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HPV E6/E7 mRNA Versus HPV DNA Biomarker in Cervical Cancer Screening of a Group of Macedonian Women

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High risk types of human papillomaviruses E6/E7 oncogenes and their association with tumor suppressor genes products are the key factors of cervical carcinogenesis. This study proposed them as specific markers for cervical dysplasia screening. The aim of the study is to compare the clinical and prognostic significance of HPV E6/E7 mRNA as an early biomarker versus HPV DNA detection and cytology in triage of woman for cervical cancer. The study group consists of 413 women: 258 NILM, 26 ASC-US, 81 LSIL, 41 HSIL, and 7 unsatisfactory cytology. HPV4AACE screening, real-time multiplex PCR and MY09/11 consensus PCR primers methods were used for the HPV DNA detection. The real-time multiplex nucleic acid sequence-based assay (NucliSENS EasyQ HPV assay) was used for HPV E6/E7 mRNA detection of the five most common high risk HPV types in cervical cancer (16, 18, 31, 33, and 45). The results show that HPV E6/E7 mRNA testing had a higher specificity 50% (95% CI 32–67) and positive predictive value (PPV) 62% (95% CI 46–76) for CIN2+ compared to HPV DNA testing that had specificity of 18% (95% CI 7–37) and PPV 52% (95% CI 39–76) respectively. The higher specificity and PPV of HPV E6/E7 mRNA testing are valuable in predicting insignificant HPV DNA infection among cases with borderline cytological finding. It can help in avoiding aggressive procedures (biopsies and over-referral of transient HPV infections) as well as lowering patient's anxiety and follow up period. ***J. Med. Virol. 87:1578–1586, 2015.***

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KEY WORDS: HPV DNA; HPV E6/E7 mRNA; PPV; specificity; cervical cancer screening

INTRODUCTION

Cervical cancer (CC) is the third most common cancer affecting women worldwide [Arbyn et al., 2008]. The incidence and mortality have been recently lowered due to the implementation of preventive cervical screening programs. Nevertheless it is still one of the most common malignant diseases and leading cause of morbidity and mortality among women worldwide [Hakama et al., 2008]. Cervical carcinogenesis is strongly associated with persistent infection with HR (high-risk) human papillomavirus (HPV) [zur Hausen, 1996; Thomison et al., 2008]. *Human papillomavirus* belongs to the *Papillomaviridae* family. Its presence is found in 99.7% cervical cancers worldwide [Walboomers et al., 1999]. The most frequent HPV genotypes in cervical cancer are HPV 16, 18, 31, 33, and 45 [Bosch et al., 2002]. Cytomorphological examination of cervical smear is widely applied, but it is not an ideal screening method for cervical cancer and its precursors (cervical intraepithelial neoplasia—CIN) due to low sensitivity of approximately 55% for detection of high-grade CIN [Koss, 1993; Robertson and Woodend, 1993; Mayrand et al., 2007; Arbyn et al., 2008].

The high risk HPV (HR-HPV) DNA testing had improved the cervical cancer screening significantly [Bovicelli et al., 2000; Sherman et al., 2003] but its positive predictive value (PPV) and the specificity are still low especially when applied on the young

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population [Kulasingam et al., 2002; Schiffman et al., 2005; Mayrand et al., 2007]. As most HPV infections are transient, HPV DNA testing could result in increased costs and patient anxiety in the follow-up of women with clinically insignificant infection [Verdoodt et al., 2013].

Former studies have shown that more specific tests should be used to minimize the over-referral for colposcopy and treatment. Many new studies propose HPV E6/E7 mRNA testing as more specific test than HPV DNA, repeated cytology, and colposcopy for the follow-up of women with borderline finings (ASC-US, LSIL) [Galarowicz et al., 2012; Koliopoulos et al., 2012; Oliveira et al., 2013; Munkhdelger et al., 2014; Zappacosta et al., 2015] and useful as a screening marker for early prediction and subsequent progression to severe dysplasia [Mockel et al., 2011; Rijkaart et al., 2012; Argyri et al., 2013].

It is well established that the viral oncogenes E6 and E7 are responsible for HPV initiated cervical oncogenesis. Only a small portion of the high risk HPV positive patients develop cervical carcinoma (CC). It is very important to predict which patients are with increased risk for cervical lesion progression. HPV E6 and E7 oncogene active transcription can be monitored directly through the detection of E6/E7 viral mRNA transcripts or proteins [Cuschieri and Wentzensen, 2008; Lie and Kristensen, 2008; Schweizer et al., 2010] or indirectly through p16 host protein expression [Benevolo et al., 2006; Carozzi et al., 2008] affected by the HR-HPV E7 protein and its up-regulation.

In May 2011 the Institute of Public Health of R. Macedonia (IPH) introduced the new NucliSENS EasyQ HPV test for screening of woman who underwent the cervical screening program. In this study we have evaluated the clinical value of this test in terms of the improved HPV detection specificity which may lead to the reduction of the repeated cytological and colposcopical evaluation.

MATERIAL AND METHODS

Study Subjects and Collection of the Samples

Samples were collected from September 2011 to March 2013 from patients that came for cervical cancer screening in private gynecological clinics in Skopje, Macedonia. Written consent was obtained from all participants. The participant received a self-administered questionnaire requesting personal data. Additional part of the questionnaire (cytological status data for >6 months ago) was filled-in by the gynecologist of the participants. The actual results from the cytological and histological analysis were obtained from the Oncology departments for all the patients respectively. The participants underwent cytology, colposcopy, and sampling for subsequent HPV testing. In cases where colposcopy suggested the presence of suspicious lesions, biopsy specimens were obtained for histopathological evaluation.

The study group consisted of 413 women between 19 and 78 years of age including seven women with unsatisfactory cytology. All of them were analyzed for presence of HPV DNA and E6/E7 mRNA. Eighty three of them consisted a group of HPV DNA positive woman for the five most common types (HPV 16, 18, 31, 33, and 45) detected previously at RCGEB, MASA laboratories. This group was included in the study to increase the power of statistical analysis. The study protocol was approved by IPH of R. Macedonia.

The cytological diagnosis was done by cytopathologists using the Bethesda classification system. Histology was performed with specimens collected by a colposcopy-directed biopsy and/or cone specimens collected by the loop excision procedure. Histology results were obtained for 15.02% (61/406). The pathologists involved in the cytological and histological assessments were not involved in testing for HPV.

Cervical specimens for nucleic acid analyses were collected with a cervical brush by standard procedures. The material was preserved in PreservCyt/ThinPrep solution (Cytyc Corporation, Boxborough, MA) or in VTM (Viral Transport Medium—PBS). Analyses of the samples were performed by the Laboratory for Virology and Molecular Diagnostics at the IPH, in Skopje, Macedonia.

Nucleic Acid Isolation

Total nucleic acid was extracted from the pellet after centrifuging the cervical specimen in VTM according to the NucliSens protocol using miniMAG platform (bioMerieux, Lyon, France). The nucleic acids were eluted in a 55 ml elution buffer and were further processed for HPV DNA testing as well as mRNA detection.

HPV DNA Detection and Genotyping

HPV DNA analyses were performed using three methods – two commercially available kits and one in house method.

Seeplex® HPV4AACE screening, assay Seegene (Seoul, Korea) is based on dual priming oligonucleotide technology (DPO^{tm}) that enables high specific priming, stable annealing and blocking of not-specific annealing of primers/probes to the target during the PCR reaction. It was used as a commercial test for the first line HPV DNA screening. The test enables differentiation between low risk HPV types (6 and 11) and 16 most frequent high risk HPV types (26, 31, 33, 35, 39*, 45, 51, 52*, 53, 56, 58, 59, 66, 68*, 73, 82) but doesn't allow specific type identification with the exception of genotyping for the two high risk HPV types 16 (500 bp band) and 18 (360 bp) and the two low risk types HPV 11 and 6 (260 bp). The others are referred as high risk (HR) types (450 bp).

Qualitative Real Time HPV Typing PCR

HPV positive samples obtained from screening tests that were not classified as HPV 16, HPV 18, or

low risk HPV 11 and HPV 6 but only as high risk types by the HPV 4 ACE test, additionally were identified using the HPV high risk genotyping multiplex real time PCR test (Sacace, Como, Italy). The test enabled the detection of the 12 most frequent HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and it is composed of four different tubes for multiplex analysis. Each tube contains a primer directed against regions of three HPV types and β-globin gene used as an internal control.

All HPV samples that were positive at the 4ACE HPV screening test and not genotyped with real time PCR, were genotyped using MY09/MY11 consensus primers located in the L1 region of the virus. MY09/MY11 positive samples were typed by restriction fragment length polymorphism (RFLP) analysis of PCR amplified fragments using *Bam*HI, *Dde*I, *Hae*III, *Hin*fI, *Pst*I, *Rsa*I, and *Sau*3 AI restriction enzymes [Bernard et al., 1994]. This method enables additional genotyping of the types not included in the real time genotyping test (HPV 26, 28, 41, 42, 46, 53, 66, 68, 72, 73, 82…).

HPV mRNA Detection

HPV mRNA was detected with the NucliSENS EasyQ HPV test according to the manufacturer's instructions. In brief, three premixes were made by the reconstitution of reagent spheres of U1A/HPV16, HPV18/31, and HPV33/45 primer/molecular beacon mixes and KCl stock solution. The reaction was started by the addition of enzymes and measured in real time in isothermal condition of the NucliSENS EasyQ analyzer at 41°C. Data analysis were performed using the NucliSENS EasyQ Director software.

The NucliSENS EasyQ HPV test includes primer pairs targeting U1A mRNA as intrinsic control to determine the sample validity. Human U1 small ribonucleoprotein (U1A mRNA) was used as a RNA integrity/adequacy internal control. When the U1A amplification was not detected, the test result was determined invalid. To evaluate the run validity, positive controls for U1A/HPV16, HPV18/HPV31, and HPV33/45 were included.

Statistic

The calculation of the test concordance between HPV DNA and HPV E6/E7 mRNA detection was done by Cohen's kappa test. The κ values of <0.20 indicate poor agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement and >0.80 indicate nearly perfect agreement.

Significance of the overall test agreement for cases with CIN2+ and for <CIN2 was calculated with McNemar's *P* value. Fisher's exact test was used for defining statistical significance for either assay clinical performance. For this purpose GraphPad scientific statistical software-QickCalck was used (http://www.graphpad.com/quickcalcs). Sensitivity, specificity, PPV and NPV were calculated using two statistic software package—Clinical calculator (http://vassarstats.net/clin1.html) and calculator from the Center for Based Medicine, Toronto (http://ktclearinghouse.ca/cebm/practise/ca/calculators/statscalc).

RESULTS

This study asseses the clinical performance of HPV E6/E7 mRNA (NucliSens EasyQ HPV assay) in comparison with DNA based test for HSIL. The examination was done on patients referred to routine screening that underwent cytological, HPV and colposcopical examination. The majority of these patients had cytological findings within the normal limits and/or low-grade disease. In seven specimens, cytology showed qualitatively-unsatisfactory material for cytological analysis. All seven patients were HPV DNA positive. Among cases with cytological defined diagnoses, 258 women (63.5%) had NILM, 26 women (6.4%) had ASC-US, 81 women (20.0%) had LSIL, and 41 women (10.1%) had HSIL.

In 15.0% (61/406) of women, histology was obtained by colposcopically directed biopsy. Ten biopsies (16.4%) were normal, 22 women (36.1%) had CIN1, 20 women (32.8%) had CIN2 and 9 women (14.8%) had CIN3. In three patients with normal cytology and colposcopically suspected findings, histology was normal in two women and CIN2 in one woman. From 26 patients with ASC-US, histology was performed in three cases, one normal and two CIN1. In the LSIL group (n = 81) biopsy was performed on 21 cases; three normal, 16 CIN1 and two CIN2.

Within the group of patients with HSIL cytology (n = 41), biopsy was performed in 34 cases: three normal, five CIN1, 17 CIN2, and 9 CIN3.

A total of 185 of the 413 (44.8%) studied woman were positive at the HPV DNA test. The cytology was unsatisfactory in seven of the 185 HPV positive women. A total of 138 (74.5%) patients were positive for the presence of the five most common types (HPV 16, 18, 33, 45, and 31) as either single or multiple HPV infection (Table I). HPV DNA multiple infections were present in 25.4% (n = 47) of all positive HPV DNA cases; 7 with HSIL, 21 with LSIL and 19 in woman with normal cytology.

HPV E6/E7 mRNA positive results relative to the entire group were 17.9% (n = 74/413). The most common type revealed by the RNA testing was HPV 16 (n = 42), followed by HPV 45 (n = 12), HPV 18 (n = 11), HPV 31 (n = 9), and HPV 33 (n = 4). Five of the E6/E7 positive cases had multiple infections. Comparison of percentage of HPV DNA and HPV E6/E7 mRNA in different cytologically defined cervical lesions is given in Figure 1. NILM group has 27.5% HPV DNA and 4.3% mRNA positivity; LSIL: 74.0% HPV DNA with 34.6% mRNA and HSIL: 90.2% HPV DNA with 73.3% mRNA positivity respectively.

TABLE I. Prevalence of the Five HPV Types (16, 18, 31, 33, and 45) through cytologicaly assessed cases

Cytology	HPV DNA positive (%)	HPV DNA (5 types) (%)	HPV E6/E7mRNA (%)
Normal n = 258	71 (27.5)	50 (19.4)	11 (4.3)
ASC-US n = 26	10 (38.5)	6 (23.1)	4 (15.4)
LSIL n = 81	60 (74.0)	42 (51.9)	28 (34.6)
HSIL n = 41	37 (90.2)	33 (80.5)	30 (73.2)
Total n = 406	178 (43.8)	131 (32.3)	73 (18.0)

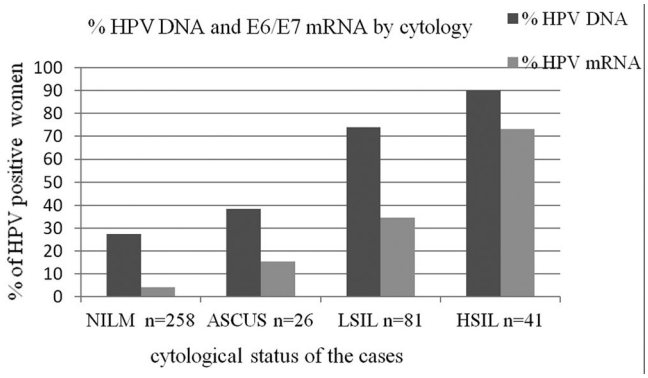

Fig. 1. Percentage distribution of HPV DNA and E6/E7 mRNA by cytological status.

HPV E6/E7 mRNA was detected in 39.5% (73/185) of the HPV DNA positive patients. The presence of mRNA is higher in HPV DNA positive patients with higher grade lesion: 15.5% (11/71) in HPV DNA positive patients with NILM; 40% (4/10) of those with ASC-US; 46.7% (28/60) of LSIL and 81.1% (30/37) of HSIL (Fig. 3).

In terms of histological findings, the decreasing grade of lesions is accompanied by a decrease of the mRNA positivity. Presence of mRNA transcripts versus HPV DNA positivity by histological grade is shown in (Fig. 2). Among the patients with normal histology 25% (1/4) of HPV DNA positive cases were HPV E6/E7 mRNA positive, while positivity of 68.2% (15/22), 90% (18/20), and 100% (9/9) was detected in CIN1, CIN2, and CIN3 cases respectively (Fig. 4).

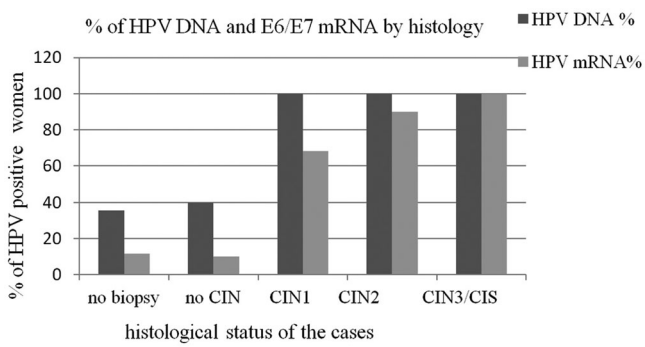

Fig. 2. Percentage distribution of HPV DNA and E6/E7 mRNA by histological grade.

The evaluation of the concordance of these two methodologies was poor in NILM, fair in ASCUS and LSIL and moderate in HSIL (k = 0.2, 0.4, 0.3, 0.5, and 0.4). The overall agreement is poorly fair (k = 0.4). In histological assessed lesions the concordance of the tests was fair in normal and no biopsy cases, substantial in CIN1 (k = 0.3, 0.3, 0.7), respectively and very good in CIN2+ (93%). McNemar's test for assessment of significance of difference between the two methods shows relevance in <CIN2 ($P = 0.004$) and no significance in CIN2+ cases ($P = 0.5$; Table II).

Cytological and histological findings were used for estimation of sensitivities, specificities, PPVs, and NPVs of positive DNA and RNA test results. In the cytology based analysis HSIL was used as cut off condition and CIN2+ findings in histology based analysis. There is a notable difference between DNA and mRNA specificity and PPV ($P < 0.0001$) in cytologically determined <HSIL lesions, as well as in histological <CIN2+ ($P = 0.017$). The results show higher sensitivity of DNA based test versus HPV E6/E7 mRNA test (90.2% vs. 73.2%) in cytologically determined findings and the mRNA based test showed better specificity and PPV (88.2%, and 41.1%/61.4%, and 20.8%, respectively; Table III). The NPV was almost the same everywhere (88.8–100%). In histology based analysis the results were similar. The HPV E6/E7 mRNA test has a significantly higher specificity and PPV (50.0% and 62.8%) versus HPV

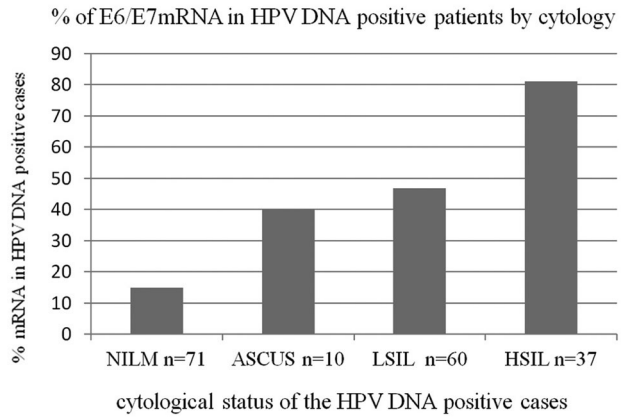

Fig. 3. HPV E6/E7 mRNA percantage in HPV DNA positive cases by cytology.

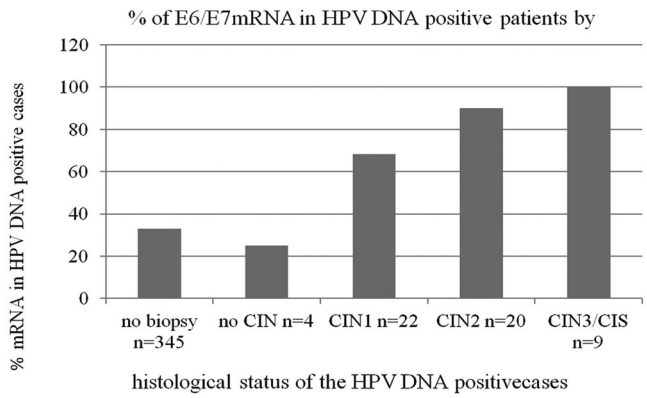

Fig. 4. HPV E6/E7 mRNA percantage in HPV DNA positive cases by histology.

DNA (18.7% and 52.7%) but lower sensitivity of 93% versus 100% respectively.

DISCUSSION

Despite the success in lowering the death rate from cervical cancer in developed countries, the implementation of more sensitive and specific methodology for its early prevention remains a challenge. The number of CC occurrence in Macedonia in 2010 was 230 (22.4 on 100,000) woman [IPH RM report, 2013]. With annual rate of mortality of 6.9 in 100,000 Macedonian women it is on second rank as mortality cause from malign diseases. Currently the first line screening in Macedonia includes the Pap testing. Guidelines for the follow up of patient with abnormal cytological smears are based on repeated cytology, HPV DNA testing and colposcopy [IPH RM report, 2013]. The low sensitivity of the cytology and specificity of HPV DNA testing requires improvement of the cervical cancer screening and follow up algorithms using more specific methods than cytology and HPV DNA testing especially in young population (21–29 years) for whom the HPV DNA testing isn't recommended.

Despite the low specificity [Cuzick et al., 2006b, 2008] for detecting CIN2+, many trials have shown the effectiveness of screening with the HR-HPV DNA test in reducing cervical cancer mortality [Cuzick et al., 2006a; Dillner et al., 2008; Sankaranarayanan et al., 2009]. DNA testing is not informative enough for differentiating transient from persistent infections. This kind of triage confers a risk of overdiagnosis and overtreatment of transient condition [Ronco et al., 2008, 2010]. Testing for HPV oncogenic activity rather than presence of HPV DNA is more relevant clinical indicator of cervical lesions development and cervical cancer. It provides clinically predictive markers to identify woman at risk of developing high grade cervical dysplastic lesions and cervical carcinoma [Varnai et al., 2008] and can be used as more specific than HPV DNA in triage of women with minor cervical lesion [Sorbye et al., 2014]. In order to improve the screening program, achieve better specificity and PP values the IPH of Macedonia introduced HPV mRNA based detection methodology.

TABLE II. The HPV DNA and E6/E7 mRNA Tests Concordance by Cytological and Histological Diagnoses

	a*	b*	Total*	%	k	95% CI	McNemar p
Cytology							
Normal	11	187	197/258	76.4	0.2	0.1–0.3	0.0001
ASC-US	4	16	20/26	76.9	0.4	0.1–0.8	0.04
LSIL	28	21	49/81	60.5	0.3	0.2–0.4	0.0001
HSIL	30	4	34/41	88.2	0.5	0.1–0.8	0.02
Histology							
No biopsy	30	224	254/345	73.7	0.3	0.2–0.4	0.0001
normal	1	6	7/10	70.0	0.3	0–0.7	0.2
CIN1	15	2	17/22	77.2	0.7	0.4–1.0	0.02
CIN2+	27	0	27/29	93.1			0.5

a*, positive concordance; b*, negative concordance; total*, total concordance; k, Cohen's kappa value; 95% CI, confidence interval.

TABLE III. Sensitivity, Specificity, PPV and NPV of the Two Tests (HPV DNA and HPV E6/E7 mRNA)

Histology					(95% CI)
HPV DNA	<CIN2	CIN2+	Sensitivity	100	85–100
Positive	26	29	Specificity	18.7	7–37
Negative	6	0	PPV	52.7	39–66
Total	32	29	NPP	100	51–100
mRNA	<CIN2	CIN2+	Sensitivity	93.1	76–98
Positive	16	27	Specificity	50.0	32–67
Negative	16	2	PPV	62.8	46–76
Total	32	29	NPP	88.9	64–98

95% CI, confidence interval.

The histological findings from this study confirm that the conventional Pap smear test commonly used for both primary and secondary screening has a relatively poor sensitivity for detection of high-grade cervical lesions. Discrepancy of the results in 24.1% between cytology and histology in this study is very similar (25.1%) with previous similar study for HPV genotyping of Macedonian women [Duvlis, 2002] done on bigger population group (1280 patients with 490 histological findings).

Histological CIN2 in one woman with normal cytology and two CIN2 in woman with LSIL cytology in this study may result in incorrectly follow-up examinations leading to serious consequences. Oppositely there is also a discrepancy in smear of detecting histological lower dysplastic condition than the one detected by cytology. This kind of overestimation of the lesion's grade could cause unnecessary anxiety in patients during the six mounts period of repeat cytology which is a prescribed follow-up step in the Macedonian algorithm for management of borderline findings (LSIL and ASC-US). The low specificity of HPV DNA testing in young population group is not very helpful in defining the actual situation of cervix uteri. That will prolong the follow-up period and the final diagnosis would have to be done by an invasive method. The oncogenity detection will be much better predictive marker and quite useful to avoid invasive procedures.

The use of Seegen's HPV ACE test as screening test for HPV DNA in this study was done based on a Korean study [Hong et al., 2009] that compares this test with HC2 (Hybrid capture 2 assay). It shows specific improvement of the test by dual primer labeling. The novel HPV4 ACE test is a valuable tool for the detection of HR HPVs and genotyping of HPV 16 and HPV18 [Hong et al., 2009]. Actually it is the same test as HC2 that is mostly used as it is FDA recommended. It gives higher analytical specificity but compared to the RNA based test related to the clinical importance the specificity is notably lower.

HPV testing in this study shows generally lower RNA positivity compared to the DNA positivity in all grades of lesions especially in normal finings. This is in accordance with a number of other previous studies [Cuschieri et al., 2005; Lie et al., 2005; Molden et al., 2005a,b; Molden et al., 2006]. Negative mRNA results in HPV DNA positive patients reflect that not all HR HPV infections express E6 and E7 which is expected in transient infections. Although the HPV DNA tests detects more than 5 HR types covered by NucliSENS EasyQ HPV assay, the last ones are most common confirmed in higher grade lesions and cancer specimens [Clifford et al., 2003; Kraus et al., 2004, 2006; Molden et al., 2005a; Cox and Cuzick, 2006; Cuzick et al., 2006a]. The fact that HPV 16 and HPV 18 are found in more than 70% cervical cancer cases [Munoz et al., 2004] justifies the use of this test in screening. Analysis of the type distribution through lesions in this study shows their presence in 60% of ASC-US and benign finings, 70% of LSIL and 85% and 100% in CIN2 and CIN3 lesions, respectively.

An overall presence of 39.5% mRNA in group of HPV DNA positive patients could be explained by transient nature of the infection or by the lower number of HPV types detected by the NucliSens EasyQ HPV assay. It can also be a consequence of episomal state of the virus in the cell, low transcriptional activity of integrated genome or occurrence of a mutation in the region covered by primers and probes [Kraus et al., 2006].

The lower rate of concordance of the results between the two tests occurs as expected in low-grade lesions. In these cases, DNA from HPV was detected more frequently than the E6 and E7 mRNA. The expectation is based on the fact that 75% of HPV DNA positive infections spontaneously cleared by host immunity and only a small percent left persistent conferring cervical lesion and transformation to cervical cancer [Cuschieri et al., 2005]. Higher rate of concordance detected in CIN2+ lesions indicates that HPV E6 and E7 mRNA is a more specific test for detection of the CIN2+ conditions. The presence of E6 and E7 transcripts in benign lesions shows that the virus is oncogenically active before development of cytologically detectable abnormality [Cattani et al., 2009] and this test can predict early the potential of oncogene transformation to severe dysplasia. Oppositely mRNA negativity in HPV DNA positivity of specimen cannot exclude possibility for afterward oncogenic activation of the virus which means that the follow-up recall will be longer [Cattani et al., 2009].

As most patients in this study were referred within the primary screening program and only a few were referred by secondary gynecology practitioners, the majority of cytological results were within the range of normal and benign findings. Inclusion of HPV positive patient group previously evaluated at RCGEB, MASA laboratories, created an increase of overall HPV positivity, but this didn't affect the final results of HPV DNA/mRNA presence in different cytological findings or the sensitivity, specificity, PPV and NPV test results.

The detection of mRNA in 34.6% of cases with CIN1 lesion and its low presence in benign and normal findings is due to possibility of regression and spontaneously resolving of the changes. Usually increasing lesion grade raises the agreement between the DNA and mRNA based test. In CIN3 lesions there is nearly perfect match pointing out that this marker is more specific in predicting the high grade changes. Two CIN2 cases in this study group were mRNA negative and HPV DNA positive, from which one is attributed to presence of HPV type 53 that is not included in the test and the second HPV 16 DNA positive failed to be detected perhaps due to low HPV copy number beneath cut off, meaning that transcriptional activity occurs but at lower levels.

Most important clinical significance of the test was obtained through statistical analysis of the sensitivity, specificity and the PPV. Results from this study yields much higher specificity and lower sensitivity of mRNA testing versus HR DNA screening at detecting CIN2+ as cut off value similarly to previous studies [Andersson et al., 2006; Molden et al., 2006; Keegan et al., 2009; Ratnam et al., 2011]. The lower sensitivity of detecting CIN2 with NucliSENS EasyQ HPV test is attributed to limited number of HPV types targeted by test. Four HSIL patients had HPV types not included in the test. If statistical analysis was restricted to these five types HPV DNA positive patients the sensitivity becomes quite similar.

Higher specificity for histological CIN2+ of the HPV E6/E7 mRNA test (50.0%) versus the HPV DNA test (18.8%) is in agreement with other similar studies. In one study of the follow up of women with ASC-US and LSIL cytology the specificity of the HPV E6/E7 mRNA test was 76% versus 18% of the HPV DNA based tests [Ovestad et al., 2011]. The PPV as clinically predictive value in this study is 40.0% for cytological and 62.0% for histological defined CIN2+ lesions which is very close to results from the mentioned study [Molden et al., 2005a] (37.5%). An Irish study of sensitivity and specificity of the HPV E6/E7 mRNA test -PreTect HPV-Proofer and the HC2 DNA test for the detection of high-grade cytology (CIN2+) [Keegan et al., 2009] shows 71.4%/75.8% and 100%/43.7%, respectively. The relatively low detection rate observed by PreTect HPV-Proofer in the whole range of cytological positive cases in this study, combined with a relatively higher specificity and PPV, suggests that PreTect HPV-Proofer might be more useful than HC2 for triage and in predicting high-grade disease. The UK study of Szarewski et al. [2008] on histologically confirmed cases shows higher specificity of the two mRNA based different tests: PreTect HPV-Proofer (73.1%) and APTIMA (42.2%) versus DNA based test: Amplicor Roche (28.4%) and HC2 (Hybrid capture 2) (21.7%). The range of the specificity detected values in several mRNA based studies varied from 46.9% to 84.9% in histological confirmed cases but it is yet about twice higher than specificity of DNA based test in mentioned studies (9.4–50.0). An Italian study [Cattani et al., 2009] shows notable difference of the specificity of the mRNA based (62.5%) versus DNA based test- HC2 test (27.5%). Recent study by Rebolj et al. [2014] also confirms better specificity of HPV mRNA based test (APTIMA: 0.35) from all three compared HPV DNA based tests (HC2: 0.22; Cobas: 0.27; and CLART: 0.32). Somewhat different results were obtained in the new study by Cuschieri et al. [2014] where specificity of the mRNA based test (APTIMA) doesn't show big difference from the DNA based tests without including age influence. In fact, specificity of mRNA based test rises in women over 30 year of age. Comparing the population group number of mentioned studies, the results from our study are obtained on a lower number of histological confirmed lesion as well as lower number of high-grade lesions. To confirm this result the study should be performed in larger population group and it needs more analysis on higher-grade lesions. A combination of more specific tests whose positivity strongly relates to the presence of a CIN2+ lesion [Dona et al., 2012] might be included in cervical cancer triage in future. Waldstrom et al. [2013] proposed the p16(INK4a)/Ki-67 dual-staining test in LSIL cytology samples that demonstrated high sensitivity similar to that of the HPV mRNA based tests in the detection of underlying high-grade disease but with enhanced specificity, especially among women aged <30 years. The sensitivity and specificity of $p16^{INK4a}$/Ki-67 immunocytochemistry from 87.3% (95% CI 78.0–93.8%) and 76.4% (95% CI 71.6–80.8%), respectively and the positive and negative predictive values from 45.7% (37.6–54.0%) and 96.4% (93.4–98.3%), respectively reported in the study by Fiji et al. [2014] indicate that this test could be also included in screening program as adjunctive to cytology instead of HRHPV DNA testing and as indirect detection of HPV E6/E7 mRNA oncogenic effect.

It should be noted that some of CIN2 lesions utilized as an endpoint in clinical assessment of the disease could also regress as well as that only 12–31% of CIN3 lesion progress to carcinoma if they are not treated [McIndoe et al., 1984; McCredie et al., 2008]. The results from the statistical analysis should take this fact in consideration in assessing the clinical values of the test. This study was unable to predict how many CIN2 lesions will regress since Macedonian woman with CIN2 lesion routinely undergo preventive conization. After the inclusion of HPV vaccination in the Macedonian mandatory schedule of vaccination, the prevalence of the most frequent HPV vaccine types—HPV16 and 18 are expect to be reduced. Therefore in the future there will be a need for the adaptation of HPV E6/E7 mRNA based testing according to the dynamic change of the geographically prevalence of the HPV types.

The substantial discrepancy between the cytology and histology of 24.1%, the low sensitivity of cytology and low specificity of HPV DNA tests as presented in this study justify the implementation of the HPV E6/E7 mRNA as a more specific test for the first line screen for cervical cancer and its use in screening algorithms. The most important benefits from the implementation of the mRNA triage of woman at risk of cervical cancer or in follow-up patients with borderline findings are: less frequent colposcopy referrals, avoidance of patient's anxiety and costs benefits.

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REFERENCES

Andersson S, Hansson B, Norman I, Gaberi V, Mints M, Hjerpe A, Karlsen F, Johansson B. 2006. Expression of E6/E7 mRNA from 'high risk' human papillomavirus in relation to CIN grade, viral load and p16INK4a. Int J Oncol 29:705–711.

Arbyn M, Bergeron C, Klinkhamer P, Martin-Hirsch P, Siebers AG, Bulten J. 2008. Liquid compared with conventional cervical cytology: A systematic review and meta-analysis. Obstet Gynecol 111:167–177.

Argyri E, Tsimplaki E, Daskalopoulou D, Stravopodis DJ, Kouikoglou O, Terzakis E, Panotopoulou E. 2013. E6/E7 mRNA expression of high-risk HPV types in 849 Greek women. Anticancer Res 33:4007–4011.

Benevolo M, Mottolese M, Marandino F, Vocaturo G, Sindico R, Piperno G, Mariani L, Sperduti I, Canalini P, Donnorso RP, Vocaturo A. 2006. Immunohistochemical expression of p16 (INK4a) is predictive of HR-HPV infection in cervical low-grade lesions. Mod Pathol 19:384–391.

Bernard HU, Chan SY, Manos MM, Ong CK, Villa LL, Delius H, Peyton CL, Bauer HM, Wheeler CM. 1994. Identification and assessment of known and novel human papillomaviruses by polymerase chain reaction amplification, restriction fragment length polymorphisms, nucleotide sequence, and phylogenetic algorithms. J Infect Dis 170:1077–1085.

Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. 2002. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 55:244–265.

Bovicelli A, Bristow RE, Montz FJ. 2000. HPV testing: Where are we now? Anticancer Res 20:4673–4680.

Carozzi F, Confortini M, Dalla Palma P, Del Mistro A, Gillio-Tos A, De Marco L, Giorgi-Rossi P, Pontenani G, Rosso S, Sani C, Sintoni C, Segnan N, Zorzi M, Cuzick J, Rizzolo R, Ronco G. 2008. Use of p16-INK4A overexpression to increase the specificity of human papillomavirus testing: A nested substudy of the NTCC randomised controlled trial. Lancet Oncol 9:937–945.

Cattani P, Zannoni GF, Ricci C, D'Onghia S, Trivellizzi IN, Di Franco A, Vellone VG, Durante M, Fadda G, Scambia G, Capelli G, De Vincenzo R. 2009. Clinical performance of human papillomavirus E6 and E7 mRNA testing for high-grade lesions of the cervix. J Clin Microbiol 47:3895–3901.

Clifford GM, Smith JS, Plummer M, Munoz N, Franceschi S. 2003. Human papillomavirus types in invasive cervical cancer worldwide: A meta-analysis. Br J Cancer 88:63–73.

Cox T, Cuzick J. 2006. HPV DNA testing in cervical cancer screening: From evidence to policies. Gynecol Oncol 103:8–11.

Cuschieri K, Wentzensen N. 2008. Human papillomavirus mRNA and p16 detection as biomarkers for the improved diagnosis of cervical neoplasia. Cancer Epidemiol Biomarkers Prev 17:2536–2545.

Cuschieri KS, Cubie HA, Whitley MW, Gilkison G, Arends MJ, Graham C, McGoogan E. 2005. Persistent high risk HPV infection associated with development of cervical neoplasia in a prospective population study. J Clin Pathol 58:946–950.

Cuschieri K, Cubie H, Graham C, Rowan J, Hardie A, Horne A, Earle CB, Bailey A, Crosbie EJ, Kitchener H. 2014. Clinical performance of RNA and DNA based HPV testing in a colposcopy setting: Influence of assay target, cut off and age. J Clin Virol 59:104–108.

Cuzick J, Clavel C, Petry KU, Meijer CJ, Hoyer H, Ratnam S, Szarewski A, Birembaut P, Kulasingam S, Sasieni P, Iftner T. 2006a. Overview of the European and North American studies on HPV testing in primary cervical cancer screening. Int J Cancer 119:1095–1101.

Cuzick J, Mayrand MH, Ronco G, Snijders P, Wardle J. 2006b. Chapter 10: New dimensions in cervical cancer screening. Vaccine 24:S3/90–S3/97.

Cuzick J, Arbyn M, Sankaranarayanan R, Tsu V, Ronco G, Mayrand MH, Dillner J, Meijer CJ. 2008. Overview of human papillomavirus-based and other novel options for cervical cancer screening in developed and developing countries. Vaccine 26: K29–K41.

Dillner J, Rebolj M, Birembaut P, Petry KU, Szarewski A, Munk C, de Sanjose S, Naucler P, Lloveras B, Kjaer S, Cuzick J, van Ballegooijen M, Clavel C, Iftner T. 2008. Long term predictive values of cytology and human papillomavirus testing in cervical cancer screening: Joint European cohort study. BMJ 337:a1754.

Dona MG, Vocaturo A, Giuliani M, Ronchetti L, Rollo F, Pescarmona E, Carosi M, Vocaturo G, Benevolo M. 2012. p16/Ki-67 dual staining in cervico-vaginal cytology: Correlation with histology, Human Papillomavirus detection and genotyping in women undergoing colposcopy. Gynecol Oncol 126:198–202.

Duvlis S. 2002. Genotypisation of HPV in female population in Republic of Macedonia. Interdisciplinary Postgraduate Studies in Molecular Biology and Genetics Engineering, University Stt Cyril and Methodius. MSc.

Fujii T, Saito M, Hasegawa T, Iwata T, Kuramoto H, Kubushiro K, Ohmura M, Ochiai K, Arai H, Sakamoto M, Motoyama T, Aoki D. 2014. Performance of p16/Ki-67 immunocytochemistry for identifying CIN2+ in atypical squamous cells of undetermined significance and low-grade squamous intraepithelial lesion specimens: A Japanese Gynecologic Oncology Group study. Int J Clin Oncol 18:18.

Galarowicz B, Jach R, Kidzierska J, Dyduch G, Zajac K, Pitynski K, Banas T, Huras H, Streb J, Dulinska-Litewka J, Hosiawa V, Juszczak M, Milewicz T. 2012. The role of mRNA E6/E7 HPV high oncogenic risk expression in colposcopy of cervical intraepithelial neoplasia (CIN). Przegl Lek 69:651–657.

Hakama M, Coleman MP, Alexe DM, Auvinen A. 2008. Cancer screening: Evidence and practice in Europe 2008. Eur J Cancer 44:1404–1413.

Hong JH, Song SH, Kim JK, Han JH, Lee JK. 2009. Comparison of the novel human papillomavirus 4 auto-capillary electrophoresis test with the hybrid capture 2 assay and with the PCR HPV typing set test in the detection of high-risk HPV including HPV 16 and 18 genotypes in cervical specimens. J Korean Med Sci 24:579–584.

IPH report. 2013. Annual IPH of Macedonia report of the results from cervical screening program in Macedonia

Keegan H, Mc Inerney, J, Pilkington L, Gronn P, Silva I, Karlsen F, Bolger N, Logan C, Furuberg L, O'Leary J, Martin C. 2009. Comparison of HPV detection technologies: Hybrid capture 2, PreTect HPV-Proofer and analysis of HPV DNA viral load in HPV16, HPV18 and HPV33 E6/E7 mRNA positive specimens. J Virol Methods 155:61–66.

Koliopoulos G, Chrelias C, Pappas A, Makridima S, Kountouris E, Alepaki M, Spathis A, Stathopoulou V, Panayiotides I, Panagopoulos P, Karakitsos P, Kassanos D. 2012. The diagnostic accuracy of two methods for E6&7 mRNA detection in women with minor cytological abnormalities. Acta Obstet Gynecol Scand 91:794–801.

Koss LG. 1993. Cervical (Pap) smear. New directions. Cancer 71:1406–1412.

Kraus I, Molden T, Erno LE, Skomedal H, Karlsen F, Hagmar B. 2004. Human papillomavirus oncogenic expression in the dysplastic portio; an investigation of biopsies from 190 cervical cones. Br J Cancer 90:1407–1413.

Kraus I, Molden T, Holm R, Lie AK, Karlsen F, Kristensen GB, Skomedal H. 2006. Presence of E6 and E7 mRNA from human papillomavirus types 16, 18, 31, 33, and 45 in the majority of cervical carcinomas. J Clin Microbiol 44:1310–1317.

Kulasingam SL, Hughes JP, Kiviat NB, Mao C, Weiss NS, Kuypers JM, Koutsky LA. 2002. Evaluation of human papillomavirus testing in primary screening for cervical abnormalities: Comparison of sensitivity, specificity, and frequency of referral JAMA 288:1749–1757.

Lie AK, Kristensen G. 2008. Human papillomavirus E6/E7 mRNA testing as a predictive marker for cervical carcinoma. Expert Rev Mol Diagn 8:405–415.

Lie AK, Risberg B, Borge B, Sandstad B, Delabie J, Rimala R, Onsrud M, Thoresen S. 2005. DNA- versus RNA-based methods for human papillomavirus detection in cervical neoplasia. Gynecol Oncol 97:908–915.

Mayrand MH, Duarte-Franco E, Rodrigues I, Walter SD, Hanley J, Ferenczy A, Ratnam S, Coutlee F, Franco EL. 2007. Human

papillomavirus DNA versus Papanicolaou screening tests for cervical cancer. N Engl J Med 357:1579–1588.

McCredie MR, Sharples KJ, Paul C, Baranyai J, Medley G, Jones RW, Skegg DC. 2008. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: A retrospective cohort study. Lancet Oncol 9:425–434.

McIndoe WA, McLean MR, Jones RW, Mullins PR. 1984. The invasive potential of carcinoma in situ of the cervix. Obstet Gynecol 64:451–458.

Mockel J, Quaas J, Meisel H, Endres AS, Schneider V. 2011. Human papillomavirus E6/E7 mRNA testing has higher specificity than liquid-based DNA testing in the evaluation of cervical intraepithelial neoplasia. Anal Quant Cytol Histol 33:311–315.

Molden T, Kraus I, Karlsen F, Skomedal H, Nygard JF, Hagmar B. 2005a. Comparison of human papillomavirus messenger RNA and DNA detection: A cross-sectional study of 4,136 women >30 years of age with a 2-year follow-up of high-grade squamous intraepithelial lesion. Cancer Epidemiol Biomarkers Prev 14:367–372.

Molden T, Nygard JF, Kraus I, Karlsen F, Nygard M, Skare GB, Skomedal H, Thoresen SO, Hagmar B. 2005b. Predicting CIN2+ when detecting HPV mRNA and DNA by PreTect HPV-proofer and consensus PCR: A 2-year follow-up of women with ASCUS or LSIL Pap smear. Int J Cancer 114:973–976.

Molden T, Kraus I, Karlsen F, Skomedal H, Hagmar B. 2006. Human papillomavirus E6/E7 mRNA expression in women younger than 30 years of age. Gynecol Oncol 100:95–100.

Munkhdelger J, Choi Y, Lee D, Kim S, Kim G, Park S, Choi E, Jin H, Jeon BY, Lee H, Park KH. 2014. Comparison of the performance of the NucliSENS EasyQ HPV E6/E7 mRNA assay and HPV DNA chip for testing squamous cell lesions of the uterine cervix. Diagn Microbiol Infect Dis 79:422–427.

Munoz N, Bosch FX, Castellsague X, Diaz M, de Sanjose S, Hammouda D, Shah KV, Meijer CJ. 2004. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer 111:278–285.

Oliveira A, Verdasca N, Pista A. 2013. Use of the NucliSENS EasyQ HPV assay in the management of cervical intraepithelial neoplasia. J Med Virol 85:1235–1241.

Ovestad IT, Vennestrom U, Andersen L, Gudlaugsson E, Munk AC, Malpica A, Feng W, Voorhorst F, Janssen EA, Baak JP. 2011. Comparison of different commercial methods for HPV detection in follow-up cytology after ASCUS/LSIL, prediction of CIN 2-3 in follow up biopsies and spontaneous regression of CIN2-3 Gynecol Oncol 123:278–283.

Ratnam S, Coutlee F, Fontaine D, Bentley J, Escott N, Ghatage P, Gadag V, Holloway G, Bartellas E, Kum N, Giede C, Lear A. 2011. Aptima HPV E6/E7 mRNA test is as sensitive as Hybrid Capture 2 Assay but more specific at detecting cervical precancer and cancer. J Clin Microbiol 49:557–564.

Rebolj M, Lynge E, Ejegod D, Preisler S, Rygaard C, Bonde J. 2014. Comparison of three human papillomavirus DNA assays and one mRNA assay in women with abnormal cytology. Gynecol Oncol 135:474–480.

Rijkaart DC, Heideman DA, Coupe VM, Brink AA, Verheijen RH, Skomedal H, Karlsen F, Morland E, Snijders PJ, Meijer CJ. 2012. High-risk human papillomavirus (hrHPV) E6/E7 mRNA testing by PreTect HPV-Proofer for detection of cervical high-grade intraepithelial neoplasia and cancer among hrHPV DNA-positive women with normal cytology. J Clin Microbiol 50:2390–2396.

Robertson JH, Woodend B. 1993. Negative cytology preceding cervical cancer: Causes and prevention. J Clin Pathol 46:700–702.

Ronco G, Giorgi-Rossi P, Carozzi F, Confortini M, Dalla Palma P, Del Mistro A, Gillio-Tos A, Minucci D, Naldoni C, Rizzolo R, Schincaglia P, Volante R, Zappa M, Zorzi M, Cuzick J, Segnan N. 2008. Results at recruitment from a randomized controlled trial comparing human papillomavirus testing alone with conventional cytology as the primary cervical cancer screening test. J Natl Cancer Inst 100:492–501.

Ronco G, Giorgi-Rossi P, Carozzi F, Confortini M, Dalla Palma P, Del Mistro A, Ghiringhello B, Girlando S, Gillio-Tos A, De Marco L, Naldoni C, Pierotti P, Rizzolo R, Schincaglia P, Zorzi M, Zappa M, Segnan N, Cuzick J. 2010. Efficacy of human papillomavirus testing for the detection of invasive cervical cancers and cervical intraepithelial neoplasia: A randomised controlled trial. Lancet Oncol 11:249–257.

Sankaranarayanan R, Nene BM, Shastri SS, Jayant K, Muwonge R, Budukh AM, Hingmire S, Malvi SG, Thorat R, Kothari A, Chinoy R, Kelkar R, Kane S, Desai S, Keskar VR, Rajeshwarkar R, Panse N, Dinshaw KA. 2009. HPV screening for cervical cancer in rural India. N Engl J Med 360:1385–1394.

Schiffman M, Khan MJ, Solomon D, Herrero R, Wacholder S, Hildesheim A, Rodriguez AC, Bratti MC, Wheeler CM, Burk RD. 2005. A study of the impact of adding HPV types to cervical cancer screening and triage tests. J Natl Cancer Inst 97:147–150.

Schweizer J, Lu PS, Mahoney CW, Berard-Bergery M, Ho M, Ramasamy V, Silver JE, Bisht A, Labiad Y, Peck RB, Lim J, Jeronimo J, Howard R, Gravitt PE, Castle PE. 2010. Feasibility study of a human papillomavirus E6 oncoprotein test for diagnosis of cervical precancer and cancer. J Clin Microbiol 48:4646–4648.

Sherman ME, Lorincz AT, Scott DR, Wacholder S, Castle PE, Glass AG, Mielzynska-Lohnas I, Rush BB, Schiffman M. 2003. Baseline cytology, human papillomavirus testing, and risk for cervical neoplasia: A 10-year cohort analysis. J Natl Cancer Inst 95:46–52.

Sorbye SW, Fismen S, Gutteberg TJ, Mortensen ES, Skjeldestad FE. 2014. HPV mRNA is more specific than HPV DNA in triage of women with minor cervical lesions. PLoS ONE 9: e112934.

Szarewski A, Ambroisine L, Cadman L, Austin J, Ho L, Terry G, Liddle S, Dina R, McCarthy J, Buckley H, Bergeron C, Soutter P, Lyons D, Cuzick J. 2008. Comparison of predictors for high-grade cervical intraepithelial neoplasia in women with abnormal smears. Cancer Epidemiol Biomarkers Prev 17:3033–3042.

Thomison J III, Thomas LK, Shroyer KR. 2008. Human papillomavirus: Molecular and cytologic/histologic aspects related to cervical intraepithelial neoplasia and carcinoma. Hum Pathol 39:154–166.

Varnai AD, Bollmann M, Bankfalvi A, Speich N, Schmitt C, Griefingholt H, Kovacs K, Klozoris C, Bollmann R. 2008. Predictive testing of early cervical pre-cancer by detecting human papillomavirus E6/E7 mRNA in cervical cytologies up to high-grade squamous intraepithelial lesions: Diagnostic and prognostic implications. Oncol Rep 19:457–465.

Verdoodt F, Szarewski A, Halfon P, Cuschieri K, Arbyn M. 2013. Triage of women with minor abnormal cervical cytology: Meta-analysis of the accuracy of an assay targeting messenger ribonucleic acid of 5 high-risk human papillomavirus types. Cancer Cytopathol 121:675–687.

Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 189:12–19.

Waldstrom M, Christensen RK, Ornskov D. 2013. Evaluation of p16 (INK4a)/Ki-67 dual stain in comparison with an mRNA human papillomavirus test on liquid-based cytology samples with low-grade squamous intraepithelial lesion. Cancer Cytopathol 121:136–145.

Zappacosta R, Gatta DM, Marinucci P, Capanna S, Lattanzio G, Caraceni D, Rosini S. 2015. Role of E6/E7 mRNA test in the diagnostic algorithm of HPV-positive patients showing ASCUS and LSIL: Clinical and economic implications in a publicly financed healthcare system. Expert Rev Mol Diagn 15:137–150.

zur Hausen H. 1996. Papillomavirus infections—A major cause of human cancers. Biochim Biophys Acta 1288:F55–F78.