EXPERT REVIEWS

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

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Objective: Colposcopy is widely used to triage women with mild cervical abnormalities. However, this approach is associated with low specificity and predictive value. The efficacy of E6/E7 mRNA test for this purpose has been demonstrated, but studies estimating its cost-effectiveness are still lacking. Given the limited healthcare financial resources, such an evaluation is a priority. Methods: We analyzed the clinical history of 432 women referred to colposcopy and colposcopy-directed biopsy for persisting ASCUS and LSIL, and compared three alternative triage protocols: immediate colposcopy; reflex HPV DNA testing and HPV DNA plus mRNA tests in sequence. Results: Molecular tests in sequence significantly reduce colposcopy referral, cost for assessed women, and cost for CIN2 detected. On the other hand, incremental cost-effectiveness ratio of this protocol was the highest. Conclusion: Our preliminary data, providing an estimation of the economic burden deriving from the introduction of E6/E7 mRNA test in the triage algorithm of patients with mild cervical abnormalities, may be useful for future healthcare policy.

KEYWORDS: ASCUS and LSIL • cost–effectiveness analysis • E6/E7 HPV-mRNA test • human papillomavirus • ICER • persistent HPV infections • triage test

Population-based screening programs, even in countries where screening is less than perfect, has significantly decreased the incidence of cervical cancer (CC) in large parts of the world. This improvement has been largely attributed to Papanicolaou (Pap) test. Despite this success, worldwide burden of cervicocarcinoma is still enormous: over 500,000 new cases diagnosed each year, and 280,000 deaths recorded [1,2].

It has been established that cervical malignancy is associated with oncogenic human papillomavirus (HPV) infection [3]. HPV-16 and -18 are the most prevalent genotypes, and together with HPV types 31, 33 and 45 account for >80% of CC worldwide [4]. However, HPV infection is a necessary but far from sufficient event during cervical carcinogenesis [5].

Invasive squamous cell cancer (SCC) of the cervix develops over time from the progression of a precursor lesion. Progression of the disease is slow and may take as long as 10–20 years before the disease becomes invasive [3]. The precursors, called cervical intra-epithelial neoplasia (CIN), are histologically graded into mild (CIN1), moderate (CIN2) and severe (CIN3). The corresponding cytological categorization, based on Bethesda system, encompasses atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL) and high-grade

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SIL (HSIL). The long natural history of CC provides the opportunity for early detection, treatment and cure. In spite of this, cytological screening has some limitations, including the low sensitivity and the poor inter-observer reproducibility of morphologic interpretation [6]. An audit of UK National Health Service Cancer Screening Programmes on CC found that CC occurred in 29% of women aged below 65 years of age, whose screening was up-to-date and in line with national guidelines [7]. ASCUS diagnosis, which is considered the border between clearly normal and clearly abnormal, as evidenced by Bethesda System terminology, is pathognomonic in this sense, underlying 38.8% of histologically confirmed high-grade lesions [8,9]. On the other hand, CDC estimates that the vast majority of HPV infections will regress within 2 years, and that only 10% of those would lead to intraepithelial lesions, which are at high risk for progression toward invasive cancer [10].

Of the million Pap smears performed each year worldwide, approximately 5% are diagnosed as ASCUS and 2.5% as LSIL. In the USA, about 2–3 million of ASCUS/LSIL are referred to colposcopic assessment each year. Cost for the management of these lesions was estimated in 3–6 billion dollars [11].

To balance the low predictive value of ASCUS, and the poor specificity of LSIL categories, some programs propose to repeat Pap test every year, thus compromising cost efficacy of CC screening [12].

HPV-DNA testing to triage ASCUS & LSIL

The weakness of cytological diagnosis animated scientific community to look for optional screening tools and predictive markers, in order to improve clinical accuracy of screening program. ASCUS and LSIL triage study, is a US National Cancer Institute multicenter randomized trial. It was designed to evaluate the best strategy to manage women who were referred to follow-up for ASCUS or LSIL cytology [13,14]. ASCUS and LSIL triage study extensively analyzed Hybrid Capture 2 assay (HC2, Qiagen GmbH, Hilden, Germany), a DNA-based assay that detects 13 oncogenic HPV types in aggregate [15]. At ASCUS threshold, HC2 demonstrated better sensitivity but equal specificity in detecting high-grade CIN, if compared with repeated Pap test, [13,16],. At LSIL threshold, HC2 showed equal sensitivity but lower specificity, in respect to repeated cytology [14,17,18]. It is clear that ASCUS and LSIL would inevitably create needless worry for women, as well as unnecessary costs for healthcare system, due to unnecessary surgical treatments [19].

In 2003, the US FDA approved the use of HC2 for primary screening [20]. Scientific evidences report the high sensitivity of HPV testing, as well as its negative predictive value (NPV) in conjunction with cytology. Primary screening with HC2 generally detects more than 90% of all CIN2+. It demonstrated 25% more sensitive than cytology at ASCUS and LSIL cut-off, although it is 6% relatively less specific [18]. In this context, it would be extremely important to not directly refer HPV-DNA-positive women to colposcopy, but to triage them with another test.

In countries where cytology is of a good quality, the current triage method is based on cytological evaluation of HPV-positive patients [18]. If Pap would test as abnormal, women should be referred to colposcopy. If cytology would be as normal, women are re-invited for a new screening round at regular interval. However, HPV-based screening should not start before 30–35 years of age, due to the high rate of infection that will spontaneously regress in younger people. Consequently, below 30 years of age cytological screening is recommended [18]. With these premises, it would be necessary to strictly monitor and coordinate HPV-based organized screening activities, as well as to clarify the uncertain aspects [20].

Knowledge about HPV-based screening is rapidly evolving, and it is possible that the previously mentioned protocol will change in the next few years [21].

The ideal test & E6/E7 oncoproteins

Presently, the challenge is how to discriminate between transient and persistent infections. In other terms, the issue would be to establish the real risk of developing CC by applying the most sensitive test (i.e., DNA testing) first, and the most specific test (the ideal test) second.

Much efforts have been done to find the ideal biomarker.

The carcinogenic potential of E6/E7 proteins was confirmed by numerous studies [22–24], which demonstrated their transforming properties as a consequence of their interaction with host cell-growth regulating proteins (i.e., p53 and pRb). E6/E7 would also be essential in maintaining malignant phenotype [25–28]. Recent data suggest the important role of E6/E7 in the inhibition of host immune response [29–31].

The pattern of HPV gene expression changes within the different layers of cervical epithelium. Viral life cycle is linked to the differentiation of squamous epithelium. In the undifferentiated basal layer, promoter and enhancer sequences are both represented. During replicative phase HPV-DNA is episomal, and E4 and E5 are the most expressed proteins. In this case, E6/E7 expression within basal layers is low but sufficient to start viral replication [32]. In the upper layers of the epithelium, E2 protein works as a repressor of E6/E7 transcription [32].

In persisting infection, high-grade lesions and cancers, HPV-DNA is often integrated into the host genome. E1/E2 region is disrupted, E6/E7 downregulation by E2 is lost and expression of E6/E7 oncogenes increases to cause transformation [28]. In such circumstances, E6/E7 proteins are expressed throughout the epithelium, and are able to block differentiation of cervical cells. This is a rare molecular event occurring during HPV life cycle and, in view of many HPV infections, is an exceptional occurrence [32]. On the other hand, continuous, deregulated and persistent activity of E6/E7 stem cells compartment would start neoplastic progression. Based on this background, it would be likely that E6/E7 expression represents an essential and indispensable requisite to develop CC [28], and the detection of such transcripts would

be useful to predict progression of cervical lesions toward malignancy [33].

Numerous in-house E6/E7 mRNA assay have been described and many commercial kits have been developed.

Aptima HPV Assay (Gen-Probe Incorporated, San Diego, CA, USA) detects 14 oncogenic HPV types and is based on target capture before transcription-mediated amplification of E6/E7 transcripts [34]. OncoTect (IncellDx, Menlo Park, CA, USA) combines FISH with flow cytometry to detect mRNA molecules on a single-cell level [35]. QuantiVirus assay (DiaCarta, Hayward, CA, USA) uses chemiluminescent detection of mRNA molecules, which are hybridized to DNA probes, and cover 13 oncogenic and 6 non-oncogenic HPV types [36]. Nuclisens EasyQ HPV assay (Biomerièux SA, Craponne, France), in certain countries distributed as PreTect HPV-Proofer assay (Norchip AS, Klokkarstua, Norway), is a multiplex nucleic acid sequence amplification technique detecting and genotypes full-length transcripts of E6/E7 oncoproteins in the same reaction. This test is based on the molecular beacon probe technology and on the real-time detection of the five most oncogenic HPV types (16, 18, 31, 33 and 45) [37].

A small nuclear specific ribonucleoprotein A (U1A) is included in the kit as internal control for RNA integrity and specimen adequacy [37]. When the target sequences are detected, fluorescent signal is emitted and analyzed by Nuclisens EasyQ HPV software in real-time.

Several studies assessed the diagnostic performances of PreTect HPV-Proofer and Nuclisens EasyQ HPV, hence designated with the collective name of mRNA test.

Cuschieri *et al.*, [38] showing an mRNA test specificity higher (81%) than that of DNA-based methods (44%), postulated the usefulness of mRNA testing in reducing unnecessary follow-up and treatment in women showing HPV infection.

A large cross-sectional study, carried out on about 4000 women older than 30 years assessed longitudinally for a minimum of 2 years, showed identical sensitivity in detecting CIN grade 2-or-worse (CIN2+) for DNA and RNA tests; the corresponding specificities were 50 and 85%, respectively [39].

Benevolo *et al.* carried out a retrospective study evaluating accuracy of mRNA test as a triage test. Stratifying patients by cytological grades, they found an mRNA test specificity of 45–82%, ranging the corresponding values for DNA testing from 4 to 29% [40].

All these data would suggest the introduction of mRNA test in the diagnostic algorithm of women with mild cervical abnormalities, in order to improve efficacy of CC prevention strategies.

CC screening in Italy

The Italian Ministry of Health establishes health-related objectives at national level, while allows their implementation to regional governments. Some Italian regions implemented CC screening programs in the 1970s, while nationwide organized programs started in 1996, following the European Commission Guidelines on Quality Assurance in Cervical Cancer Screening [41]. Presently, an organized CC screening program using liquid-based cytology (LBC) at 3-year interval is recommended in Italy for women aged 25–64 years [42]. In this country, the total annual cost for HPV-related disease is between €200 and €250 million [43]. The annual cost associated with the management of abnormal Pap smears, which were repeated after initial screening and were attributable to HPV was estimated to be €6.3 million [44]. Most of these costs were attributable to women with ASCUS and LSIL.

Repeating Pap testing and/or HPV-DNA testing in women with negative results would markedly affect costs of screening. Moreover, disadvantages to triage women with minor cytological abnormalities with a test having low specificity would include risks of overdiagnosis and overtreatment with possible subsequent adverse events, unnecessary psychological stress and healthcare costs [45]. In view of this evidence, WHO recommends to base decision-making processes not exclusively on price, but on quality/price ratio (cost–effectiveness ratio), in order to allow a more efficient delivery of healthcare interventions [46].

Currently, there are no studies estimating the cost–effectiveness of E6/E7 mRNA test in triaging ASCUS and LSIL. Given the limited nature of health resources, such evaluation becomes a priority.

The objective of the study was to bridge this foregoing gap, by analyzing the economic burden deriving from the introduction of mRNA test within the triage algorithm of patient reporting ASCUS and LSIL cytological diagnosis.

A new study

We derived data via literature review, and carried out a systematic search of the following electronic databases: MEDLINE (PubMed), CDC, NIHR Health Technology Assessment Program, covering the period 1990–2014. We also searched literature from other relevant sources, including the Italian Ministry of Health, the Italian National Institute of Health, WHO, the International Agency for Research on Cancer as well as other sites dedicated to scientific publications in socioeconomic and healthcare fields (e.g., Research Papers in Economics). Moreover, 25 national and international guidelines about management and treatment of abnormal Pap test were identified and reviewed. These included the European Guidelines for Quality Assurance in Cervical Cancer Screening [47], the Italian Society of Cervical pathology and Colposcopy [47], the Italian Society for Gynaecology and Obstetrics [48] and the Italian Group for Cervical Cancer Screening (GISCi) [42].

Ethical aspects

The investigators respected the prevailing norms of Good Clinical Practice as well as the requisites of the Declaration of Helsinki (1975, 2008 revision). The study was performed in agreement with the standards of the ethics review board of 'SS Annunziata' Hospital, and was tacitly approved by the Ethical Committees of 'G. d'Annunzio' University.

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Neither the first name nor surname or any other type of data indicating the identification of the patients has been registered, since identification has been made by numeric code.

Study design & sources

Using a retrospective incidence-based approach, we derived a clinical prediction model to evaluate the diagnostic performances of E6/E7 mRNA testing, and to compare costs and effectiveness of three alternative strategies for the management of ASCUS/LSIL.

1. Protocol 1: immediate colposcopy (extensively used in Italy, as well as in other countries) [42,47].
2. Protocol 2: triage with reflex HPV-DNA test.
3. Protocol 3: triage by reflex mRNA testing, performed on HPV-DNA-positive women.

Based on GISCi survey [42], we made the following assumption for our analysis:

- Protocol 1, if no lesion would be detected at colposcopy, patient should be referred to cytology at 6 months;
- Protocol 2, patients testing HPV-positive should be referred to colposcopy. Women showing HPV-DNA-negative result should return to normal screening intervals [47].
- Protocol 3, we hypothesized that patients showing DNA/mRNA positivity should refer to colposcopy and histological assessment of any visible lesion. Women showing CIN2- should repeat mRNA at 6 months. Patients with CIN2+ should refer to surgical treatment. Women with DNA-positive/mRNA-negative result should repeat DNA testing at 1 year (Figure 1).

Population

Case series were extracted from the electronic database of the public institution 'ASL 2 Abruzzo', by analyzing the clinical history of those patients who underwent colposcopy and colposcopy-directed biopsy from January to December 2013.

According to the ongoing protocol, these patients were referred for a colposcopy because of cytological evidence of LSIL or more severe; persisting ASCUS at 6-month repeated cytology and surveillance after excision for CIN2+ [47–49]. Following the aim of the study, we selected those patients who were referred for a colposcopy for persisting ASCUS and LSIL, and who also had a colposcopy-directed biopsy performed.

An additional inclusion criteria was the availability of residual liquid-based cervical specimen, stored at room temperature (RT) in the Laboratory of Advanced Diagnostic Techniques in Cellular Pathology (placed within the Surgical Pathology Unit), in accordance with the protocol of the Regional Cervical Cytology Biobank.

Exclusion criteria were:

- treatment for cervical lesion in the previous 5 years, or a history of any type of cancer;
- undergoing a surgical or ablative treatment during baseline colposcopy, except biopsy;
- hysterectomy;
- HIV positivity or other causes of immunodeficiency and
- pregnancy.

A written informed consent was obtained from all patients included in the study.

Since data regarding the management of cervical lesions after the baseline histological diagnosis were not available, we derived these from a literature review. Sixteen guidelines about treatment of pre-invasive lesions were identified. The review of these documents revealed that management practice of CINs is extremely varying [50]. In order to reflect the Italian national trend, we chose to apply data from 2006 GISCi survey [42,51]. For CIN2-, 63% of cases had no immediate treatment (follow-up cytology and colposcopy at 6 months), 20.3% had disruptive treatment (laser vaporization and diathermocoagulation) and 16.7% had excisional treatment (i.e., loop electrical excision procedure, cold blade or surgical conization); 100% of CIN2+ had excisional treatment.

Cytological & histological diagnosis

LBC result of ASCUS and LSIL was reported using Bethesda Reporting System (version 2001).

Histological diagnosis was classified as Cervical Intraepithelial Neoplasia grade 1, CIN1; CIN grade 2, CIN2; CIN grade 3, CIN3 and Invasive squamous cell carcinoma (SCC), according to 2003 WHO guidelines.

Two independent surgical pathologists, separately and blinded to all other study results, revised tissue slides and established the final diagnosis, basing on the highest CIN grade present within each sample. Benign cases (ectropion) as well as CIN1 were referred here as less than CIN2 (CIN2-). CIN2, CIN3 and SCC were referred here as CIN2+.

Only patients whose histological diagnosis reached consensus were finally included in the study.

In our analysis, we assumed that both colposcopy ad biopsy were 100% sensitive. In this view, histological diagnosis from biopsies taken under colposcopic control was accepted as a verification of disease status, and was regarded as the gold standard. Moreover, negative colposcopy was considered as sufficient ascertainment for absence of disease.

In accordance with recent studies, CIN2+, instead of CIN3+, was considered as the outcome threshold to calculate all diagnostic performances. CIN2+ would reflect the current community standard for referral to treatment, while CIN3+ is considered a research end point [39,40,50]. Arbyn *et al.* in their meta-analysis demonstrated that results do not change [45].

In this way, we reflected the current standard for treatment.

Molecular tests

HPV-DNA and mRNA testing were retrospectively performed on cervical cytological samples, stored at RT in PreservCyt solution (Hologic, Marlborough, MA, USA).

An aliquot (4 ml) of each LBC sample was removed to perform HPV-DNA testing by using the commercially available HC2 system, which detects 13 oncogenic HPV types

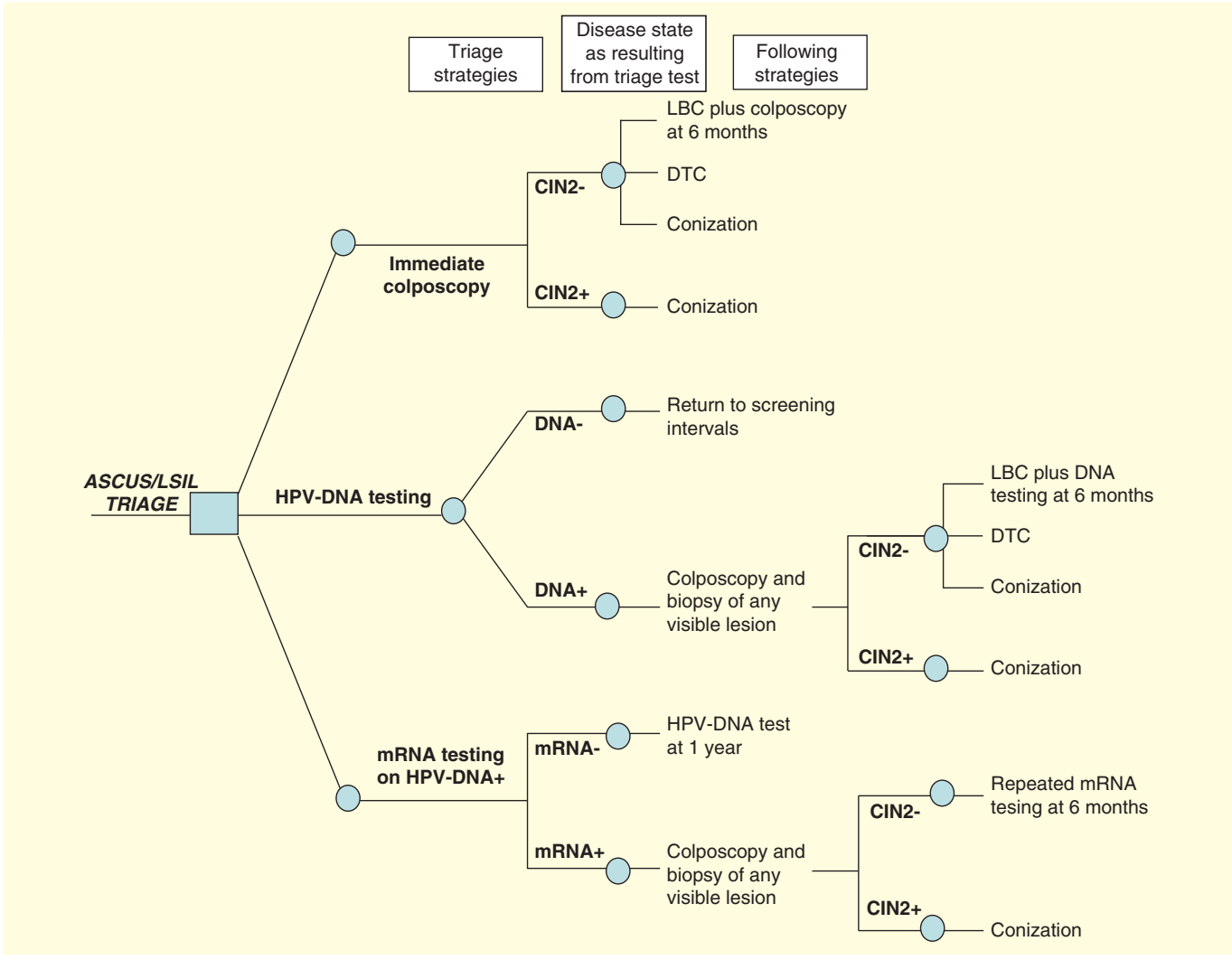

Figure 1. Decision tree used to determine the cost–effectiveness of the three different strategies to triage ASCUS/LSIL. The strategies were: immediate colposcopy; reflex HPV-DNA testing; reflex mRNA testing performed on HPV-DNA-positive cases.
ASCUS: Atypical squamous cells of undetermined significance; CIN: Cervical intra-epithelial neoplasia; DTC: Diatermocoagulation; HPV: Human papillomavirus; LBC: Liquid-based cytology; LSIL: Low-grade squamous intraepithelial lesion.

(16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). In accordance with manufacturer's protocol, HC2 reactions were read by a luminometer, which provided a relative quantification of each individual sample in comparison to the mean of a series of positive controls containing 1 pg/ml of HPV-DNA (corresponding to 100,000 HPV-16 genomes/ml or 5000 HPV copies per reaction). The cutoff of 1 relative light unit was used to classify a specimen as positive or negative.

A second aliquot (3 ml) from each residual LBC specimen was transferred into a fresh 10 ml tube for nucleic acids extraction by silica extraction technology (NucliSENS EasyMAG automated technology BioMèrieux, France); 15 µl of nucleic acids from each specimen was used to perform mRNA testing (Nuclisens EasyQ HPV, BioMèrieux, France), in accordance with the manufacturer's instructions.

Statistical analysis

By standard methods, the authors calculated the prevalence of HPV-DNA and mRNA positivities; 2 × 2 tables were used to correlate results from molecular tests with histological diagnosis, and chi square or Fisher's exact test was used to assess the association between variables. Concordance between histopathological diagnosis and molecular tests were calculated by Kappa statistics. According to the criteria of Landis and Koch, the K values were divided into six scales of strength of agreement: poor (<0.00), slight (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80) or almost perfect (0.81–100) [52]. Odds ratio (OR) was employed to assess the association of HPV-DNA test and mRNA test with histological outcome.

Cochran–Armitage test was used to assess the trend of DNA and mRNA tests results in relation with the severity of cervical lesion.

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Table 1. Distribution of studied cases by histological diagnosis, according to age, cytological report, human papillomavirus-DNA testing and E6/E7 mRNA testing.

Age category (years)	Histological diagnosis		Total (%)
	CIN2- (%)	*CIN2+ (%)*	
≤30 years	123 (33.6)	32 (48.5)	155 (35.9)
>30 years	243 (66.4)	34 (51.5)	277 (64.1)
Cytology			
ASCUS	153 (41.8)	17 (25.8)	170 (39.4)
LSIL	213 (58.2)	49 (74.2)	262 (60.6)
HPV-DNA test			
Negative	218 (59.6)	1 (1.5)	219 (50.7)
Positive	148 (40.4)	65 (98.5)	213 (49.3)
E6/E7 mRNA test			
Negative	325 (88.8)	3 (4.5)	328 (76)
Positive	41 (11.2)	63 (95.5)	104 (24)
Total	**366 (84.7)**	**66 (15.3)**	**432**

ASCUS: Atypical squamous cells of undetermined significance; CIN2+: Cervical intraepithelial neoplasia grade 2-or-worse; CIN2-: Less than CIN2; LSIL: Low-grade squamous intraepithelial lesion.

Accuracy parameters (sensitivity, specificity) of each test separately, as well as comparison of accuracy parameters were assessed by receiver operating characteristic analysis (receiving operating curve [ROC] curves), regarding histological diagnosis as the gold standard. Areas under the ROC curves with 95% CIs were estimated to assess differences in performances between molecular tests, and McNemar test was used for statistical significance. Positive predictive value (PPV) and NPV were also calculated, and results given with 95% CIs.

Statistical analyses were performed by using SPSS software (SPSS for Windows, Inc., Chicago, IL, USA), version 15.0. In all analyses, probability values $p < 0.05$ was regarded as significant.

Costs measures

Micro-costing techniques have been used to estimate cost-per-case of the three alternative triage options. Cost-per-case represents the present value of the total direct medical costs that accrued from the time of cytological diagnosis to follow-up [53]. Direct medical costs were modeled over a short period (1 year). Direct non-medical costs, and patient time costs were not included in the analysis, and given the short period, cost and effectiveness were not discounted [54].

Unit costs for the relevant procedures associated with both clinical and surgical management of cervical lesions were derived from the official 2013 National Italian Tariff Formularies [55]. Cost data were reported on hospital Disease-Related Groups (DRGs) basis and outpatient tariffs.

The DRG system aggregates all activities, including surgical interventions, drugs administered, materials and personnel for each individual diagnosis and stipulated the reimbursement tariff, which corresponds to the sum of all interventions provided, to be paid to the hospital. Outpatient costs included the cost of diagnosis, intervention and treatments for all women, as reimbursed to the local territorial healthcare service.

Given the large variation in terms of DRG and outpatient tariffs within the Italian Regions, mean national values were calculated.

It was assumed that excisional treatment for CIN2+ (loop electrical excision procedure, cold blade or surgical conization) was performed in hospital as inpatient procedures [56]. In our Institution, conization is usually done under general anesthesia and the patient need to stay in hospital overnight (hospitalization). Disruptive treatments for CIN2- (laser vaporization, cryotherapy and diathermocoagulation) were considered as outpatient procedures.

A cost for gynecologic examination was added to the cost of each diagnostic procedure. All costs were reported in 2013 Euros [55]. Costs for each alternative strategies of triage were modeled on the basis of GISCi survey [50].

Cost analysis & outcome measures

Cost analysis has been conducted comparing the costs of protocol 1 with the other two alternative strategies. Effectiveness was defined as the number of CIN2+ detected.

The relative performances of the whole alternative management strategies were expressed as incremental cost–effectiveness ratios, which were calculated as the incremental cost divided by the incremental effectiveness of protocols 2 and 3 compared with the benchmark protocol 1.

In this context, we use the term cost-effective to refer to the incremental cost–effectiveness ratio associated with a CIN2+ outcome, rather than to life expectancy or quality-adjusted life years.

Results

A total of 2611 women underwent to colposcopy between January and December 2013. Consensus during revision of histological slides was not reached for 29 (1.1%) patients. For 2150 (82.3%) women, residual LBC specimens were not available.

Finally, 432 (16.6%) patients were included in the study. The median time from collection of LBC samples were 11.3 months. The mean age ± standard deviation (SD) was 37.4 ± 11.4 years (range 19–81). One hundred and fifty-five women (35.9%) were 30 years of age or below, and two hundreds and seventy-seven (64.1%) were above 30 years of age; 86.3% of patients aged between 25 and 64 years.

Table 1 shows the distribution of patients by final histological outcome (CIN2- and CIN2+) according to age, cytological diagnosis (ASCUS or LSIL), HPV-DNA and mRNA testing. In all specimens, HPV-DNA and mRNA testing gave an interpretable result.

Table 2. Diagnostic performances of each molecular test separately and in sequence. CIN2+ was considered as the worse outcome.

Molecular testing	Diagnostic performances (95% CI)			
	Sensitivity	*Specificity*	*PPV*	*NPV*
DNA test	98.5% (91–100)	59.6% (54.5–64.5)	30.5% (24.3–36.7)	99.5% (98.7–100)
mRNA test	95.5% (86.8–98.9)	88.8% (85.1–91.6)	60.6% (51.2–70)	99.1% (98–100)
DNA plus mRNA[†]	95.4% (86.6–98.9)	76.4% (68.8–82.5)	63.9% (54.4–73.5)	97.4% (94.5–100)

[†]mRNA test performed on HPV-DNA-positive patients. (DNA test vs mRNA testing, p = 0.013; DNA test vs co-testing, p = 0.013; mRNA test vs co-testing, p = 0.84).
PPV: Positive predictive value; NPV: Negative predictive value.

Overall, 366 CIN2- and 66 CIN2+ were detected among the 432 patients. No SCC has been found.

The PPV of the different cytological categories was 10% for ASCUS and 18.7% for LSIL, respectively.

HPV-DNA positivity was detected in 49.3% (n = 213/432) of the patients. Of those, 44.1% (94/213) aged 30 years of age or below, while 55.9% (119/213) aged above 30 years.

Among HPV-DNA-positive cases, 30.5% (n = 65/213) showed CIN2+ and 69.5% (n = 148/213) showed CIN2-. HPV-DNA positivity rate was 98.5% among CIN2+ and 40.4% among CIN2-, respectively.

Within study population, 50.7% of women (n = 218/432) resulted DNA-negative. HPV-DNA negativity rate was 59.6% among CIN2- and 1.5% among CIN2+ patients (Cochran–Armitage trend test, $p < 10^{-4}$). A positive DNA test result conferred a CIN2+ OR risk of 95.7 (95% CI: 18.7–489.6). Overall percent agreement between DNA test and histological diagnosis was 65.5% (Cohen's kappa value: 0.30).

mRNA-positive results has been found in 24% (n = 104/432) of the specimens, which corresponded to 49 women (47.1%) aging 30 or below, and to 55 patients (52.9%) above 30 years of age. Among mRNA-positive cases, 60.6% (n = 63/104) had CIN2+ diagnosis, while 39.4% (n = 41/104) had CIN2-. The proportion of mRNA-positive results increased with the severity of histological diagnosis, being 11.2% among CIN2-, and 95.5% among CIN2+ (Cochran–Armitage trend test, $p < 10^{-4}$).

The percentage of women testing negative for mRNA was 75.9% (n = 328/432). Particularly, among CIN2-, mRNA negativity rate was 88.8%. mRNA test result was associated with CIN2+ diagnosis with an OR of 166.5 (95% CI: 54.1–512). Overall percent agreement between mRNA test and histological diagnosis was 89.8% (Cohen's kappa value: 0.68).

Accuracy parameters of each test separately are represented in Table 2. mRNA test improved specificity and PPV of DNA testing. Differences were statistically significant (McNemar test, p = 0.013). ROC curves analysis showed an area under the curve reaching 0.79 (95% CI: 0.75–0.83) for DNA test and 0.921 (95% CI: 0.89–0.95) for mRNA test (Figure 2). Difference reached statistical significance (p < 0.0001).

Diagnostic performances of molecular tests in sequence, when mRNA testing was applied on DNA-positive specimens, are also represented in Table 2. The combination DNA+ *plus* mRNA significantly improved specificity and PPV of DNA test alone (McNemar test, p = 0.013). Co-testing demonstrated a slightly lower specificity than that of mRNA alone, but difference did not reach statistical significance (McNemar test, p = 0.84).

Correlation between molecular test results and final outcome is highlighted in Figure 3. Overall percent agreement between co-testing and histological diagnosis was 82.2% (Cohen's kappa value: 0.63).

Table 3 shows results and costs analyses related to protocols 1, 2 and 3.

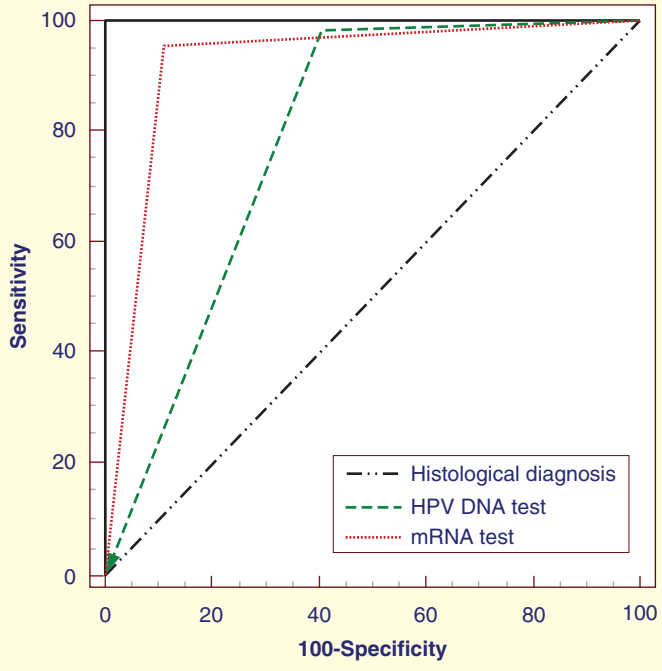

Figure 2. Summary of receiver operating curves comparing sensitivity and specificity of histological diagnosis (continuous tick line), mRNA test (continuous thin line) and HPV-DNA testing (dashed line) in detecting CIN2+.
AUCs reach 0.79 (95% CI: 0.75–0.83) for DNA test and 0.921 (95% CI: 0.89–0.95) for mRNA test (p < 0.0001). AUC of histological result is 1.0. Bisector line indicates a reference AUC threshold of 0.5.
AUC: Area under the curve; CIN2+: Cervical intraepithelial neoplasia grade 2-or-worse; HPV: Human papillomavirus; ROC: Receiver operating curve.

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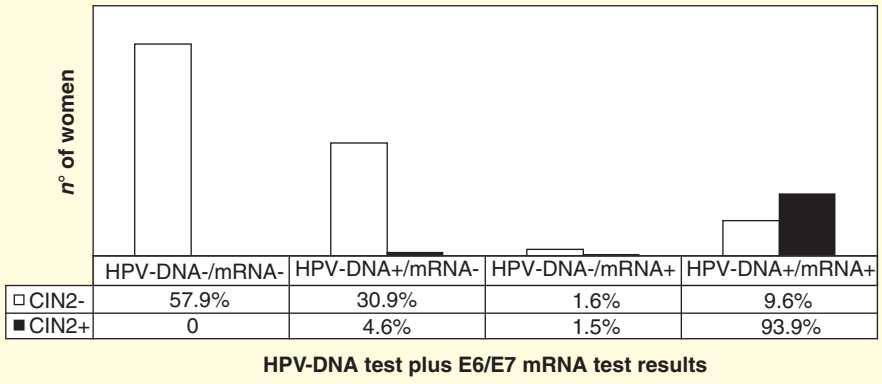

Figure 3. Correlation between histological diagnosis and molecular test results, obtained by the application of HPV-DNA and E6/E7 mRNA tests in sequence.
CIN2+: Cervical intraepithelial neoplasia grade 2-or-worse; CIN2-: Less than CIN2; HPV: Human papillomavirus.

According to GISCi survey, we simulated that 63% of CIN2- cases would have conservative management and follow-up with LBC *plus* colposcopy (protocol 1) or LBC *plus* HPV-DNA testing at 6 months (protocol 2), 20.3% would have diathermocoagulation and 16.7% excisional treatment (conization). For CIN2+ we simulated conization in 100% of the cases.

Current protocol was associated with a cost of €508.80 per assessed woman and of €3330 per CIN2+ detected. Compared with colposcopy triage, HPV triage reduced the number of colposcopies by 50.7%, reduced CIN2+ detection by 1.5% and was associated with a cost of €459.6 per assessed woman and of €3054 per CIN2+ detected.

Compared with immediate colposcopy, triage by HPV-DNA/mRNA combination reduced the number of colposcopies by 77.5%, reduced CIN2+ detected by 6.1% and was associated with a cost/assessed woman of €410.5, and of €2860 per CIN2+ detected.

With respect to HPV-DNA triage, co-testing reduced colposcopy referral by 54.5% and reduced CIN2+ detected by 4.6%.

Compared with current protocol, HPV-DNA triage showed a cost/assessed woman which decreased of €49.2, and was associated with a loss of 1 CIN2+. In this case, ICER was €327. In relation with current protocol, triage with co-testing reduced the cost per assessed women of €98.3, missed 4 CIN2+ and was associated with an ICER of €685 (Figure 4).

Expert commentary

Triage and follow-up of a large number of women with ASCUS and LSIL is associated with extensive costs, anxiety and possible adverse obstetric effects for patients [57]. Most of direct and indirect costs should be reduced, since most of these lesions will spontaneously regress over time. Additional methods to correctly identifying those women who will require further investigation or surgical procedures are then necessary.

HPV testing has been widely proposed as triage test. However, the mere identification of viral genome cannot allow distinction between transient and persistent infections, thus lacking in specificity.

The candidate biomarker should demonstrate high level of specificity in defining the different stages of cellular changes associated with HPV infection (persistence, progression or clearance). Since persistent integration of viral genome into the host genome has proven to be a key factor in CC development, a test revealing persistence of HPV infection would also give highly predictive information regarding the outcome of the lesion [58].

The most important consequence of the integration of HPV into the host genome is the continuous and deregulated expression of E6/E7 viral oncogenes. Persistent expression of E6/E7 oncoproteins is therefore a necessary step during cervical carcinogenesis. E6/E7 mRNA testing demonstrated a high-pooled specificity for the detection of CC precursors [55]. However, before introducing this test into clinical practice, deeper clinical validation and economic studies are necessary [59].

The present large retrospective analysis has been carried out within a clinical setting in which colposcopy is used as standard triage method, following European guidelines for quality assurance in cervical screening recommendations [47,48]. Although we are conscious that some underestimation might have occurred, since the sensitivity of colposcopy is considered as suboptimal, we adopted colposcopy-directed biopsy as the gold standard for the presence of CIN2+ [59,60].

Our data confirmed the low PPV of ASCUS and LSIL cytologic categories, the limited improvement of PPV of cytology by DNA testing (30.5 vs 14.5%) and the good sensitivity of HPV-DNA testing in detecting CIN2+ (98.5%) [58]. Our findings are in line with results from other studies [59,60].

In our study, mRNA triage of HPV-DNA-positive cases showed a good sensitivity (95.5 vs 98.5%), an excellent specificity (88.8 vs 59.6%) and almost double PPV (60.6 vs 30.5%) if compared with DNA triage alone. Our data also agreed with the findings of Molden *et al.*, demonstrating mRNA sensitivity and specificity of 85 and 86%, respectively [39], while different from the study by Benevolo *et al.* [40]. We have four possible explanations for these discrepancies. First, the inclusion criteria: Benevolo *et al.* included women having baseline mRNA test results, while we selected patients based on persisting ASCUS and LSIL cytological diagnosis. Second, Benevolo *et al.* considered ASCUS and LSIL as separate cytological categories. In their series, mRNA sensitivity ranged from 63 to 94% for ASCUS and 47 to 75% for LSIL, while mRNA specificity was in the order of 73–89% for ASCUS and 70–81% for LSIL [40]. Conversely, we referred test accuracy to pooled ASCUS and LSIL diagnosis, which were jointly considered as low-grade cervical abnormalities [45]. Third otherwise by us, Benevolo *et al.* collected and pooled data from four different clinical settings. Fourth, unlike

Table 3. Direct medical costs and number of CIN2+ detected, associated with the three different protocols.

Procedure	Unitary cost (€)[†]	Protocol 1	Cost	Protocol 2	Cost	Protocol 3	Cost
Diagnostic							
Colposcopy	10.74	432	4639.7	213	2287.6	97	1041.8
Cervical biopsy	14.10	432	6091.2	213	3003.3	97	1367.7
Histological evaluation of cervical biopsy	24.79	432	10709.3	213	5280.3	97	2404.6
LBC	31.29	–	–	–		–	
HPV-DNA test	81.60	–	–	432	35251.2	432	35251.2
E6/E7 mRNA test	122.57	–	–	–		213	26107.4
Gynecologic examination	13.63		10944.9	–	4579.7	–	–
Follow-up with LBC + colposcopy	–	231[‡]	12857.5	8638.8	–	–	–
Follow-up with LBC + DNA test	–	–	9708.9	93[§]	10498.8	–	–
Follow-up with DNA test	–	–	–	–	–	116	9465.6
Follow-up with mRNA test	–	–	–	–	–	35	4290
Treatments		–	–	–	–	–	–
DTC/laser vaporization/ cryotherapy	37.18	74	2751.3	30	1115.4	–	–
LEEP, cold blade or surgical conization	–	–	–	–	–	–	–
Day hospital	913.85	–	–	–	–	–	–
Full hospitalization	1,516.84	127	192638.7	90	136515.6	62	94044.1
Total cost	–	–	***219783.8***	–	***198531.8***		***177352.6***
CIN2+ detected	–	66	–	65	–	62	–
Cost per assessed woman	–	–	508.8	–	459.6	–	410.5
Cost per CIN2+ detected	–	–	3330	–	3054.3	–	2860.5

[†]Direct cost for procedure, medical and surgical treatments, in Euro (2013 Euro).
[‡]231 represents 63% of CIN2-, which were followed at 6 months with LBC plus colposcopy according with GISCi survey.
[§]93 represents 63% of CIN2-, which were followed at 6 months with LBC plus HPV-DNA test according with GISCi survey.
DTC: Diatermocoagulation; GISCi: Italian Group for Cervical Cancer Screening; LBC: Liquid-based cytology; LEEP: Loop electrical excision procedure.
Data taken from official 2013 National Italian Tariff Formularies [44,53].

Benevolo *et al.*, we calculated diagnostic accuracy of mRNA testing performed on the same residual liquid-based samples, which were used to prepare cytological slidex (reflex mRNA test).

Along with a good specificity, it is essential for a triage test to display a high sensitivity, in order to not miss patients with significant disease. In our findings, women having mRNA-positive result showed OR for underlined CIN2+ that was higher than that of women with a positive DNA result (166.5 vs 95.7). Since, OR encloses both sensitivity and PPV, the higher value we found would signify the overall better performances of mRNA test in identifying women who need further work-up. On the other hand, the risk of having CIN2+ despite a negative mRNA test was higher than that shown by patients with negative colposcopy or negative DNA test result. It is clear that co-testing increases the efficiency of colposcopy triage, but a negative result in this case does not provide the same safety risk of a negative DNA testing (NPV of 99.5% and 97.4, respectively).

Two studies documented results obtained by the application of mRNA test on SCC. In both analyses, mRNA test missed one cancer [60,61]. It is our opinion that, in such cases, DNA-positive/mRNA-negative patients should not return to normal screening interval, but should repeat DNA testing after 1 year.

In our study, mRNA triage of DNA-positive cases detected 93.9% of the overall CIN2+, versus 98.5% of CIN2+ detected by DNA test alone. This difference is in agreement with the

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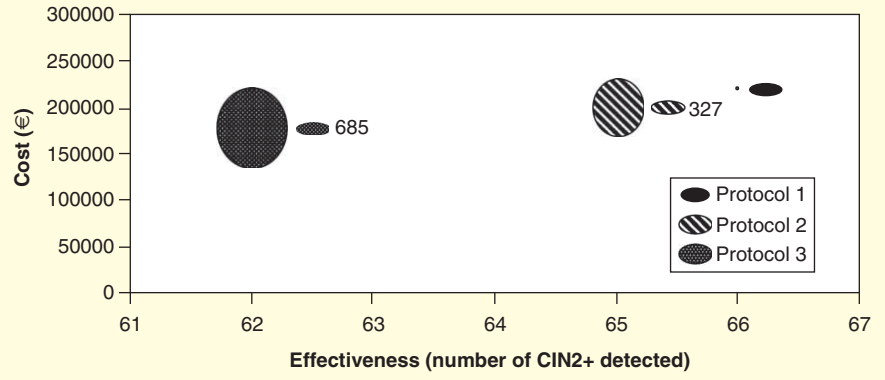

Figure 4. Bubble chart showing cost–effectiveness of the three alternative protocols to manage women reporting ASCUS and LSIL diagnosis. Cost values on Y axis express the global economic burden (in Euros) of the three different strategies. effectiveness on X axis is defined as the number of CIN2+ detected by the three different protocols. The area of bubble is proportional to the ICER. ICERs were calculated as the ratios between charge in cost and charge in effectiveness of protocol 2 (€327) and 3 (€685) in relation to protocol 1. Since protocol 1 is considered as the reference strategy, ICER has not been calculated.
ASCUS: Atypical squamous cells of undetermined significance; CIN2+: Cervical intraepithelial neoplasia grade 2-or-worse; ICER: Incremental cost–effectiveness ratio; LSIL: Low-grade squamous intraepithelial lesion.

cumulative proportion of cancers attributable to the 5 most common HPV types versus all 13 carcinogenic HPV types [61]. It has been repeatedly suggested that the increased specificity of mRNA testing is the direct result of the detection of only 5 oncogenic HPVs, rather than the 13 detected by HC2. This hypothesis is supported by data from studies investigating accuracy of Aptima assay, which targets all HPV types detected by HC2 plus the additional type 66. Compared with five HPV types mRNA test, Aptima demonstrated a high level of sensitivity. On the contrary, specificity was lower and did not differ from that of HC2 [62].

Analyzing results obtained by the application of mRNA test on HPV-DNA-negative cases too, we found that 1 CIN2 + resulted HPV-DNA negative but mRNA positive. This discrepancy would be due to the high analytical sensitivity of mRNA test, which relies on a RT-PCR platform, and to its ability to locate target sequences, which are not deleted during HPV integration. Vice versa, almost all DNA assays would detect L1 region that is deleted when viral genome is integrated into the host genome. As a consequence, HPV-DNA-based tests fail in identifying 4–25% of persistent infections and CC cases [62]. In agreement with other studies, in current analysis colposcopy and histological diagnosis were considered as gold standard, and colposcopy without biopsy was accepted as absence of cervical lesion. It was consequently assumed that colposcopy-directed biopsy was 100% sensitive. Although in accordance with Kim *et al.* [63], this assumption is imperfect and may influence the estimation of the accuracy of the different triage tests. In this context, the effectiveness of alternative strategies would be surely lower with respect to colposcopy triage, and this fact would in turn affect ICER index.

In our analysis, we demonstrated that triage with co-testing reduced colposcopy referral by 77.5% in comparison with current protocol, and by 54.5% in comparison with protocol based on HPV-DNA test alone. Therefore, both cost per assessed woman and cost per CIN2+ detected would decrease. Concerning this, the economic burden of protocol 3 demonstrated to be the lowest. The assumption that mRNA test has been performed as reflex test, on residual cytological specimen from the original liquid-based sample, contributed to further reduce cost, since costs of office visit and separate sample collection had been avoided.

Despite a positive cost/assessed woman and cost/CIN2+ detected analysis, the higher ICER found in relation to protocol 3 would highlight the need to also consider the rate of regressing CIN2+, and the less than perfect performances of colposcopy and biopsy as predictive marker for disease. These findings must to be viewed in the context of an overall CC screening program, in which most of the Pap abnormal diagnoses are ASCUS and LSIL and where the vast majority of CIN2+ are destined to regress. In this context, one of the weaknesses of our analysis is the lack of information regarding the longitudinal outcome of women with CIN2+ missed by co-testing. To hypnotize the biological behavior of such CIN2+, genotyping by real-time multiplex PCR (by Anyplex II HPV28, Seegene, Korea) was performed on residual LBC samples (data not shown). HPV-35 has been detected in first case, HPV-58, -51 and -73 in the second and HPV-52 in the third. In CCs originating from central and eastern Europe, the frequency of HPV-35, -39, -51, -52, -56, -58, -59 and -68 was significantly lower than that of HPVs 16, 18, 31, 33 and 45 [64]. Consequently, the missing of a minority of CIN2+, among which no invasive squamous cell cancer was detected, and most of which will be destined to regress, might not have a major negative impact on screening efficacy and may be acceptable if associated with a major cost sparing. If the cost of molecular testing would further decrease with technological progress, this protocol would be able to become the most cost-effective procedure in the future.

Another limitation of this analysis would consist in its modeling on an intermediate outcome (CIN2+). In this case, we were not able to estimate the cost per life saved or quality-adjusted life year gained. On the other hand, analyses which are able to estimate long-term outcome, would rely on secondary data, and would only postulate hypothesis on previous steps [63]. Our aim for future research should be to fill some of these gaps, as well as to reduce some uncertainties associated with the present estimates.

The present cost analysis is valid for the Italian scenario, in which cost data based on DRG tariffs did not include the actual direct costs incurred by hospital and outpatients practice.

Indirect costs, as well as direct non-medical costs (anxiety and fear of patients, days off from work, etc.) were also not included in this study, due to the lack of availability of published Italian data.

Many cost–effectiveness analyses prefer the human capital approach because they focus on the economic implication of disease, and ignore to quantify aspects of diminished health as 'pain and suffering', and other intangibles (such as anxiety and possible adverse obstetric effects among patients) [65].

In summary, the introduction of mRNA test in the clinical work-up of women with ASCUS and LSIL would certainly increase the diagnostic accuracy of HPV-DNA testing, thereby reducing overdiagnosis and overtreatment of minor cytological abnormalities. Compared with alternative triage strategies, co-testing would also reduce psychological distress and cost for women having transient infection.

In summary, the introduction of E6/E7 mRNA test in the triage of ASCUS and LSIL would lead to clinical and economic advantages that the correct identification of women with a true risk of developing CC would entail. As a counterpart, a level of caution must be exercised, due to a minority of CIN2+ which could be missed by this test. As suggested by our protocol, a rational proposal for a future application of mRNA test in women with ASCUS and LSIL would be to refer mRNA-positive women to colposcopy, and to repeat DNA test after 1 year in DNA-positive/mRNA-negative patients.

Although with the above listed limitations, we are confident that our data would provide a suitable estimation of the economic burden that mRNA test would entail in triaging ASCUS and LSIL, and would be useful for future healthcare policy in this sense.

Five-year view

Much effort has been done to identify and validate tools able to triage women with ASCUS and LSIL cervical abnormalities. Most of these markers have not yet passed the first phases of validation, but their number is expected to expand, as more genomic and proteomic studies will appear in the near future.

In the future, primary screening will most probably shift from cytology to HPV-DNA testing. Even more, new studies will be needed to validate mRNA test as triage test of women who resulted HPV-DNA positive at primary screening.

The reduction of CC risk obtained by HPV vaccination will be an additional dimension that would be considered in looking for the test with the highest PPV. Most national vaccination programs are currently under analysis but several issues still need to be addressed, in matter of overall potential and impact of vaccination on for public health [66]. First of all, the effects on female psychology. If vaccinated women will believe to be at no further risk of CC and will leave screening programs, the last impact of vaccination on the incidence of CC will be invalided [67].

In this context, it is therefore important that both women and healthcare professionals do not perceive HPV vaccination as an immediate alternative to CC screening. Only integrating vaccination strategy into screening programs would maximize benefits offered by HPV vaccine, and would lead to a further decrease of CC prevalence, incidence and mortality. In a background of vaccinated and well-screened population, the remaining CIN2+ would have a greater risk for progression [68,69].

In conclusion, moving clinical practice from cellular level toward molecular level, would not only allow the better identification of cervical precancerous, but would also prevent cervical abnormalities at the stage of molecular changes. Using mRNA testing, the management of women with HPV infection would be based on risk categories rather than on specific assay results.

This tailored approach gives hope for the improvement of effectiveness of CC prevention and for a significant reduction of screening costs.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.

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Key issues

- Persistent infection with oncogenic human papillomavirus is a key factor that could drive cervical intraepithelial lesions to progress toward invasive cancer.
- Atypical squamous cells of undetermined significance and low-grade squamous intraepithelial lesion cytologic categories have a low positive predictive value for CIN2+.
- Human papillomavirus-DNA test demonstrated low specificity and poor positive predictive value.
- mRNA test would better identify infections at high risk of persistence.
- mRNA test demonstrated high clinical performance.
- The introduction of mRNA test raises concern regarding how to best manage women with borderline or minor cytologic abnormalities.
- It would be of great importance to economically evaluate the possible introduction of mRNA test in the triage of patients with atypical squamous cells of undetermined significance/low-grade squamous intraepithelial lesion.
- The addition of mRNA test to diagnostic work-up presently in use would lead to clinical and economic advantages.

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