to 3-5 years

In the event that the primary screening was performed with Pap cytological test, the triage of ASC-US and L-SIL lesions is possible by conducting cell expression identification tests of p16ink4a immunomarkers or p16ink4a/Ki67 dual test.

A promising objective for research is to investigate which will be the effects of nonavalent HPV vaccine vs bivalent and quadrivalent HPV vaccines on the presence and progression of PCL.

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All authors report no potential conflict of interest.

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