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Article

p16/ki67 and E6/E7 mRNA Accuracy and Prognostic Value in Triaging HPV DNA-Positive Women

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Abstract

Background: The study presents cross-sectional accuracy of E6 and E7 (E6/E7) mRNA detection and p16/ki67 dual staining, alone or in combination with cytology and human papillomavirus (HPV)16/18 genotyping, as a triage test in HPV DNA-positive women and their impact on cervical intraepithelial neoplasia (CIN2+) overdiagnosis. **Methods:** Women aged 25-64 years were recruited. HPV DNA-positive women were triaged with cytology and tested for E6/E7 mRNA and p16/ki67. Cytology positive women were referred to colposcopy, and negatives were randomly assigned to immediate colposcopy or to 1-year HPV retesting. Lesions found within 24 months since recruitment were included. All *P* values were 2-sided. **Results:** 40 509 women were recruited, and 3147 (7.8%) tested HPV DNA positive; 174 CIN2+ were found: sensitivity was 61.0% (95% confidence interval [CI] = 53.6 to 68.0), 94.4% (95% CI = 89.1 to 97.3), and 75.2% (95% CI = 68.1 to 81.6) for cytology, E6/E7 mRNA, and p16/ki67, respectively. Immediate referral was 25.6%, 66.8%, and 28.3%, respectively. Overall referral was 65.3%, 78.3%, and 63.3%, respectively. Cytology or p16/ki67, when combined with HPV16/18 typing, reached higher sensitivity with a small impact on referral. Among the 2306 HPV DNA-positive and cytology-negative women, relative CIN2+ detection in those randomly assigned at 1-year retesting vs immediate colposcopy suggests a -28% CIN2+ regression (95% CI = -57% to +20%); regression was higher in E6/E7 mRNA-negatives ($P_{interaction}$ = .29). HPV clearance at 1 year in E6/E7 mRNA and in p16/ki67 negative women was about 2 times higher than in positive women ($P_{interaction}$ < .001 for both). **Conclusions:** p16/ki67 showed good performance as a triage test. E6/E7 mRNA showed the highest sensitivity, at the price of too high a positivity rate to be efficient for triage. However, when negative, it showed a good prognostic value for clearance and CIN2+ regression.

ARTICLE

Human papillomavirus (HPV) DNA-based screening has been shown to be more effective than cytology-based screening, and its implementation is fundamental for cervical cancer elimination (1,2). It is replacing cytology-based screening in most high income countries and is now also the recommended strategy for low-income countries (3).

Positivity to HPV DNA is too high and prevalence of lesions in HPV DNA-positive women too low to refer all HPV DNA-positive women to colposcopy (4). It is therefore necessary to apply a triage test to identify those women who need immediate colposcopy and those who should undergo surveillance. To date, cytology, alone or in combination with partial genotyping,

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Table 1. Accrual timing, number of women, type of HPV DNA test, and HPV positivity rate, by NTCC2 study recruiting center

Recruiting center	Age range, y	No. of recruited women	HPV DNA assay	Date start	Date stop	HPV DNA positive (%)
Center						
Umbria	35-64	15 145	Cobas	04/2013	07/2016	898 (5.9)
Veneto	25-59	7478	Cobas	05/2015	07/2016	447 (6.0)
	25-59	2596	HC2	09/2014	05/2015	198 (7.6)
Florence	34-59	1049	Cobas	06/2016	10/2016	101 (9.6)
	34-59	7136	HC2	06/2015	06/2016	710 (9.9)
Turin	30-59	7105	HC2	02/2016	01/2017	793 (11.2)
Total included in the present analyses	25-64	40 509	—	04/2013	01/2017	3147 (7.8)
Total Cobas of which HPV16 and/or 18	25-64	23 672	—	04/2013	10/2016	1446 (6.1)
Total HC2	25-59	16 837	—	09/2014	01/2017	1701 (10.1)
Trento[a]	35-60	618	HC2	07/2016	10/2016	33 (5.3)
Total recruited	25-64	41 127	—	04/2013	01/2017	3180 (7.7)
Total Cobas	25-64	23 672	—	04/2013	10/2016	1446 (6.1)
Total HC2	25-60	17 455	—	09/2014	01/2017	1734 (9.9)

[a]Data from Trento are not included in the analyses reported in this work. HPV = human papillomavirus; NTCC2 = New Technologies for Cervical Cancer screening 2.

is the only triage strategy recommended by European (4) and US guidelines (5,6). Several biomarkers have been proposed as triage tests (7), including those targeting molecular changes due to overexpression of the viral oncogenes E6 and E7 (E6/E7), which are technologically well developed and mature for implementation, if proven to have better performance (8-17).

HPV DNA-based screening can also potentially increase false-positive histology results (18) and especially unnecessary treatments of regressive lesions. High-grade cervical intraepithelial neoplasia (CIN), particularly CIN2, is highly regressive, and its treatment is associated with negative pregnancy outcomes (19). Therefore, the ability of biomarkers to predict the persistence of lesions is important. Very little evidence is, however, available about the association between biomarkers' positivity and the probability of CIN2+ regression or virus clearance.

The clinical performance of a triage test depends on its accuracy as well as the algorithm of which it is part (eg, the sequence of tests, the interval for surveillance, and the test to be used in surveillance). In some of the most widespread algorithms, including that adopted by Italian screening programs (Supplementary Figure 1, available online), HPV DNA-positive/cytology-negative women are recalled after 12 months to be retested for HPV DNA (4,6,20,21) and return to routine screening if negative, whereas, if positive, they are referred to colposcopy. With such algorithms, the association between being negative to the triage test and clearance of HPV DNA is a strong determinant of overall colposcopy referral (22). However, direct information on how biomarkers predict clearance is lacking.

In the New Technologies for Cervical Cancer screening 2 (NTCC2) study, all of the HPV DNA-positive women were tested for cytology and putative biomarkers (E6/E7 mRNA and p16/ki67). Furthermore, HPV DNA-positive/cytology-negative women were randomly assigned to immediate colposcopy or to 1-year HPV DNA retesting. This design permits a comparison of the performance of these biomarkers, alone or in combination with cytology and partial genotyping, as a triage test and of their ability to predict CIN2+ regression and virus clearance.

Methods

Setting and Recruitment

Women were recruited from 5 Italian HPV DNA-based organized cervical cancer screening programs using active call and recall inviting systems (see Table 1). Colposcopies and follow-up visits were managed with scheduled appointments at the screening program facilities.

Study Design

The NTCC2 trial aimed at evaluating different biomarkers (E6/E7 mRNA [Aptima, Hologic, San Diego, CA]; p16/ki67 expression [Roche Diagnostics, Basel, Switzerland]) as putative triage tests for HPV DNA-positive women. Specific objectives were 1) to compare the cross-sectional accuracy of cytology, HPV E6/E7 mRNA, and p16/ki67 as triage tests; 2) to measure the value of these tests to predict CIN2 regression and HPV clearance in 1 year; and 3) to measure the 5-year CIN2+ and CIN3+ cumulative detection in HPV DNA-positive/triage-negative women.

To assess point 2, HPV DNA-positive and cytology-negative women were randomly assigned to immediate colposcopy or 1-year HPV DNA retesting. Here, we report on points 1 and 2. Data include all the CIN2+ found up to 24 months from recruitment, so that the assessment of HPV DNA-positive women is complete, and all the information necessary for the purpose is presented. Analyses include women recruited in 4 of the 5 centers (see Table 1).

Women aged 25-59 years who reside in program areas and are undergoing a new screening episode were eligible for the study (see Figure 1). In Umbria, women aged 60-64 years were also recruited and excluded from long-term follow-up but not from the present analyses. Exclusion criteria were pregnancy and treatment for a CIN2+ lesion in the previous 5 years. All women meeting the inclusion criteria were asked to provide written informed consent to participate in the trial. Women who refused were screened by HPV DNA according to routine practice. In these analyses, original histological diagnoses are considered. In all centers, p16/ki67 staining for histology was used only for equivocal cases.

Sample Size

The planned 60 000 women provided a 95% confidence interval (CI) of +/- 0.5 of 1000 of the CIN2+ cumulative incidence at 5 years in HPV-positive E6/E7 mRNA-negative women under the following assumptions: a cumulative incidence of 1 of 1000 in all the E6/E7 mRNA-negative women, 50% of the E6/E7 mRNA-

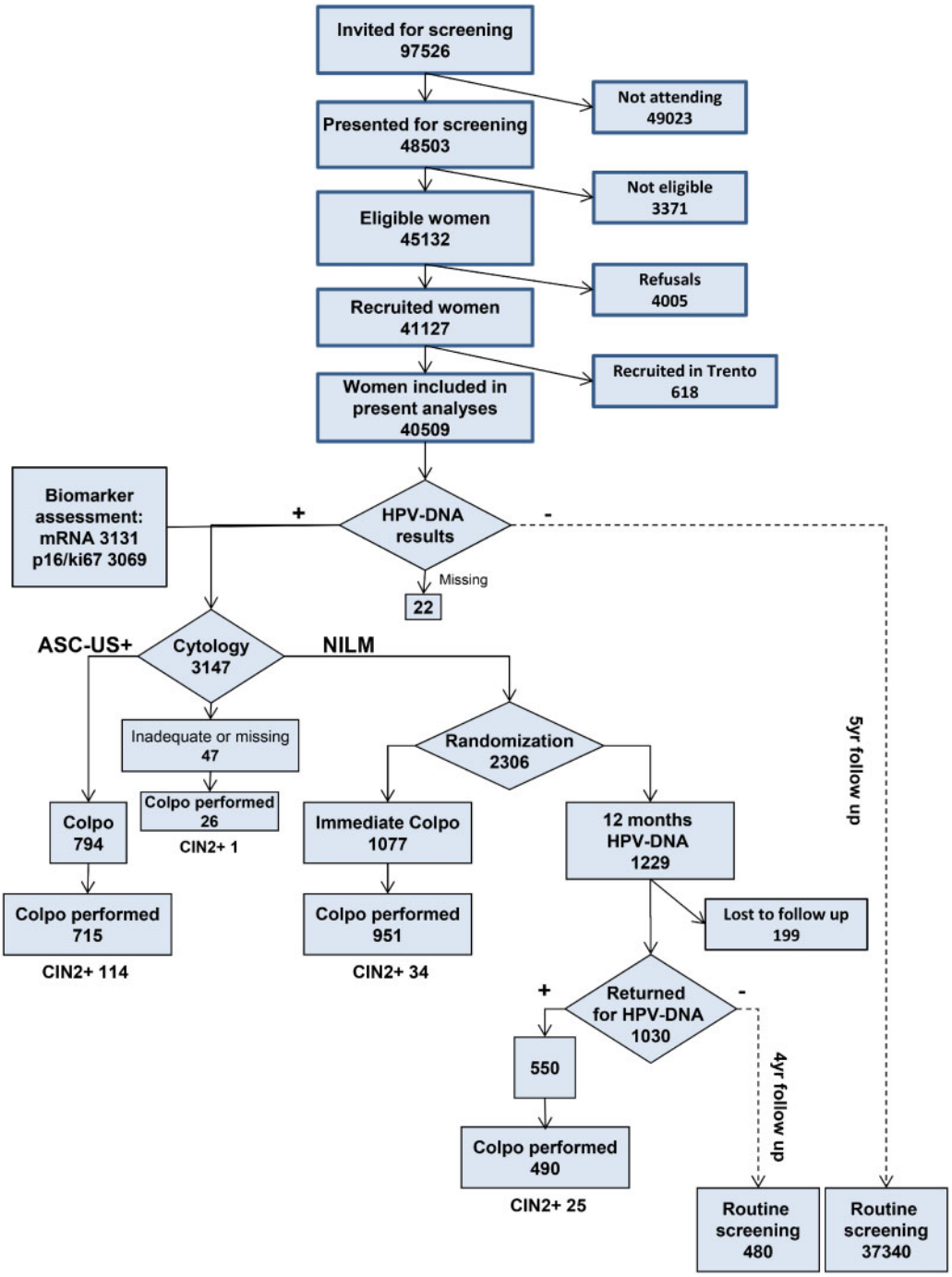

Figure 1. Study flowchart reporting recruitment process, randomization, and primary endpoint results. Women were invited for screening by the standard program management system. Participating women were assessed for eligibility and, if eligible, were asked to participate; those willing signed informed consent. All women underwent screening with human papillomavirus (HPV) DNA test with reflex cytology if positive; those included in the study and positive for HPV DNA were also tested for biomarkers (E6/E7 mRNA, p16/ki67). Women with atypical squamous cells of undeterminde significance (ASC-US) or more severe cytology were referred to colposcopy; those negative for intraepithelial lesion or malignancy (NILM) were randomized to immediate colposcopy or to 1-year retesting (the routine protocol in Italy; Supplementary Figure 1, available online). If HPV DNA positive at retesting, women were referred to colposcopy. CIN = cervical intraepithelial neoplasia; colpo = colposcopy.

negative women who developed a lesion in the following 5 years were HPV DNA positive at recruitment, and 70% completed follow up. This sample size would give an estimate of more than 400 CIN2+ lesions at baseline, in the hypothesis of a detection of 7 of 1000; with this number of CIN2+ the study would have more than 90% power to observe as statistically significantly different (alpha 0.05) two biomarkers with sensitivity 70% and 80%, respectively(McNemar 2-tail test, under the hypothesis of correlation ≥ 0.01). This sample size would have given 62% power to detect as statistically significant ($P < .05$) an 80% regression of the HPV DNA-positive E6/E7 mRNA-negative CIN2+ in the 1-year control arm vs the immediate colposcopy arm when assuming that 7% of the CIN2+ found in HPV DNA-positive women are negative to E6/E7 mRNA and that the total detection rate with HPV is 6 of

1000. Unfortunately, the actual detection was much lower (see below).

Primary Testing

All women were tested for HPV DNA. Cervical samples were collected in PreservCyt solution (Thin Prep, Hologic) and tested locally. Two different tests were used, based on the collection period and center (see Table 1): 1) COBAS 4800 HPV test (Roche), which separately reports positivity for HPV 16 and 18 and for at least 1 of the remaining genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68); 2) Hybrid Capture 2 (HC2, Qiagen, Hilden, Germany) for the pooled detection of 13 high-risk types (same as COBAS except HPV66). DNA was extracted using the QIAsymphony DSP HPV Media Kit (Qiagen), and hybridization was done by the Rapid Capture System (RCS) (Qiagen) according to manufacturer's instructions.

Management of HPV DNA-Positive Women

All HPV DNA-positive women had reflex cytology. Liquid-based slides (Hologic) were prepared and interpreted in local screening pathology laboratories using the Bethesda 2001 classification (23). All biomarker analyses were performed on the same cervicovaginal sample.

Cytology-positive women were referred to colposcopy, as in routine practice (Supplementary Figure 1, available online). HPV DNA-positive/cytology-negative women were randomly assigned, with 1:1 ratio, to immediate colposcopy or to repeat HPV DNA test after 12 months. Women were referred to colposcopy if still positive or returned to a new screening round if negative (20). Gynecologists performing colposcopies and colposcopy-guided biopsies were blind to biomarkers' results. Women with CIN2+ were treated almost exclusively with loop electrosurgical excision procedure.

Randomization Procedures

Women were randomly assigned using locally implemented systems nested in the screening management software. Random assignment was automatically activated when the result of cytology was uploaded to or registered in the screening database. Arm allocation automatically determined the next steps. Unfortunately, the randomization procedure failed to work for a few short periods in 2 centers because of routine software updates, and in these periods, all women were allocated to 1-year retesting (Supplementary Table 1, available online).

Biomarkers

Biomarkers were assessed only in HPV DNA-positive samples.

The Aptima HPV Assay test (Hologic) detects E6/E7 viral mRNA from 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, detected as a pool). The assay was performed by the Panther system, according to manufacturer's instructions, as previously described (24). Signal-to-cutoff ratios 0.5 or higher were considered as positive.

Slides for P16/Ki67 immunostaining were prepared from the same liquid-based cytological sample, after having obtained a slide for cytology, using a ThinPrep 2000 or 5000 processor (Hologic) and were immunostained using the CINtec PLUS kit (Roche), according to manufacturer's instructions, in 4 laboratories (Florence; Umbria; Turin, which also stained the slides from Trento; and Regina Elena Institute, Rome, for samples collected in Veneto).

A plan for harmonizing interpretation criteria was implemented before the start of the trial (24). All slides were read by 3 centers, blind to cytology and histology results, and classified as positive, negative, or inadequate. Samples were scored as positive when double immunoreaction was revealed within at least 1 cell. Slides with less than 5000 squamous epithelial cells were considered as inadequate but recorded as positive if showing p16/ki67 immunopositive cells. Here, we used the majority diagnosis. If only 2 readings were informative, we considered the interpretation made in the first reading.

Statistical Analyses

For stand-alone cytology, E6/E7 mRNA, and p16/ki67, we estimated, among HPV DNA-positive women, the proportion of test positives, sensitivity for histology-proven CIN2+ and CIN3+, specificity for less than CIN2, immediate and 1-year referral to colposcopy when referring women positive for each putative triage test immediately, and the remaining after 1-year if still HPV DNA positive (Supplementary Figure 1, available online) and its positive predictive value. Both raw values and values adjusted for unequal arm size are reported (Supplementary Methods, available online).

We estimated the same parameters also for the combined use (colposcopy referral of women positive to either test; see Supplementary Methods, available online) of HPV16/18 partial genotyping (obtained from the Cobas test, thus available only for about half of study participants) and cytology and of p16/ki67. Other combinations with weaker rationale are reported in Supplementary Table 2 (available online). Missing or invalid samples were excluded from analyses. In the case of test combinations, women with at least 1 valid positive test were always kept because they would in any case be referred to colposcopy (Supplementary Methods, available online). We computed sensitivity and specificity of E6/E7 mRNA, p16/ki67, and biomarker combinations including only women randomly assigned to immediate colposcopy (Supplementary Methods, available online). All 95% confidence intervals of proportions were obtained from the exact binomial distribution.

To study the regression of CIN2+, we compared CIN2+ detection in the 1-year repeat arm (p1) to that in the immediate colposcopy arm (p0) and used (p1-p0)/p0 as an estimate (see Supplementary Methods, available online). Thus, the estimate is based only on cytology-negative women. For each of the 2 studied biomarkers, we report estimates for biomarker-positive and -negative women at baseline. In addition, we computed the 2-sided *P* value for the arm-biomarker interaction in a logistic model as an estimate of the probability that a similar or larger difference in the relative risk would occur under the null hypothesis of equal regression in biomarker positives and negatives. Given the low power of the study for this comparison, we did not set a statistical significance threshold, and no formal statistical test was performed. Noncompliers to colposcopy in both arms and noncompliers to 1-year test repeat in the retesting arm were excluded. To evaluate whether biomarker positivity was a predictor of HPV DNA persistence, we computed the relative probability of clearance with 95% confidence intervals.

Ethics

The study protocol was approved by the S. Giovanni Battista University Hospital, Turin, Italy, on June 20, 2012 (N. CEI513) and by the local committees of all recruiting centers. The study has been registered (Clinicaltrials.gov registration number: NCT01837693, NTCC2 study) (25).

Results

Accuracy of Triage Strategies

The 4 centers included in this analysis recruited 40 509 women (Figure 1), including 600 aged 60 years and older. Of the 40 487 women with a valid HPV DNA test, 3147 (7.8%) were positive (Table 1). Cytology was available for 3137, positive in 794 (25.3%) and unsatisfactory in 37 (1.2%); positivity for type 16/18 was 27.0% (391 of 1446 tested with Cobas); E6/E7 mRNA was available for 3131 and positive in 2092 (66.8%); p16/ki67 was available for 3069 of whom 822 (26.8%) were positive and 164 (5.3%) unsatisfactory.

Among the 2306 HPV DNA-positive/cytology-negative women, 1077 (46.7%) were randomly assigned to immediate colposcopy and 1229 (53.3%) to 12-month HPV DNA retesting (Figures 1 and 2); their characteristics are reported in Supplementary Table 1 (available online). Delay in colposcopy was longer in the cytology-negative women randomly assigned to immediate colposcopy than in those cytology-positive (median time from HPV DNA testing to colposcopy 93 vs 70 days, respectively). Overall, 174 CIN2+ were diagnosed (including 95 CIN3 and 1 AIS).

The sensitivity of atypical squamous cells of undetermined significance or more severe (ASC-US+) cytology was just 61.0% (95% CI = 53.6% to 68.0%) for CIN2+ and 68.2% (95% CI = 60.6% to 75.2%) for CIN3+, with 76.6% (95% CI = 74.5% to 78.5%) specificity for less than CIN2. E6/E7 mRNA had very high sensitivity (94.4%, 95% CI = 89.1% to 97.3%, and 96.9%, 95% CI = 90.0% to 99.3%, for CIN 2+ and CIN3+, respectively) but just 34.4% (95% CI = 31.9% to 37.0%) specificity for less than CIN2. With p16/ki67, dual-staining sensitivity (75.2%, 95% CI = 68.1% to 81.6%, and 80.6%, 95% CI = 70.9% to 88.3%, for CIN2+ and CIN3+, respectively) was lower than with E6/E7 mRNA but higher than with cytology, whereas specificity (74.8%, 95% CI = 72.4% to 77.1%) was much higher than with E6/E7 mRNA and similar to that of cytology (Table 2). Immediate referral to colposcopy was 25.6% for cytology, 66.8% for E6/E7 mRNA, and 28.3% for p16/ki67, whereas colposcopy referral after 1-year HPV-DNA retesting was 39.7%, 11.4%, and 35.0%, respectively (Table 3).

When referring to immediate colposcopy, for all women who were either cytology- or HPV16/18-positive, sensitivity reached 93.8% (95% CI = 82.8% to 98.7%) for CIN2+ and 91.9% (95% CI = 74.0% to 99.0%) for CIN3+, but specificity was only 57.4% (95% CI = 53.2% to 61.4%). Results were similar for p16/ki67 combined with HPV16/18 genotyping (Table 2; Supplementary Table 2, available online).

The estimated overall proportion of HPV DNA-positive women who would have been referred to colposcopy either immediately or after 1 year with the assumed algorithm was slightly lower with p16/ki67 (63.3%) than with cytology at ASC-US threshold (65.3%). Overall, referral was highest for E6/E7 mRNA assay (78.3%) despite very limited (11.4%) referral after 1-year retesting. The overall positive predictive value was similar for all biomarkers: 9.5% for cytology, 8.3% for E6/E7 mRNA, and 10.1% for p16/ki67 (Table 3; Supplementary Table 3, available online).

Regression of CIN2+ and Clearance of HPV DNA

Among all HPV DNA-positive/cytology women, the CIN2+ detection was 2.6% (25 of 971) and 3.6% (34 of 951) in the 1-year retesting and immediate-colposcopy arm, respectively (Figure 1). Thus, the estimated overall 1-year CIN2+ variation was -28% (ie, that 28% of them regressed) with 95% confidence interval from a -57% regression to a +20% increase. Point estimates of regression were smaller in women who were biomarker positive at baseline (-20%, 95% CI = -53% to +35%) and -22% (95% CI = -61% to +56%) for E6/E7 mRNA and p16/ki67, respectively, than in biomarker negatives (-76%, 95% CI = -97% to +110%) and -39% (95% CI = -72% to +33%) for E6/E7 mRNA and p16/ki67, respectively, but confidence intervals were very wide. The *P* value for interaction was .29 and .67 for E6/E7 mRNA and p16/ki67, respectively) (Figure 2).

Moreover, among HPV DNA-positive/cytology-negative women, the clearance of HPV DNA after 12 months in mRNA-negative women (268 of 409 = 65.5%) was 1.9 times (95% CI = 1.7 to 2.2) that in mRNA-positive women (211 of 617 = 34.2%; $P_{interaction} < .001$), and clearance in p16/ki67-negative women (393 of 768 = 51.2%; $P_{interaction} < .001$) was 1.9 times (95% CI = 1.5 to 2.5) that in p16/ki67-positive women (48 of 182 = 26.4%) (Figure 2).

Discussion

Consistent with available data, we observed that the sensitivity of p16/ki67 was slightly higher than that of cytology (10-12) and that E6/E7 mRNA sensitivity was close to that of HPV DNA testing (13,15,17), but that more than 60% of HPV-DNA positive women were also E6/E7 mRNA positive. Combined use of cytology or p16/ki67 and HPV 16-18 typing increases sensitivity with a modest loss of specificity (11,26).

Based on the relative detection of CIN2+ in the 2 study arms (Figure 2), our data suggest that, overall, less than 1 of 4 cytology-negative CIN2+ regress in 1 year. Similar estimates were obtained from the ASCUS-LSIL Triage Study (ALTS) (27) and in a systematic review (28). In principle, some of the lesions detected in women randomized to 12-month retesting might not yet have been detectable at baseline, leading to a possible underestimate of regression. We estimated almost no regression in women positive for p16/ki67 or E6/E7 mRNA, whereas regression was estimated to be 70% and 40% in women E6/E7 mRNA and p16/ki67 negative, respectively. These data suggest that the 2 biomarkers under study may predict CIN2+ clearance, which is also plausible given what we know about the molecular pathogenesis of the disease. The association was, however, far from being statistically significant, likely because of the small number of CIN2+ found.

Conversely, our data clearly show that HPV infections in women negative to the p16/ki67 test or the E6/E7 mRNA assay have strongly increased probability of clearing within 12 months. Low persistence of HPV infection in tissues not overexpressing these 2 viral oncogenes is consistent with their functional suppression activity on p53 and Retinoblasoma protein and suggests that such tissues have little or no malignant potential, thus logically supporting high regression of only apparently abnormal histological findings.

HPV DNA persistence after a negative p16/ki67 or E6/E7 mRNA test was also lower than after a normal cytology. This reduces colposcopy referral if screening algorithms based on HPV DNA retesting of women negative to the triage test are applied. With retesting after 12 months, as currently done in Italy,

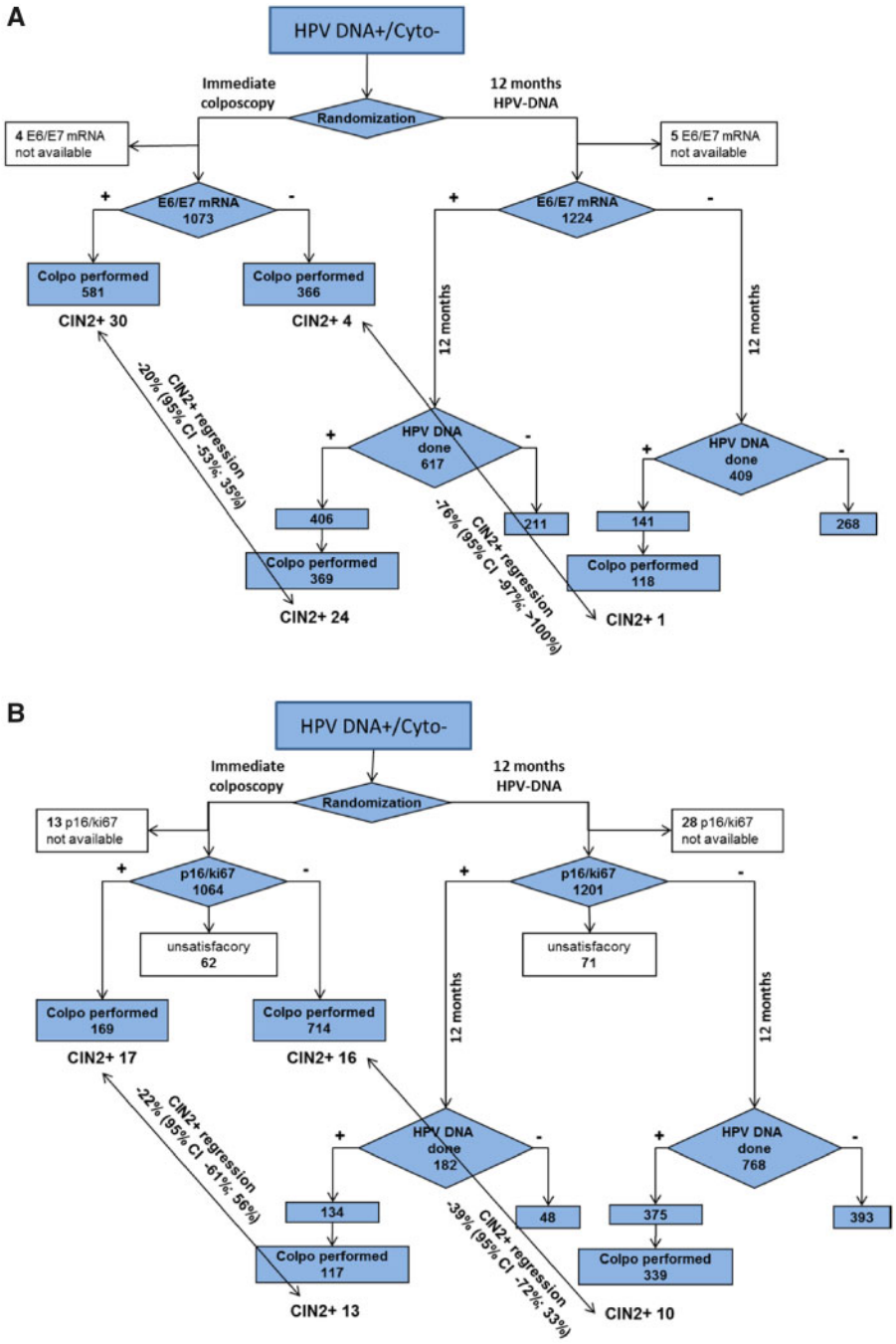

Figure 2. Results by randomization arm and biomarker results. Flowchart of human papillomavirus (HPV) DNA-positive/cytology-negative women randomly assigned to immediate colposcopy or 1-year HPV retesting, distinguishing between E6/E7 mRNA-positive and E6/E7 mRNA-negative women (**A**) and distinguishing between p16/ki67-positive and p16/ki67-negative women (**B**). Baseline and up to 24 months of results are reported. Among the 2306 HPV-positive/cytology-negative women randomly assigned, 2297 had a valid E6/E7 mRNA test, and 2132 had a valid p16/ki67 test. **Dotted arrows** show the comparison between cervical intraepithelial neoplasia grade 2 or more severe (CIN2+) detection in the 2 arms; for the 2 biomarkers, the ratio between detection in the 1-year referral arm vs that in the immediate colposcopy arm represents an unbiased estimate of lesion regression in 1 year. Here, it is reported as reduction (negative percentage) of the CIN2+ proportion found in the immediate colposcopy arm. In panel **(A)**, overall CIN2+ detection was 3.6% in the immediate colposcopy referral arm (34 of 951) and 2.6% in the 1-year referral arm (25 of 971), resulting in a 28% reduction (95% CI from a 57% reduction to a 20% increase). CI = confidence interval; colpo = colposcopy; cyto = cytology.

the total referral (immediate and at 12 months) would be similar with p16/ki67 or cytology as a triage test, despite higher immediate referral with the former. Conversely, total referral with E6/E7 mRNA would be more than 10% higher because of very high initial referral.

We acknowledge that, given the study design, we estimate HPV DNA persistence after negative p16/ki67 or E6/E7 mRNA just from cytology-negative women. Actually, cytology positivity occurred together with p16/ki67 negativity in only 11.6 % of tested women and only in 3.7% with E6/E7 mRNA negativity.

Table 2. Accuracy of different triage tests. Sensitivity for CIN2+ and CIN3 and specificity for CIN2+, with relative 95% confidence interval (CI) of cytology, E6/E7 mRNA assay, and p16/ki67 dual staining

Triagestrategy	Tested[a]	Test positive	TP	FP	TN	FN	Sensitivityfor CIN2+ (95% CI), %		Specificityfor <CIN2+ (95% CI), %		Sensitivity forCIN3+ (95% CI), %	
							Raw	Adj[b]	Raw	Adj[b]	Raw	Adj[b]
CytologyASC-US+[c]	2636	715	114	601	1862	59	65.9 (58.3to 72.9)	61.0 (53.6to 68.0)	75.6 (73.9to 77.3)	76.6 (74.5to 78.5)	71.9 (61.8to 80.6)	68.2 (60.6to 75.2)
E6/E7mRNA	2650	1784	167	1617	859	7	96.0 (91.9to 98.4)	94.4 (89.1to 97.3)	34.7 (32.8to 36.6)	34.4 (31.9to 37.0)	96.9 (91.1to 99.4)	96.9 (90.0to 99.3)
p16/ki67[c]	2471	714	131	583	1720	37	78.0 (70.9to 84.0)	75.2 (68.1to 81.6)	74.7 (72.9to 76.5)	74.8 (72.4to 77.1)	85.1 (76.3to 91.6)	80.6 (70.9to 88.3)
Cytology ASC-US+or HPV16/18 typing	1203	548	47	501	650	5	90.4 (79.0to 96.8)	93.8 (82.8to 98.7)	56.5 (53.6to 59.4)	57.4 (53.2to 61.4)	96.2 (80.4to 99.9)	91.9 (74.0to 99.0)
p16/ki67or HPV16/18 typing	1121	521	44	477	595	5	88.8 (77.8to 96.6)	90.1 (76.9to 96.5)	55.5 (52.5to 58.5)	53.7 (49.9to 57.5)	100.0 (86.8to 100)	100.0 (85.8to 100)

[a]All women with valid test and complete assessment. ASC-US = atypical squamour celss of undeterminde significance; Adj = adjusted; CIN2+ as endpoint. CIN = cervical intraepithelial neoplasia; FN = false negative; FP = false positive; HPV = human papillomavirus; TN = true negative; TP = true positive.
[c]Only valid test included.
[b]Adjusted for completeness of follow-up. Formulas used for raw and adjusted estimates are in Supplementary Methods (available online).

Thus, even if clearance were lower in these women, the underestimation of overall referral would be minimal.

In 2 centers, more women were assigned to the conventional arm because of software malfunctioning. Because the staff could not manipulate randomization, the resulting bias could at most be unequal center and season distributions in the study arms. Excluding all the women recruited during periods of possible malfunctioning had virtually no effect on results.

Some women who were randomly assigned to immediate colposcopy were assessed more than 6 months after HPV DNA testing. This led to underestimating the overall regression of triage- cytology-negative CIN2+ and to a dilution of its association with p16/ki67 and E6/E7 mRNA negativity.

In this study, 2 liquid-based slides, 1 for cytology and 1 for p16/ki67, were prepared for each HPV DNA-positive woman. The slide for cytology was prepared first, before the slide used for p16/ki67, because it was used for actual management of the woman. This may explain the relatively high proportion of unsatisfactory dual-stained slides.

NTCC2 was implemented in population-based organized screening programs. This, along with the high proportion of eligible women who accepted being enrolled, guarantees high applicability of results to routine activity. Computerized management, centralization of procedures in dedicated facilities, availability of fail-safe systems, and systematic computerized registration of results enabled high adherence to protocols and completeness of follow-up (including very high participation to retesting and colposcopy), thus assuring high-quality data.

Finally, the main limit was the much lower prevalence of CIN2+ than that assumed in power calculations. Indeed, although 30% fewer women than planned were recruited, the low CIN2+ prevalence was the main reason for the low power, which was mainly due to an unexpectedly high proportion of women previously screened by HPV testing. Detection was particularly low at 1-year follow-up because of the higher sensitivity of cytology than that observed in previous trials (29).

Considering practical conclusions, p16/ki67 is more sensitive than cytology and entails similar colposcopy referral even when keeping the same interval to retesting, thus leading to increased efficiency. High sensitivity would plausibly guarantee the safety of prolonged intervals to retesting, which would further increase efficiency.

The E6/E7 mRNA assay has very high sensitivity, but its immediate colposcopy referral was higher than the total (immediate plus delayed) referral with cytology. Its sensitivity was so high that only 7 lesions were missed, and we estimate that most of these may regress in 1 year. This makes E6/E7 mRNA a good candidate as a primary screening test. Thus, if overdiagnosis is an issue, the relevance of the biomarker really depends on what being positive implies. Results from the long-term follow-up of this study and of other ongoing cohorts (16, 30-34) are necessary to determine the longest safe interval after E6/E7 mRNA-negative primary testing.

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Table 3. Immediate, at 1-year retesting, and overall estimated referral rate and positive predictive value (PPV) for cytology, E6/E7 mRNA assay, p16/ki67 dual staining, and combinations of biomarkers

Triage strategy	No. tested	No. test positive	Immediate		1-year retest		Overall	
			Colposcopy referral, %	PPV, %	Colposcopy referral, %	PPV, %	Colposcopy referral, %	PPV, %
Cytology ASC-US+[a]	3100	794	25.6	16.2	39.7	5.1	65.3	9.5
E6/E7 mRNA[b]	3131	2092	66.8	9.5	11.4	0.8	78.3	8.3
p16/ki67[a,b]	2905	822	28.3	18.5	35.0	2.9	63.3	10.1
Cytology ASC-US+ or HPV 16/18 typing[b]	1446	638	44.1	8.3	31.4	1.8	75.5	5.6
p16/ki67 or HPV 16/18 typing[b]	1446	614	42.5	8.5	30.4	1.7	72.8	5.7

[a]Only valid test included. ASC-US = atypical squamour celss of undeterminde significance; HPV = human papillomavirus.
[b]Values are estimated assuming that HPV DNA-positive women are referred to immediate colposcopy according to test or combination of tests specified in table lines and the remaining to colposcopy after 12 months if still HPV DNA positive.

Notes

Role of the funder: The providers did not have any role in study design and conduction, data analysis, or the decision to submit data for publication.

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Data Availability

Individual participant data that underlie the results reported in this article, after deidentification, are available for investigators whose proposed use of the data have been approved by the S. Giovanni Battista University Hospital Ethic committee, Turin, Italy, to achieve aims in the approved proposal. Proposals should be directed to paolo.giorgirossi@ausl.re.it and to comitatoetico@cittadellasalute.to.it. To gain access, data

requestors will need to sign a data access agreement. The study protocol is freely available online.

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