# Comparison of Cytology, Colposcopy, HPV Typing and Biomarker Analysis in Cervical Neoplasia

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**Abstract.** *Background: Cervical cancer is the leading cause of mortality among women worldwide, despite existing prevention programs. In light of the recent development of anti-HPV vaccines, the aim of this study was to evaluate concurrently the efficacy of four methods for risk assessment (cytology, colposcopy, HPV molecular typing and detection of biomarkers in cervical biopsies) in an attempt to define the most efficient combination. Patients and Methods: The studied group included 62 women with abnormal Pap tests and cervical lesions ranging from cervicitis and condylomas to intraepithelial neoplasias and invasive cancer. All women underwent full colposcopy assessment and colposcopically-taken biopsies were selected for histological examination, immunohistochemical identification of p16, p53, Bcl-2 biomarkers, as well as molecular detection and typing of HPV genomes. Results: Cytology and colposcopy showed very high sensitivity in detecting CIN and cancer (91.7% and 94.4%, respectively), but low specificity (34.6% and 50%, respectively). The detection of the 3 biomarkers reached an impressive sensitivity (83.3%) and a moderate specificity (65.4%). HPV detection and typing achieved 77.8% sensitivity, and the highest specificity of 80.8% in detecting CIN and cancer cases. HPV DNA testing had the highest positive prognostic value (84.9%; confidence interval, CI: 67.4%- 94.3%) and cytology the lowest (66.0%; CI: 51.2%- 78.4%). Coupled HPV typing and colposcopy proved to be the most efficient combination, increasing sensitivity to 97.2% and negative prognostic value to 92.3%. The estimation of cervical neoplasia or cancer in women with high-risk HPV types increased approximately 15-fold (odds ratio, OR: 14.70; CI: 4.30-50.09, p<0.001), ~23-fold in the case of combined positive biomarkers (OR: 23.18; CI: 4.97- 104.23, p<0.001), and 35-fold in case of colposcopically detected cervical neoplasia (OR: 35.00; CI: 5.16- 225.07, p<0.001). Conclusion: The most efficient combination among all tested methodologies was found to be HPV typing with colposcopy.*

Cervical cancer is the second most frequent type of cancer and the leading cause of mortality among women worldwide (1). Human papillomavirus (HPV) is considered to be the main etiological factor for the development of cervical intraepithelial neoplasias (CIN) and cervical cancer, since the viral DNA is detected in about 97% of cases (2). HPV types have been characterised and classified according to their neoplastic ability as high-risk and low-risk types (3). High-risk HPV types (HR-HPV), such as 16, 18, 31 and 33, are associated with cervical cancer or advanced precancerous stages CIN stages II and III, which are cytologically characterized as high-grade squamous intraepithelial lesions (HGSIL) (4-6). On the other hand, low-risk HPV types (LR-HPV), such as 6 and 11, are mostly associated with benign genital lesions such as condylomas and CIN I, characterized as low-grade squamous intraepithelial lesions (LGSIL) which rarely progress to cancer (4, 6, 7).

The incidence of cervical cancer mortality rates varies in different geographic regions depending on existing screening routines in regard to cytology, colposcopy, or HPV DNA testing for identifying the population at risk (8, 9). Papanicolaou (Pap) cervical cytology examination has a relatively low sensitivity of 50 to 75% in detecting HGSIL, with high discrepancies between laboratories (10-12). Furthermore, about 10% of Pap smears classified as LGSIL or atypical squamous cells of undetermined significance/ atypical glandular cells of undetermined significance (ASCUS/AGUS) in reality have a high-grade disease (13-

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15). On the other hand, colposcopy may detect almost all cases of high-grade CIN, but has limited specificity and reproducibility in patients with minor cytological abnormalities (16-18). These limitations and the fact that HPV DNA is present in almost all cases of cervical cancer indicate that molecular testing for viral genomic sequences in a cervical sample might improve the efficacy of screening for this type of malignancy (19, 20). Molecular detection of the virus should be followed by HPV typing, which provides important information for prognosis and best treatment strategy (21). Nevertheless, since >90% of HPV infections are transient, the challenge is to identify those infected women who are at risk of developing high-grade lesions and cervical cancer without overtreating those with infections that are likely to resolve (22).

In recent years, some investigators have focused on the identification of molecular biomarkers of dysplastic cervical cells infected by high-risk HPV types as new tools for prognosis of malignancy. It is well established that high-risk viruses contribute to carcinogenesis predominantly through the action of the viral oncogenes E7 and E6, which interfere with function of host cell cycle regulatory proteins p16 and p53, respectively (23-25). Specifically, p16, a cellular tumor suppressor protein, regulates by slowing down the cell cycle. The expression of p16 is influenced by the retinoblastoma pRb, a tumor supressor protein, through negative feedback control. The E7 oncoprotein binds to and inactivates the (pRb) protein, promoting the $G_1$/S-phase of the cell cycle, leading to the enhanced expression of p16 (26, 27). The E6 oncoprotein binds to the tumor suppressor gene product p53 and inactivates it, resulting in increased cell proliferation and reduced apoptosis, both characteristic features of cervical cancer (28-30). Apoptotic cell death is blocked by the increased transcription of survival genes such as *Bcl-2* (31-33). Therefore, the expression of p16, p53 and Bcl-2 may be used as sensitive and specific biomarkers for cells with active expression of HPV oncogenes and may prove to be of great importance in future screening, prognosis and treatment of cervical intraepithelial lesions and cancer.

In this study, the efficacy of immunohistochemical detection of p16, p53, and Bcl-2 protein was examined in comparison to well-established screening methods of cytology, colposcopy and HPV typing in various premalignant and malignant cervical lesions. In the light of our findings, we discuss the best possible combination of methods in an attempt to improve the accuracy of prognostic screening for cervical cancer.

## Patients and Methods

*Patients*. The case group was selected out of a cohort of women who visited the 1st Department of Obstetrics and Gynecology, University of Athens, during a publicized cervical cancer-screening program, which included routine gynecological examination and Pap testing. All women with abnormal Pap smears were referred to the Colposcopy and Cervix Pathology Unit of the Department for further investigation. Sixty-two women participated in the study after informed consent.

*Cytological evaluation (Pap test)*. Cervical cell scrapings were collected with a cytobrush from the ectocervix and endocervix. The cytobrush was rolled onto 2 separate glass slides which then were spray-fixed. Smears were classified by a cytologist, using the Bethesda classification system (34).

*Colposcopy and sample collection*. All patients with abnormal Pap smears underwent a full colposcopic assessment of the anogenital area using 5% acetic acid. The observed cervical appearance was recorded for each participant. Punch biopsies were obtained from all women for histological examination, immunohistochemical analysis and HPV typing.

*Histological evaluation*. Four sections of biopsies were prepared. One of them was stained with hematoxylin- eosin and analyzed by a single pathologist. Tissues were classified according to the CIN classification system as either normal cervix (including cases of chronic inflammation), mild dysplasia (CIN I), moderate dysplasia (CIN II), severe dysplasia or carcinoma *in situ* (CIN III), invasive squamous carcinoma, or adenocarcinoma (35). The pathologist had no clinical information or results of cytology, colposcopy or HPV testing.

*Immunohistochemical investigation for molecular biomarkers*. Three sections of biopsies were used for immunohistochemical detection of p16, p53 and Bcl-2 proteins. For the identification of p16, the section was incubated with monoclonal primary antibody against it (p16 F-12: sc-1661, dilution 1:500; Santa Cruz Biotechology Inc., Santa Cruz, CA, USA) as described elsewhere (36). Tonsil tissue, which strongly expresses p16, was used as positive control, while tonsil tissue without primary antibody treatment was used as negative control. A positive reaction for p16 was quantitatively determined when the nucleus in the cancer cells was stained more strongly than stroma cells. For the detection of p53 protein, the sections were incubated with monoclonal primary antibodies against it, which detects both wild-type and mutant forms of the gene product (NCL-p53-D07, diluted 1:200; Novocastra, Newcastle, UK), using standard immunohistochemical methodology, as described previously (37). A breast carcinoma with strong p53 expression was used as positive control, while a negative control was processed in the same manner, using phosphate-buffered saline (PBS) instead of the primary antibody. For the Bcl-2 protein detection, the sections were deparafinized in xylene, rehydrated in ethanol and were incubated with monoclonal primary antibodies against Bcl-2 (C-2-SC-7382, diluted 1:200; Santa Cruz Biotechnology Inc.). Colorectal carcinoma, which strongly expresses Bcl-2, was used as positive control. The same tissue was used as negative control using PBS instead of primary antibody (38). All samples were independently reviewed by two investigators blindly.

*Molecular detection of HPV*. Biopsies for HPV typing were immediately placed in sterile tubes containing RNAlater™ solution (Ambion Inc., USA) and were stored at –20˚C until DNA

extraction. The samples were given numbers and blindly processed for HPV evaluation. Total DNA was isolated using the Nucleospin™ DNA isolation kit (Macherey-Nagel, Germany). PCR amplification for HPV DNA was performed using the consensus primers MYO9/11 located at the L1 open-reading frame of more than 50 viral types (39). A positive (HPV type 16) and a negative control (blank sample) were used for each amplification. The PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized with ultraviolet transilluminator. A second nested PCR was used for detection of HPV DNA in samples with low viral load.

*Molecular typing of HPV.* HPV typing was performed by restriction fragment length polymorphism (RFLP) analysis. The amplification products were digested with restriction endonucleases *DdeI, HinfI, PstI* and *RsaI* (Takara, Japan), according to manufacturer's instructions. The digested products were analysed on 3% Metaphor™ (Bio Whittaker Molecular Applications, USA) agarose gel electrophoresis and the HPV types were determined according to previously published restriction patterns (40). In addition, the accuracy of HPV typing by RFLP analysis was examined by DNA sequencing of 10 samples. All nucleotide sequences were searched in GenBank and each of them was considered a match if it was found to have more than 80% nucleotide similarity to a known HPV type sequence, as previously suggested (41).

*Statistical analysis*. Statistical comparisons were performed using the SAS version 9.0 software package (SAS Institute Inc., USA). Sensitivity, specificity, positive and negative predictive values for neoplasia were calculated for cytology, colposcopy, HPV molecular typing and detection of biomarkers separately and in combinations, in an effort to assess for each methodology the detection rate of histologically confirmed CIN I, CIN II/III and cervical cancer samples. For this purpose, samples were considered positive for neoplasia by cytology when presenting with the diagnosis of ASCUS/AGUS and above, and positive by colposcopy when presenting with the diagnosis of CIN I and above. A sample was considered positive for neoplasia by HPV typing if a high-risk type was detected and positive for biomarkers if any of p16, p53 or Bcl-2 was expressed. The 95% confidence intervals (95% CI) were computed based on the binomial distribution. The two-tailed Fisher's exact test was applied in order to assess the screening efficacy of each method alone, as well as of their combinations. The Mantel-Haenszel method was applied for the calculation of odds ratios (OR) and their respective 95% CI. A $p$-value of $\leq 0.05$ was considered as the criterion for statistical significance for these analyses.

## Results

*Sample characteristics*. A cohort of 62 women aged 19-79 years (mean 40.7±13.4) participated in this study. Regarding their age distribution, 2 (3.2%) women were aged 20 years or less, 14 (22.6%) aged 21-30, 16 (25.8%) aged 31-40, 18 (29.0%) aged 41-50, 7 (11.3%) aged 51-60, 4 (6.5%) aged 61-70 and 1 (1.6%) aged more than 70 years. Thirty-four women (54.8%) reported that they were non smokers, 5 (9.6%) smoked fewer than 10 cigarettes per day, 9 (14.5%) smoked 10-19 cigarettes per day, 11 (17.7%) smoked 20-30 cigarettes per day and 3 (4.8%) smoked more than 30 cigarettes per day. One woman was previously diagnosed with systemic lupus erythematosus, while another had hepatitis C and reported the use of drugs on a constant basis.

*Cytological, colposcopic and histological findings*. The results from all screening tests performed and their correlation with histological grades are summarised in Table I, while Table II presents all screening categories in combination. Cytology indicated that 31 women had precancerous HGSIL and cancer, while histology showed that only 23 of them were actually premalignant CIN II/III and carcinoma cases (Table I). Interestingly, among the 6 women with ASCUS/AGUS cytology 4 were histologically detected with cervical cancer (Table I). Colposcopy findings corresponded well with histological classification in severe cases. Among the 31 CIN II/III and cancer cases that colposcopy detected, 29 women had histologically verified premalignant CIN II/III and carcinomas (Table I).

Out of the 50 specimens (60.2%) with the cytological classification of ASCUS/AGUS and above, 45 (90%) specimens were verified by histology (Table II). Among the 47 (75.8%) women who were classified as having CIN or cancer during colposcopy, only 34 (72.3%) retained the same classification by histological assessment (Table II).

*HPV typing*. DNA sequences of high-risk HPV types (HR-HPV types) were found in 33 (53.2%) of women, while low-risk HPV types (LR-HPV types) were found in 19 (30.6%) women (Table I). Among the 33 women with HR-HPV types, 28 (84.8%) were diagnosed with CIN or invasive cancer by histology, 32 (97.0%) had the same diagnosis by colposcopy, 31 (93.9%) were diagnosed with ASCUS/AGUS and above by cytology and 27 (81.8%) had at least one positive biomarker. Among the 19 women with LR-HPV types, 5 (26.9%) were diagnosed with CIN or invasive cancer by histology, 12 (63.2%) had the same diagnosis by colposcopy, 13 (68.4%) were diagnosed with ASCUS/AGUS and above by cytology and 8 (42.1%) had at least one positive biomarker (Table I). Among the 10 women with no HPV DNA detection, 3 (30.0%) were diagnosed with CIN or invasive cancer by both histology and colposcopy, 6 (60.0%) were diagnosed with ASCUS/AGUS and above by cytology and 4 (40.0%) had at least one positive biomarker.

Among all positive samples for the HR-HPV types, 25 (75.8%) were detected in CIN II/III and cervical cancer lesions, and HPV 16 and 18 DNA sequences were detected in 23 (69.7%) of them (Table I). Specifically, HPV 16 DNA was detected in 16 (48.5%) of positive HR-HPV women and among them 14 (87.5%) were diagnosed with CIN II/III and cancer. HPV 18 DNA was detected in 7(21.2%) of HR-HPV positive cases and all 7 (100%) of them were detected in cervical cancer lesions (Table I). All HPV 16 DNA-positive

Table I. *Association of cytology, colposcopy, HPV typing and biomarkers with cervical lesions of different histological grades.*

| | Histology | | | | | | |
|---|---|---|---|---|---|---|---|
| Cytology | Normal (n=1) | Cervicitis (n=3) | Condyloma (n=22) | CIN I (n=7) | CIN II/III (n=12) | Cancer (n=17) | Total (n=62) |
| Negative | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Coelocytosis | 0 | 0 | 9 | 2 | 0 | 1 | 12 |
| ASCUS/AGUS | 0 | 1 | 1 | 0 | 0 | 4 | 6 |
| LGSIL | 0 | 1 | 9 | 3 | 0 | 0 | 13 |
| HGSIL | 1 | 1 | 3 | 2 | 11 | 4 | 22 |
| Cancer | 0 | 0 | 0 | 0 | 1 | 8 | 9 |
| Colposcopy | | | | | | | |
| Negative | 1 | 3 | 3 | 1 | 0 | 0 | 8 |
| Condyloma | 0 | 0 | 6 | 1 | 0 | 0 | 7 |
| CIN I | 0 | 0 | 12 | 4 | 0 | 0 | 16 |
| CIN II/III | 0 | 0 | 1 | 1 | 12 | 3 | 17 |
| Cancer | 0 | 0 | 0 | 0 | 0 | 14 | 14 |
| HPV Typing | | | | | | | |
| Negative | 0 | 2 | 5 | 2 | 0 | 1 | 10 |
| LR Types | | | | | | | |
| 6 | 1 | 0 | 4 | 1 | 0 | 1 | 7 |
| 11 | 0 | 1 | 0 | 1 | 1 | 0 | 3 |
| 44 | 0 | 0 | 3 | 0 | 0 | 0 | 3 |
| 53 | 0 | 0 | 2 | 0 | 1 | 0 | 3 |
| 55 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 66 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 67 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| HR Types | | | | | | | |
| 16 | 0 | 0 | 1 | 1 | 6 | 8 | 16 |
| 18 | 0 | 0 | 0 | 0 | 0 | 7 | 7 |
| 31 | 0 | 0 | 0 | 1 | 2 | 0 | 3 |
| 33 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| 51 | 0 | 0 | 1 | 1 | 0 | 0 | 2 |
| 52 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| 58 | 0 | 0 | 1 | 0 | 1 | 0 | 2 |
| 59 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Biomarkers | | | | | | | |
| Negative | 0 | 3 | 14 | 4 | 1 | 1 | 23 |
| p16 | 0 | 0 | 1 | 1 | 4 | 8 | 14 |
| p53 | 1 | 0 | 6 | 2 | 1 | 0 | 10 |
| Bcl-2 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| p16 + p53 | 0 | 0 | 0 | 0 | 2 | 7 | 9 |
| P16 + Bcl-2 | 0 | 0 | 0 | 0 | 3 | 1 | 4 |
| P53 + Bcl-2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |

LR Types: Low-risk HPV types; HR types: high-risk HPV types; ASCUS, atypical squamous cells of undetermined significance; AGUS, atypical gradular cells of undetermined significance; LGSIL/HGSIL, low-grade/high grade squamous intraepithelial lesion.

samples were classified as ASCUS/AGUS and above by cytology and CIN or cancerous by colposcopy. Fifteen of them (93.8%) were definitively classified as CIN or cancerous by histological assessment and in 14 (93.3%) of them, the p16 biomarker was expressed (Table I).

*Biomarkers*. Biomarkers were detected in 39 (62.9%) cervical samples; in one third of these samples (35.9%), more than one biomarker was expressed (Table I). Among the 39 women with positive biomarkers, 30 (76.9%) were diagnosed with CIN or invasive cancer by histology, while 36 (92.3%) had the same diagnosis by colposcopy and the diagnosis of ASCUS/AGUS and above by cytology (Tables I and II). Biomarkers p16 and p53 were expressed in 27 (69.2%) and 20 (51.3%) out of all biomarker-positive specimens respectively. Among all p16-positive samples, 26 (96.3%) were definitively diagnosed with CIN and invasive cancer by histology, 27 (100%) had the same diagnosis by colposcopy and 26 (96.3%) had the diagnosis of ASCUS/AGUS and above by cytology. Among all p53-positive samples, 12 (60%) were definitively diagnosed with CIN and

Table II. *A combination of all screening result categories relating to the final histological diagnosis.*

| Screening result category | | | | Histology | | | | |
|---|---|---|---|---|---|---|---|---|
| HR-HPV | Cytology | Colposcopy | Biomarkers | No CIN or cancer (n=26) | CIN I (n=7) | CIN II/III (n=12) | Cancer (n=17) | Total (n=62) |
| – | – | – | – | 5 | 0 | 0 | 0 | 5 |
| – | – | – | + | 1 | 0 | 0 | 0 | 1 |
| – | – | + | – | 2 | 1 | 0 | 0 | 3 |
| – | – | + | + | 1 | 0 | 0 | 0 | 1 |
| – | + | – | – | 5 | 1 | 0 | 0 | 6 |
| – | + | – | + | 2 | 0 | 0 | 0 | 2 |
| – | + | + | – | 3 | 0 | 0 | 0 | 3 |
| – | + | + | + | 2 | 2 | 2 | 2 | 8 |
| + | – | – | – | 0 | 1 | 0 | 0 | 1 |
| + | – | – | + | 0 | 0 | 0 | 0 | 0 |
| + | – | + | – | 0 | 0 | 0 | 0 | 0 |
| + | – | + | + | 0 | 0 | 0 | 1 | 1 |
| + | + | – | – | 0 | 0 | 0 | 0 | 0 |
| + | + | – | + | 0 | 0 | 0 | 0 | 0 |
| + | + | + | – | 2 | 1 | 1 | 1 | 5 |
| + | + | + | + | 3 | 1 | 9 | 13 | 26 |

HR-HPV: High-risk human papillomavirus types; CIN, cervical intraepithelial neoplasia. The + sign, where applied, indicates a sample with the diagnosis of HR-HPV detection during HPV typing, or ASCUS/AGUS and above for cytology, or CIN I and above for colposcopy, or the expression of either p16, p53 or Bcl-2 during biomarker testing.

invasive cancer by histology, 17 (85%) had the same diagnosis by colposcopy and 18 (90.0%) had the diagnosis of ASCUS/AGUS and above by cytology. On the other hand, biomarker Bcl¬-2 was detected in only 6 (15.4%) out of all biomarker-positive specimens. Among the Bcl¬-2-positive specimens, 5 (83.3%) were definitively diagnosed with CIN and invasive cancer by histology and 6 (100%) had the same diagnosis by colposcopy and the diagnosis of ASCUS/AGUS and above by cytology.

Among the 14 women with double expression of biomarkers, 13 (92.9%) were diagnosed with CIN or invasive cancer by histology, while all of them (100%) had the same diagnosis by colposcopy and were also diagnosed with ASCUS/AGUS and above by cytology. Among the 23 women with no positive biomarker, 6 (21.1%) were diagnosed with CIN or invasive cancer by histology, 11 (47.8%) had the same diagnosis by colposcopy and 14 (60.9%) were diagnosed with ASCUS/AGUS and above by cytology.

*Screening efficacy of all tests.* The screening efficacy of all tests (including the combinations of colposcopy with either HPV typing or biomarkers and the combination of HPV typing and biomarkers) are shown in Table III. Colposcopy, as well as its combination with HPV typing and the combination of HPV typing with biomarkers identified women with a definite histological diagnosis of CIN or cancer with a sensitivity of 94.4% (CI: 80.0%-99.0%), the highest of all tests performed. HPV typing was shown to have the lowest sensitivity, namely 77.8% (CI: 60.4%-89.3%) and the highest specificity of all tests (80.8%, CI: 60.0%-92.7%) for detecting cases with CIN or cancer. Cytology was the test with the lowest specificity for this diagnosis (34.6%, CI: 17.9%-55.6%). HPV typing was attributed with the highest positive prognostic value (84.9%, CI: 67.4%-94.3%) and cytology the lowest (66.0%, CI: 51.2%-78.4%). In terms of negative prognostic value for CIN or cancer, the combination of HPV typing with colposcopy was shown to be the most efficient test (92.3%, CI: 64.2%-99.6%), while HPV typing alone had the lowest value (72.4%, CI: 52.5%-86.6%).

*Risk of CIN and cancer.* The risks for a definitive diagnosis of CIN or cancer in women with cytology findings of ASCUS/AGUS and above, colposcopy findings of CIN or cancer, HR-HPV type detection and positive biomarker expression per se or in combinations are shown in Table IV. There is an increased risk of a definitive diagnosis of CIN or cancer in women with positive HR-HPV results, either as a single test (OR: 14.70, CI: 4.30-50.09; $p<0.001$), or in combination either with biomarkers (OR: 23.18, CI: 4.97-104.23; $p<0.001$), or with colposcopy (OR: 35.00, CI: 5.16-225.07; $p<0.001$). In the latter combination, the 35-fold increased risk for CIN or cancer that was noted was the highest among all tests performed.

Table III. *Comparison of screening efficacy for CIN and cervical cancer between cytology, colposcopy, HPV typing and biomarker assessment.*

| Screening tool | Sensitivity (95% CI) | Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) |
|---|---|---|---|---|
| Cytology | 91.7% (76.4%-97.8%) | 34.6% (17.9%-55.6%) | 66.0% (51.2%-78.4%) | 75.0% (42.8%-93.3%) |
| Colposcopy | 94.4% (80.0%-99.0%) | 50% (30.4%-69.6%) | 72.3% (57.1%-83.9%) | 86.7% (58.4%-97.7%) |
| HPV typing | 77.8% (60.4%-89.3%) | 80.8% (60.0%-92.7%) | 84.9% (67.4%-94.3%) | 72.4% (52.5%-86.6%) |
| Any biomarker | 83.3% (66.5%-93.0%) | 65.4% (44.4%-82.1%) | 76.9% (60.3%-88.3%) | 73.9% (51.3%-88.9%) |
| HPV typing & colposcopy | 97.2% (83.8%-99.9%) | 50.0% (30.4%-69.6%) | 72.9% (57.9%-84.3%) | 92.3% (64.2%-99.6%) |
| Any biomarker & colposcopy | 94.4% (80.0%-99.0%) | 38.5% (20.9%-59.3%) | 68.0% (53.2%-80.1%) | 83.3% (50.9%-97.1%) |
| HPV typing & biomarkers | 94.4% (80.0%-99.0%) | 57.7% (37.2%-76.0%) | 75.6% (60.1%-86.6%) | 88.2% (62.3%-97.9%) |

PPV, Positive prognostic value; NPV, negative prognostic value.

Table IV. *Risk of CIN and cervical cancer based on assessment by cytology, colposcopy, HPV typing and biomarkers.*

| | | Histological detection of CIN I and above | | |
|---|---|---|---|---|
| Referral tool | Criteria | OR | 95% CI | *P-value*[a] |
| Cytology | ASCUS and above | 5.82 | (1.48-22.50) | 0.020 |
| Colposcopy | CIN I and above | 17.00 | (3.67-75.97) | <0.001 |
| HPV typing | Any HR-HPV | 14.70 | (4.30-50.09) | <0.001 |
| Biomarkers | Any biomarker | 9.44 | (2.92-30.46) | <0.001 |
| HPV typing+ colposcopy | Any HR-HPV or CIN I and above | 35.00 | (5.16-225.07) | <0.001 |
| Biomarkers + colposcopy | Any biomarker or CIN I and above | 10.63 | (2.29-47.78) | 0.002 |
| HPV typing + biomarkers | Any HR-HPV or any biomarker | 23.18 | (4.97-104.23) | <0.001 |

[a]Two-tailed Fisher's exact test. OR, odds ratio; CI, confidence interval; ASCUS, atypical squamous cells of undetermined significance; HR-HPV, high-risk human papillomavirus type; CIN, cervical intraepithelial neoplasia.

## Discussion

Prevention programs for cervical intraepithelial neoplasia, which are mostly based on cytological examination have achieved a 22% reduction in incidence and mortality of invasive cervical cancer but still with limitations due to low sensitivity and the false- negative rates reported by numerous studies (8, 9, 11, 12). In order to assess the issue of screening, this study concurrently evaluated four different methods for the detection of CIN or cervical cancer, namely cytology, colposcopy, HPV molecular typing and the expression of three biomarkers (p16, p53, Bcl-2) in cervical biopsy samples, each in comparison with histology examination results. The goals of this study were to define the most efficient identification of women at high risk for developing cervical cancer, to reduce the inherent errors of each screening methodology applied and to validate the most favourable combination of all methods, in terms of efficacy and efficiency for the detection of the disease.

Initially the validity of each test was evaluated in comparison with histological findings. Cytology alone identified the population at risk with a high sensitivity (91.7%) but the lowest (34.6%) specificity rate, mostly because it failed to identify the at-risk individuals among the ASCUS and LSIL categories who may be most susceptible to developing serious cervical disease. These findings are similar to those reported by previous studies (13, 42). HPV typing was shown to have the highest specificity of all tests (80.8%), with sensitivity as low as 77.8%. A possible explanation for this underestimation might be the existence of very few HPV infected cells or their total absence from the site of excision of the cervical samples taken for HPV testing.

The prevalence of HPV infection increased with the severity of cervical lesions, in accordance with other studies

(19, 43, 44). Surprisingly, colposcopy showed the highest sensitivity rate (94.4%) but a limited specificity (50%). Although colposcopy might theoretically detect almost all cases of high-grade CIN, the benefit is uncertain for patients with minor cytological abnormalities because of its low reproducibility and specificity. The screening efficacy of biomarkers p16, p53 and Bcl-2 displayed high sensitivity (83.3%) but an inadequate specificity (65.4%).

This study clearly indicated that the overall sensitivity and specificity of combined methods are enhanced and thus could be used in cervical cancer screening programs. Specifically, colposcopy combined with HPV molecular typing identified women with CIN and cancer with an optimal balance between sensitivity (97.2%) and specificity (80.8%). HPV typing gave the highest positive prognostic value (84.9%) and in terms of negative prognostic value for CIN or cancer, it seems that HPV typing with colposcopy is the most efficient combination, in agreement with other studies (46). Therefore, in a clinical setting of trained specialists, the use of colposcopy along with HPV testing could be useful for identifing women with ASCUS, AGUS, LSIL, or more serious cervical lesions and in reducing health care costs by reducing visits to doctor, avoiding repeated Pap tests, biopsy sampling and unnecessary treatments (47).

The alternative test of combined HPV testing and detection of biomarkers has shown a great potential in identifying women with CIN or cancer with a sensitivity of 94.4%. Of the three biomarkers studied, p16 showed the greatest diagnostic utility because of its expression in 69.2% of biomarker-positive specimens diagnosed with CIN or cancer, while p53 was expressed in 51.3% of all biomarker-positive specimens (48-50). Although there is an increased correlation between HPV positivity and p16 expression, it should also be noted that p16 was also expressed in a number of HPV-negative cases. This fact could indicate the existence of a *p16* up-regulation mechanism independent of HPV E6, E7 oncogenes. This may limit the use of p16 alone as a diagnostic biomarker, but in combination with other markers its diagnostic validity may be enhanced as has also been suggested by other investigators (48, 51, 52). Larger studies are needed to identify precisely the role of each biomarker in primary cervical screening due to the fact that HPV testing in combination with various biomarkers will increase the cost in cervical cancer screening programs.

Eight HR-HPV types were detected in more than half of the studied women and the great majority among them (84.8%) were diagnosed with CIN or invasive cancer. HPV 16 and 18 DNA sequences were present in two thirds (69.7%) of positive HR-HPV samples. These findings, which are in agreement with previous studies, clearly indicated that the great majority of women at risk for cervical malignancy could be identified early, if a larger population is screened regularly (19, 45). Recently, the development of the prophylactic vaccine for the most common HPV types 6, 11, 16 and 18, promises prevention of infection by immunization of the population before the onset of sexual activity (53). Despite the fact that the currently available vaccine may protect against only two HR-HPV types (16 and 18), this study as well as previous studies in the Greek population, suggest that more than a third of women with high-risk types would be protected against cervical cancer if vaccinated (54, 55). The increased rate of detection of 16 and 18 among HR-HPV types in the present study, as opposed to a previous one of our group (54), may reflect the selection process of more severe cases in the present cohort of patients. The development of new vaccines targeting a broader range of HR-HPV types based on the distribution of viral types across populations may eventually protect all women at risk. Until then, a screening program for cervical neoplasia may prevent new cases, using either cost-effective but less efficient cytology, or a highly effective combination of colposcopy and molecular typing of HPV DNA.

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