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ORIGINAL ARTICLE

A population of 1136 HPV DNA-HR positive women: expression of p16 INK4a / Ki67 Dual-Stain Cytology and cytological diagnosis. Histological correlations and cytological follow up

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Key words

HR-HPV (High Risk- Human Papilloma Virus) • p16/Ki67 +/- (p16 INK4a /Ki67 Immunostaining positive/negative) • HC2 (Hybrid Capture 2 - Qiagen, Hilden, Germany) • VVP CIN2+ (Positive Predictive Value for CIN2 or more) • NPV CIN2+ ((Negative Predictive Value for CIN2 or more)

Summary

Objective. The objectives of this study were to evaluate, in a selected HR-HPV positive population, the clinical performance of the p16/ki67 immunostaining in all the cytological diagnoses, as a reflex test of triage HPV-cytology, and assess the usefulness of p16/ki67-staining to classify CIN1 according to its risk of progression/regression in order to plan a personalized follow-up.
Methods. Our analysis was in consecutive cases of 1136 women aged 25-64 years, asymptomatic, HR-HPV DNA HC2 tested positive in a HPV-screening program, from February to December 2011. All the women had a cervical sample, in the Thin Prep, used for cytological diagnosis and for p16/Ki67 dual- staining. Histological correlations were 442. We studied the follow-up of two years of 387 cases, especially the biological behaviour of 316 low-grade lesions.
Results. p16/Ki67 dual-staining increases the VPP CIN2+ and NPV CIN2+, especially in atrophy/dystrophy, in ASC-US and LSIL. In follow-up of 387 cases, 71 CIN2+ and 316 CIN1, 69 CIN2+, after surgical treatment, had a negative follow up; two cases of CIN2 (p16/ki67-) without invasive treatments, had a spontaneous regression. Among the 316 CIN1, progression was observed in 10 women (4 p16/Ki67 + and 6 p16/Ki67 -); regression in 260 women (64 p16/Ki67 + and 196 p16/Ki67 -); 46 women had a persistent LSIL (9 p16/Ki67 + and 37 p16/Ki67 -). It seems no significant differences in the biological behaviour in relation to the expression of the two biomarkers.
Conclusions. p16/Ki67 immunostaining increases sensitivity of cytology in some diagnostic categories. After follow up of two years, a personalized and adequate treatment does not seem still possible. Further studies and trials are required to improve the management of the cervical lesions in HPV-based screening strategies.

Introduction

Epidemiological evidence shows that cervical cancer is related to sexual activity and associated to HR-HPV infection. Several authors compared specificity and sensibility of HPV test, cytology, and biomarkers to identify and manage high lesions of the cervix [1-8 27]. Many methods have been proposed and studied to improve the performance of HPV testing, providing different levels of evidence. As confirmed by "European guidelines for quality assurance in cervical cancer screening Second Edition Supplements. 2015", the validity relative to all new strategies triage may be: a) Cross-sectional because this is relevant for the decision of referring women to colposcopy; b) Longitudinal, to assess the risk of CIN2, CIN3, and cancer over time [9].
Numerous biomarkers proteins have been identified to increase the specificity of HPV-HR testing [1 2, 4-7 10-12]. Many of these proteins are involved in cell cycle regulation, such as p16 protein, signal transduction, DNA replication, and cellular proliferation. The altered expression of these proteins is a consequence of the binding of the HR-HPV E6 and E7 oncogenes to host regulatory proteins, resulting in the degradation of the p53 tumor suppressor gene product and the inactivation of the retinoblastoma protein leading to the deregulation of the cell cycle [2 13 14].
The Ki67 antigen, a high molecular weight non-histonic protein, is generally accepted as the most reliable marker

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of proliferating cells. It is expressed in all phases of the cell cycle, except G0 [15]. The interaction of E6 and E7 HPV DNA in the host cell disturbs the cell cycle, expressing themselves by the abnormal expression of proteins, including the Ki-67 [11]. In normal cells, the expression of p16 and Ki67 is mutually exclusive [3 16].
The objectives of this study were to evaluate the clinical performance of the p16/ki67 immunostaining in all the cytological diagnoses, as a reflex test of triage HPV-cytology, to identify samples of patients with high- grade cervical intraepithelial neoplasia [4 6 7 17 18], and assess the usefulness of p16/ki67 staining to classify cervical intraepithelial neoplasia grade 1 according to its risk of progression/regression [19] in order to plan a personalized follow-up.

Materials and methods

We conduced our analysis in consecutive cases of 1136 women aged 25-64 years, asymptomatic, HR-HPV DNA HC2 tested positive in a HPV-screening program, from February to December 2011. All the women had a cervical sample. The cytological specimen, collected in the Thin Prep® Pap Test (Hologic, Marlborough, Massachusetts Inc.), was used for cytological diagnosis and for p16/Ki67 dual staining. Thin-layer cytology slides were prepared using TP2000 slide Processor, according to manufacturer's protocol, stained according Papanicolaou method. The cytological diagnoses were made according to the Bethesda 2001 Cervical Cytology Classification System.
A second cytology slide was prepared from residual liquid-based material, using TP2000 slide Processor. Ten cases were excluded in this second examination because of the minimum squamous cellularity criteria as specified in the Bethesda System 2001.
The p16/Ki67 immunostaining was conducted using the CINtec®PLUS Kit (Roche mtm laboratories, Heidelberg, Germany) by monoclonal mouse anti-Human p16INK4a antibody, Clone E6H4TM, and monoclonal rabbit anti-Human Ki-67 antibody, Clone 274-11 AC3. Two Chromogen solutions were necessary: DAB Chromogen (3, 3'-diaminobenzidine Chromogen solution) and Fast Red Chromogen solution. Two Biologists analyzed all cases, individually and without knowing the cytological diagnosis. A positive result was, within the same cell, a brown nuclear and cytoplasmatic staining and red nuclear staining indicative of p16 and Ki67 expression [7]. The presence of one or more double-immunoreactive cells was regarded as a positive test outcome, irrespective of morphology. Slides without any double-stained cells were called negative for p16/ki67 dual-stain cytology [18].
A positive result for only one of the biomarkers (p16 or Ki67), in the same cytological slide, was regarded as a positive control of the immunocytochemical reaction.
The investigation with p16/Ki67 dual-staining did not alter the screening protocol. In fact, all the women with abnormal cytology were called for colposcopy and, in line with current clinical practice, gynecologists were aware of Cytology and HPV test results but blinded to any dual-stained cytology results [16].
Among all the 464 women with cytological abnormalities, 442 women agreed further study and were referred to colposcopy. At least one biopsy for further diagnostic was taken in all women. All histological samples were fixed in 10% buffered formalin and embedded in paraffin wax by conventional techniques. Serial section were stained with haematoxylin-eosin and classified by two certified pathologists.
The immunohistochemical investigation with primary monoclonal mouse antibody clone E6H4 TM (p16) and primary monoclonal mouse antibody clone MM1 (Ki67) were carried out on histological sections.

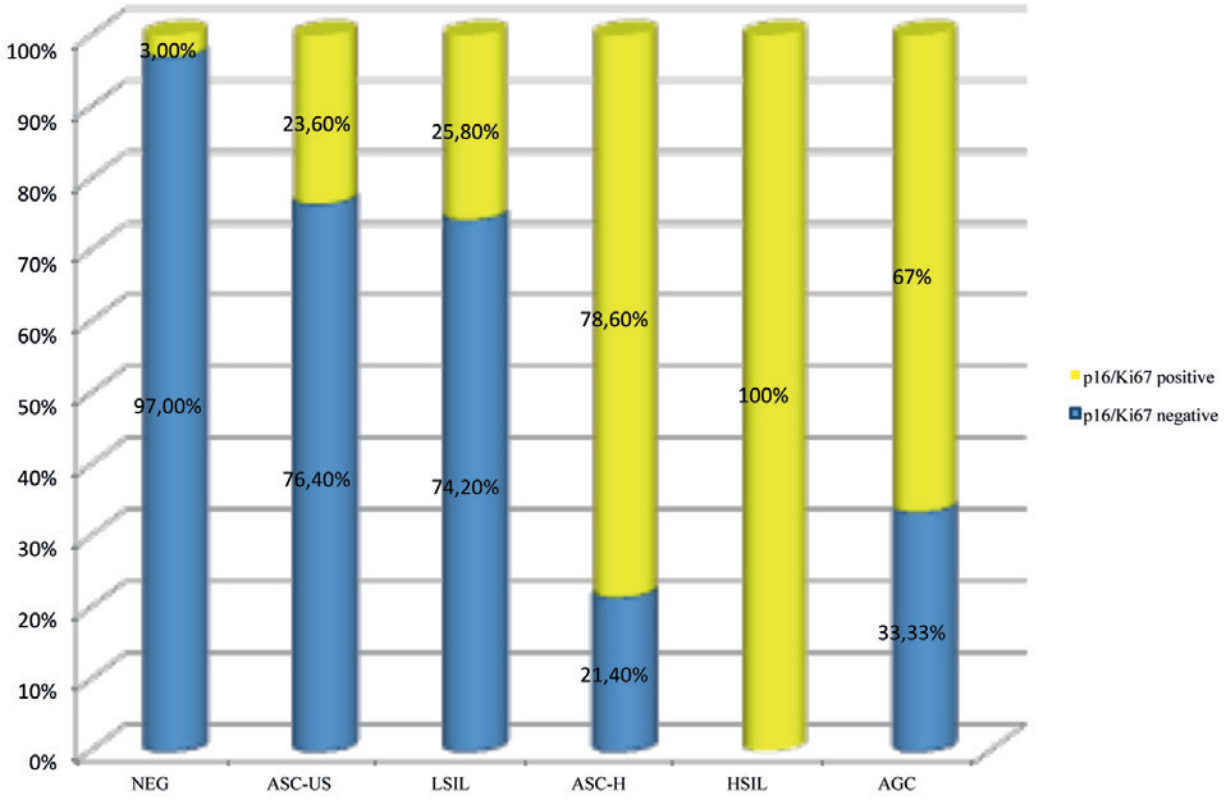
Results

All 1136 women had a cytological diagnosis with the following results: 662 had negative for intraepithelial lesion or malignancy, 177 had ASC-US, 236 had LSIL, 28 had ASC-H, 20 had HSIL, 3 had AGC and 10 had inadequate according to the Bethesda 2001 Cervical Cytology Classification System.
In the second phase, the immunostaining for p16/Ki67 was done on residual liquid-based material of all samples, except ten cases because of the minimum squamous cellularity.
Intersecting the results of the two types of analysis, cytology and p16/ki67 dual-staining, the following results were obtained (Fig. 1).
Cells showing co-expression of p16/ki67 were present in all cytological categories, with different percentages. p16/Ki67 double staining positivity (p16/Ki67 +) increased from the lowest (ASC-US, LSIL) to highest cytology categories (ASC-H, HSIL). More specifically p16/Ki67 + was: 23, 6% in ASC- US, 25, 8% in LSIL, 78.6% in ASC-H, 100% in HSIL and 67% of AGC. Among cytological negative cases a percentage (3.00%) showed elements in which the mechanism of cell regulation has been altered, due to HPV infection [16], even if the morphology does not yet seem atypical.
In this research Thin Prep (TP) monolayer slides was easily adaptable to the p16/Ki67 immunocytochemical assay. The methanol based fixative used by the TP system is capable of morphological preservation and maintaining the integrity of cellular protein 21, improving immunostaining efficiency [20].

Histological correlations

Among all the 464 women with cytological abnormalities, after three to eight month, 442 patients have accepted the invitation to more deepening examination. They were referred to colposcopy and biopsy sampling for further diagnostic follow-up in according to the screening protocol. In this study, biopsies were done in 155 women with cytological diagnosis of ASC-US, in all the

Fig. 1. Cytological diagnosis and CINtec® PLUS investigation: distribution of p16/Ki67 co-expression in 1136 cytological specimens.



patients with LSIL, ASC-H, HSIL and AGC, respectively 236, 28, 20 and 3 cases. The women did not come for more deepening examinations were 22.

The histological diagnoses were obtained: 55 negative; 316 condyloma/low-grade intraepithelial neoplasia CIN1; 47 high-grade intraepithelial neoplasia CIN2; 22 high-grade intraepithelial neoplasia CIN3; 2 Endocervical Adenocarcinoma in Situ.

For the cyto-histological correlation analysis we considered one endpoint CIN2 or more (CIN 2+), according to the current screening programs.

The Positive Predictive Value for CIN2+ (PPV CIN2+) in the cytological diagnoses was: ASC-US 9.7%; LSIL 9.3%, ASC-H 50%, HSIL 90.0%. The Negative Predictive Value for CIN2+ (NPV CIN2+) was calculated only for categories cytological of ASC-US and LSIL, which resulted to be respectively 90.3% and 90.7%.

In addition we evaluated the clinical performance of p16/ki67 as a test reflex of triage HPV-cytology to identify samples of patients with high-grade lesions. So, we calculated the hypothetical PPV and NPV of cytological categories based on p16/Ki67 double staining positivity (p16/Ki67 +) or negativity (p16/Ki67 -). The Positive Predictive Value for CIN2+ (PPV CIN2+) of ASC-US p16/Ki67 + was 45.5%, of LSIL p16/ki67 + was 43.2%; of ASC-H was 59.1%, of HSIL was 90, 0%.

Similarly, the Negative Predictive Value for CIN2+ (NPV CIN2+) of ASC-US p16/Ki67 - was 96.9%, of LSIL p16/Ki67 - was 97.2%.

In Figure 2 and Figure 3, the cytological and histological cases, that expressing the two biomarkers.

Follow-up

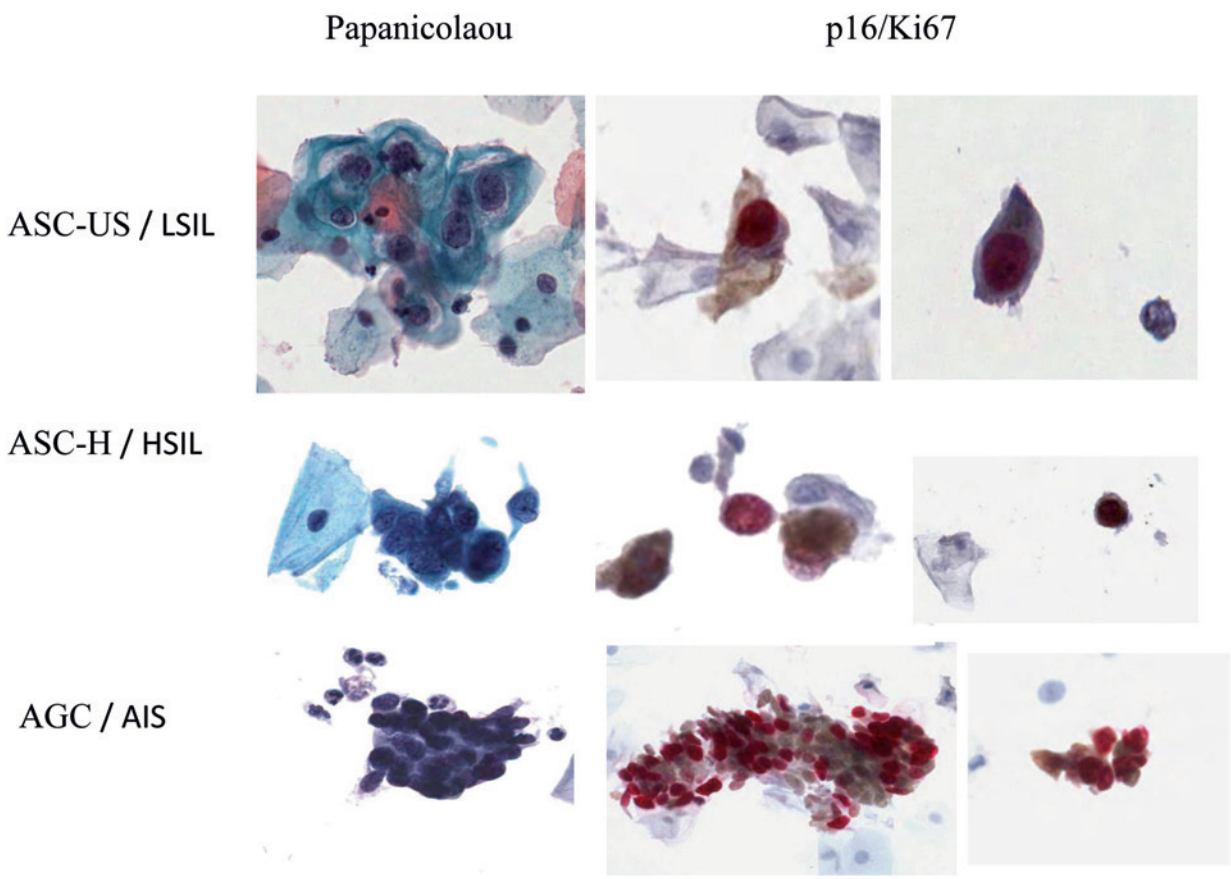
This study has considered cytological follow up of 387 cases, including 71 high-grade lesions and 316 low-grade lesions, until 31 December 2013, two years after the first diagnosis.

As regards the 71 women with high-grade lesions, excisional or ablative therapeutic interventions to remove the abnormal tissue were made on 69 cases (45 CIN2; 22 CIN3; 2 Endocervical Adenocarcinoma in Situ), according to the screening protocol. All these women have a negative follow up, through periodic checks with vaginal cytology specimens.

Only two women did not undergo treatments because pregnant and their high-grade lesions (CIN2) regressed spontaneously. Both had, as first diagnosis, ASCUS p16/Ki67 -.

In accordance with the screening protocol, the 316 low-grade lesions were followed with periodic checks, without surgical ablative treatment. Their evolution for two

Fig. 2. Cytological cases expressing the two biomarkers.



years after the first diagnosis has been studied, evaluating the following outcomes: progression was defined as a histological diagnosis of cervical intraepithelial neoplasia grades 2-3, regression as a negative cytology, and persistence as a cytological result of low-grade squamous intraepithelial lesion.

Overall 10 low-grade lesions progressed to high-grade lesions with a histological diagnosis of CIN2+, 260 regressed spontaneously in two years, and 46 resulted persistent low-grade lesions.

In order to understand the possible significance of p16/ki67 as markers of progression / regression, we subdivided the 316 low-grade lesions according to the results of Immunohistochemistry on the first cytological specimen and we have separately studied their biological behavior.

In this group of 316 cases, we had identified 77 cases p16/Ki67 + at initial cytological analysis and 239 cases p16/Ki67 -.

After two years, among the 77 low-grade lesions p16/Ki67 +, 4 (5.2% of the total of p16Ki67 +) progressed to CIN2+, 64 (83.1%) regressed and 9 (11.7%) were persistent low-grade lesions.

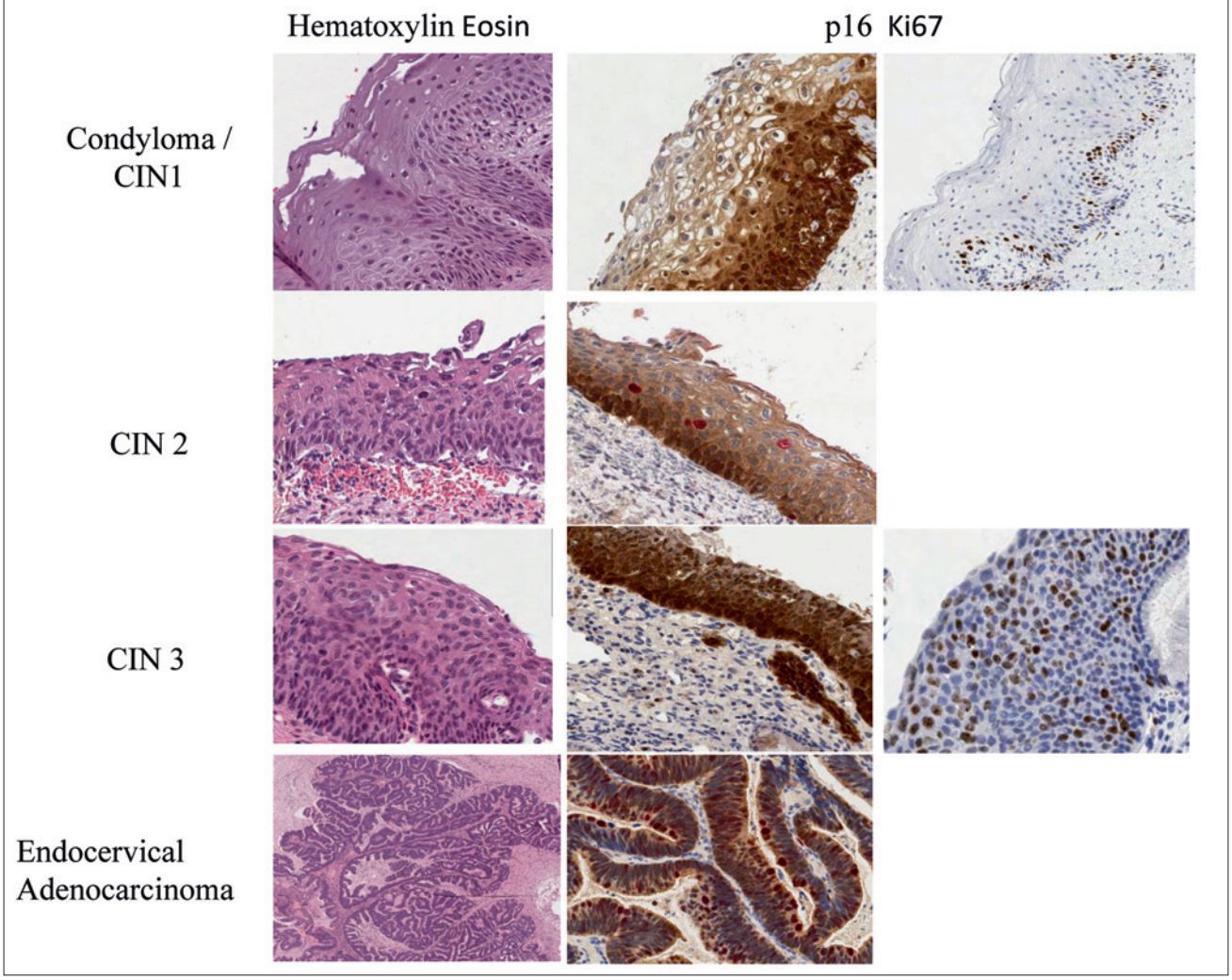
Similarly the study of the 239 low-grade lesions p16/Ki67 – showed 6 (2.5% of the total of p16Ki67 -) progressed to CIN2+, 196 (82.0%) regressed and 37 (15.5%) were persistent low-grade lesions (Tab. I).

Tab. I. Results of the biological behavior of 316 Low Grade Lesions. Follow up of two years.

	IIC results	TOTAL CASES (% of the total p16/Ki67 results*)
Progression to CIN2 + (10 cases)	p16/Ki67 positive	4 (5.2 %**)
	p16/Ki67 negative	6 (2.5 %***)
Regression to a normal cytology (260 cases)	p16/Ki67 positive	64 (83.1 %**)
	p16/Ki67 negative	196 (82.2 %***)
Low-grade lesion persistent (46 cases)	p16/Ki67 positive	9 (11.7 %**)
	p16/Ki67 negative	37 (15.5%***)

* The percentage value refers to the total number of cases CINtec®PLUS positive** or CINtec®PLUS negative***

Fig. 3. Histological cases expressing the two biomarkers.



Discussion

In the earlier part, the study examines the clinical performance of the p16/ki67 immunostaining in all the cytological diagnoses, as a reflex test of triage HPV-cytology.

The immunocytochemical investigation with CINtec®PLUS, in effect, can increase, the sensitivity of a single cytology test for the detection of cervical intraepithelial neoplasia of grade 2 or higher in the different diagnostic categories, by increasing of the Positive Predictive Value for CIN2 + and, even more, the Negative Predictive Value for CIN2+.

In women HPV positive/Pap cytology negative, p16/Ki67 dual-stained cytology may identify underlying CIN2+ [3]. Many authors consider the dual-stained cytology as a biomarker combination indicative of transforming HPV infections [16], even if the morphology seems still not atypical. In our experience, evaluating the cost / benefit ratio in the cases with negative cytology, the immunocytochemical investigation with CINtec® PLUS can be a valuable support in the clinical picture of atrophy / dystrophy (menopause) in which the differential diagnosis among atypical squamous metaplasia and high-grade lesion needs to be done, avoiding a false negative diagnosis. Similarly, the investigation with the double-staining is not necessary in the cytological diagnosis of ASC-H and HSIL, because the sensitivity of cytology is already high and does not require further examination. Women with ASC-H (atypical squamous cells, high-grade squamous lesion cannot be excluded), HSIL (high grade squamous intraepithelial lesion), or a more severe finding at cytology triage should be referred to colposcopy without further observation or testing [9].

In agreement with literature, the results of this study show that p16/Ki67 dual-staining cytology may be useful in women with cytological diagnosis of ASC-US or LSIL.

In cases p16/ki67+, the Positive Predictive Value for CIN2+ (PPV CIN2+) of ASC-US was 45.5%, of LSIL was 43.2%; of ASC-H was 59.1%, of HSIL was 90, 0%,

with an important percentage variation of +35.6% for ASC-US and +33.3% for LSIL.
In cases p16/ki67-, it seems to be more significant the Negative Predictive Value for CIN2+ (NPV CIN2+) for ASC-US and LSIL that is respectively 96.9% and 97.2%, with a percentage variation of + 6.6% for ASC-US and + 6.5% for LSIL. These values are similar to NPV 96%, referred in the literature [21 22]. Because of the high NPV, the number of colposcopy could potentially decrease for this group of women, but some authors describe, however, few women, with low-grade lesions p16 negative, which may progress to CIN 3 [5].
In glandular lesions, the double-immunostaining can be an aid in the differential diagnosis among Endocervical Adenocarcinoma in Situ (AIS) and benign endocervical glandular lesions [26], as well as ProExC [12]. The morphology-independent interpretation of p16/Ki67 dual-staining cytology testing, has a higher reproducibility compared to the p16 single-staining cytology approach, which needs of further morphological interpretation [7 10 17]. In this study, the diagnostic agreement in the interpretation of the results of Immunohistochemistry was 100% between the diagnoses made individually by two or more biologists and pathologists.
Various combinations of cytological, molecular and / or histopathological test results must be integrated in order to determine the risk of an individual woman for precancer / cancer and – according to the level of risk – its proper management [12].
In the second part of our study, we evaluated the usefulness of p16 / Ki67-staining CIN1 to classify according to its risk of progression / regression in order to plan a personalized follow-up. So we studied the follow-up of two years on a group of 387 women, evaluating the biological behavior of cervical lesions without surgery.
Most of high-grade lesions are more likely to persist rather than regress [23]. Cases of spontaneous regression are described among the high-grade lesions p16/Ki67 -, more frequently among CIN2, indicating the presence of a small subset of HSIL with low proliferative activity. Unfortunately, studies about spontaneous regression of CIN2 are difficult because of surgical treatment (conization) of these cervical lesions. In this study 69 cases high-grade lesions (45 CIN2; 22 CIN3; 2 Endocervical Adenocarcinoma) after surgical treatment, have a negative follow up, through periodic checks with vaginal cytology specimens.
Only two women did not undergo treatments because pregnant and their high-grade lesions (CIN2) regressed spontaneously. They had a first cytological diagnosis of ASCUS p16/Ki67 -.
The study about spontaneous regression of high-grade lesions requires additional clinical trials.
The majority of HR-HPV infection induces low grade lesions, which spontaneously regress, without treatment, within one to two years of exposure and less than 10% eventually progress to high-grade lesion or invasive cancer [14].
Many studies support a correlation between protein biomarkers, especially p16 expression and distribution pattern into the cell (diffusely or focally positive), and disease progression in low grade lesions. Statistical analyses showed a significant association between diffuse p16 staining and progression to CIN3, as well as between p16 negativity and regression at follow up [24]. Many studies show that CIN1 lesions p16 – rarely progress and may benefit from a less intensive follow up [19]; but few women, with p16 negative expression, may progress to CIN 3 [5].
In our follow up study of 316 low-grade lesions, only 10 lesions progress to high-grade lesions with a histological diagnosis of CIN2 after two years since the first diagnosis. Among these, the percentage of low-grade lesions, which in the first cytological analysis was p16/ki67 + is 5.2%; while the percentage of low-grade lesions p16/ki67 – which has progressed to a high-grade lesion, is 2.5%. 260 cases have a spontaneous regression, without the need for further treatments. The percentage of low-grade lesions, which in the first cytological analysis was p16/ki67 + and which later regress, is not very different from that p16/ki67 -. They are respectively of 83.1% and 82.2%.
The management of persistent lesions isn't yet standardized. Some women cannot psychologically tolerate periodical controls for a long time, and they prefer a decisive excision treatment, to prevent the development of more serious lesions.
In this study, 46 low-grade lesions are persistent. The different percentages of low-grade lesions, which in the first cytological analysis was p16/ki67 + or p16/ki67 -, slightly higher in cases p16/Ki67 -, do not seem significant because of the limited number of cases. p16/Ki67 dual-staining cytology testing does not seem to be an indicator for the persistence of the low-grade lesion. Other authors studied the possible correlation between additional biomarkers, such as p16, Ki67, E-cadherin, ProExC but the used biomarkers are not helpful to differentiate between persistent CIN and no persistent lesions [12 25]. A longer follow-up, perhaps, may be useful to better understand the biological behavior of these lesions.
In the oncogenesis of cervical carcinoma many events are necessary. The induction of chromosomal instability, accumulations of mutation and the status of the individual host's immune system influence the biological behavior, regression or progression, of cervical lesions [24 28].
According to literature [9], we think that currently available data are not yet sufficient to recommend performance of these and other triaging markers for management and follow up of HPV positive women. A review of the emerging evidence and an update of the current recommendations is required in the near future, to reduce over-treatment and plan a personalized follow-up.

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