Comparison of HPV-positive triage strategies combining extended genotyping with cytology or p16/ki67 dual staining in the Italian NTCC2 study

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Summary

Background Each high-risk HPV genotype has different oncogenic potential, and the risk of CIN3+ varies according to genotype. We evaluated the performance of different strategies of HPV-positivity triage combining cytology, p16/ki67 dual staining (DS), and extended genotyping.

Methods Samples from 3180 consecutive women from the NTCC2 study (NCT01837693) positive for HPV DNA at primary screening, were retrospectively analyzed by the BD Onclarity HPV Assay, which allows extended genotyping. Genotypes were divided into three groups based on the risk of CIN3+. HPV DNA-positive women were followed up for 24 months or to clearance.

Findings Combining the three groups of genotypes with cytology or DS results we identify a group of women who need immediate colposcopy (PPV for CIN3+ from 7.8 to 20.1%), a group that can be referred to 1-year HPV retesting (PPV in those HPV-positive at retesting from 2.2 to 3.8), and a group with a very low 24-month CIN3+ risk, i.e. 0.4%, composed by women cytology or DS negative and positive for HPV 56/59/66 or 35/39/68 or negative with the Onclarity test, who can be referred to 3-year retesting.

Interpretation Among the baseline HPV DNA positive/cytology or DS negative women, the extended genotyping allows to stratify for risk of CIN3+, and to identify a group of women with a risk of CIN3+ so low in the next 24 months that they could be referred to a new screening round after 3 years.

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Keywords: Human papillomavirus; HPV genotyping; Cervical cancer screening; Triage; HPV DNA testing; Accuracy; Cervical intraepithelial neoplasia; p16/ki67 dual staining

Introduction

Most international guidelines recommend HPV DNA as the primary test for cervical cancer screening[1–6] and molecular testing is replacing cytology-based screening. However, the HPV DNA test is less specific than the pap test and thus it requires the use of a triage test to reduce

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Research in context

Evidence before this study

We last searched PubMed and MedLine databases on July 20, 2023. Moreover, we consulted the official websites of Cervical Cancer Screening guidelines in many European countries as well in the USA. We used search terms including "Human Papillomavirus", "HPV genotyping", "cervical cancer screening", "triage", "HPV-DNA testing", "accuracy", "Cervical Intraepithelial Neoplasia".

All the results from cohort studies are consistent in identifying different oncogenic risk according to different HPV genotypes. The oncogenic risk is quantified as the risk of having or developing a CIN3 in the next 3 or 5 years. How to use this risk stratification in screening algorithms, in combination with other biomarkers such as cytology or p16/ki67 dual staining (DS), is still unclear. In particular, previous studies did not quantify how the use of genotyping may affect overall colposcopy referral, detection of CIN2+, and risk of overtreatment.

Added value of this study

Data from the NTCC2 study with 24-month follow up showed that extended genotyping combined with cytology or with DS, may identify a group of women with very low risk of CIN3+, i.e. about 0.4%. Moreover, our study, thanks to its randomized design, allows the evaluation of CIN2+ regression in one year. Our data suggest a relevant regression of the high-grade CIN occurring in the women who were infected by HPV genotypes with low oncogenic potential and who were cytology- or DS-negative. Therefore, given their low risk of CIN3+ and the high regression of CIN2+, these women could be invited for a new screening round after 3 years without further assessments, substantially reducing the colposcopy workload.

Implications of all the available evidence

The findings of the present study, together with the results of other large screening cohorts, may contribute to establishing new protocols for cervical cancer screening, to reduce the colposcopy burden, and to avoid unnecessary treatments. Moreover, all the available evidence may help update the international cervical cancer screening recommendations. In particular, the combined use of triage biomarkers may help implement the risk-based management of HPV-positive women.

the unnecessary referral to colposcopy and the risk of overtreatment. So far, cytology, with or without partial genotyping, is the only triage assay recommended by European[4] and US guidelines.[5,6]

Other biomarkers, alone or in combination with each other, are being evaluated as possible triage tests, such as the E6/E7 mRNA overexpression, the dual staining for p16/ki67 (DS),[7–9] partial or extended genotyping,[10] and the methylation status of cellular/viral genes.[11] Nevertheless, there is no consensus yet on which is the best strategy to triage HPV positivity in order to safely lower the colposcopy referral and unnecessary treatments. It has been shown that cervical precancerous lesions, to a higher extent the CIN2 but also the CIN3,[12,13] are highly regressive, so it is essential to evaluate the accuracy and clinical utility of a biomarker for identifying persistent lesions. A combination of biomarkers, instead of a stand-alone test, might increase the accuracy as well as the clinical utility of triage.

It has been shown that the oncogenic potential of the 12 HPV genotypes considered to be High Risk (HR) by the IARC[14] is different for each genotype, likely depending on different interactions with cellular proteins and on their ability to downregulate or evade the host immune system.[15] Consequently, different genotypes have different longitudinal risks for the development of CIN2+ lesions and cancer.[16–18] HPV16 has been recognized as the most oncogenic genotype both in cross-sectional and longitudinal studies. Thus, numerous trials have evaluated the performance of partial genotyping for HPV16/18 in cervical screening[10,19–22] and, as a result, partial genotyping for HPV16/18 has been introduced to triage HPV-positive women in several countries (USA,[5] Canada,[23] Australia[24]). Less has been published about the risk for precancerous lesions of other HR-types using extended genotyping assays.[16,25,26] In addition, these studies have been conducted using different genotyping methods with different performances.[27] The Onclarity HPV assay has shown a good performance and has been clinically validated for screening.[28–32]

Studies comparing different triage tests[7,33] or comparing cytology with different thresholds of positivity,[1] showed that the accuracy of the triage test scarcely influences the efficiency of a given screening algorithm. Only a major change in the screening algorithm can reduce the overall burden of colposcopies, which includes immediate and delayed referrals.[34–36] In order to change the screening algorithms maintaining the high safety of those currently recommended by the guidelines adopted in the USA and other countries,[4,5] it is necessary to identify, among HPV-positive women, a group of women with low enough risk of CIN3+ that they can be referred to screening after 2 or 3 years. This could be more easily obtained by classifying triage results into three groups, based on their risk level: the highest risk level requiring immediate colposcopy, the intermediate risk level requiring short-term HPV retesting, and the lowest risk level with sufficiently low risk to be safely referred to a new HPV testing at longer interval (3-level strategies). This 3-level strategy has the potential to be more efficient than the 2-level strategies distinguishing only those requiring immediate colposcopy and those requiring 1-year HPV retesting. The US

guidelines also suggest a fourth group with a very high risk of prevalent lesions, for which expedited treatment is an option.[5]

The New Technologies for Cervical Cancer Screening 2 (NTCC2) randomized clinical trial reported the accuracy of HPV E6/E7 mRNA testing, DS and cytology to triage HPV DNA-positive women.[7] We now retrospectively genotyped specimens from the NTCC2 biobank to evaluate 2- and 3-level triage strategies based on combining genotyping with cytology or DS, particularly as for their colposcopy referral rate and delay in detecting high-grade intraepithelial lesions.

Methods

NTCC2 study design and population

The NTCC2 study design and main results have been previously published.[7] Briefly, 41,127 women aged 25–59 years were prospectively and consecutively recruited from five Italian organized HPV DNA-based cervical cancer screening centres. Cervical samples were collected in PreservCyt solution (Thin Prep, Hologic, Bedford, Massachusetts, USA) and HPV DNA results were obtained by the HC2 (Qiagen, Hilden, Germany) or the Cobas 4800 test (Roche Diagnostics, Basel, Switzerland). All baseline HPV DNA-positive women were triaged by liquid-based cervico-vaginal cytology. They were also tested with E6/E7 mRNA assay (APTIMA; Hologic) and CINtec PLUS assay (Roche Diagnostics), even though they were managed only based on cytology results as for the Italian screening protocol. Cytology and DS methods and interpretation were described elsewhere.[7,37] Cytology-positive women, at the ASC-US threshold, were referred to immediate colposcopy, whereas cytology-negative women were randomly assigned with a 1:1 ratio to immediate colposcopy or to repeat HPV DNA test after 1 year. Women were randomized using locally implemented systems nested in the screening management software. Randomization was automatically activated when the result of cytology was recorded in the screening database. In the present analyses, we report results for HPV DNA-positive women followed up for 24 months or to clearance. For each enrolled woman, a 2 mL aliquot of the cervico-vaginal sample was stored at –80 °C in a dedicated biobank.

Extended genotyping

Extended genotyping was retrospectively obtained by means of the Onclarity HPV Assay (Becton & Dickinson, Sparks, MD, USA), which is a real-time multiplex PCR-based assay targeting the E6/E7 region of the HPV genome. The ISPRO (Florence, Italy), and the Center for Cervical Cancer Screening of Turin (Turin, Italy) laboratories performed the analyses according to the manufacturer's protocol. Before beginning the analyses, a training course was organized for the dedicated staff. We used 0.5 mL of the 2 mL of Thin Prep cervico-vaginal sample stored in the biobank. Nucleic acid extraction was carried out by BD Fox extraction and BD Viper Extraction Reagent, followed by a multiplex RT PCR assay for the qualitative detection of 14 HR-HPV types: individual results for genotypes 16, 18, 45, 31, 51 and 52, and pooled results for genotypes 33/58, 35/39/68, and 56/59/66. The human β-globin gene served as the internal control for sample adequacy and assay performance. BD Viper LT software automatically performed the interpretation of results. The presence or absence of clinically relevant HPV DNA was determined by the number of PCR cycles (CT), which was then compared with a pre-established threshold. The positivity threshold was defined as 38.4 CT for HPV16 and 34.2 CT for other HPV types and the internal control.

Statistical analyses

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 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.

We reported: the colposcopy referral rate, overall and divided into immediate and after 1-year HPV-retesting, as a percentage of HPV-positive women; the positive predictive value (PPV), overall and separately for immediate and after 1-year retesting; the sensitivity for CIN2+ and CIN3+ of the strategies in which we regarded as positive all those cases referred to immediate colposcopy and to 1-year retesting. Accordingly, we assumed 100% sensitivity for the 2-level strategies. Moreover, we reported the proportion of CIN2+ and CIN3+ detected at immediate colposcopy and at 1-year referral, and the observed 24-month risk of CIN3+ in women referred to a 3-year screening round.

In the analyses, we took into consideration raw data for single infection (i.e. single-channel positivity) and multiple infections (i.e. positivity for two or more

channels). Genotyping has been classified in two different ways: partial genotyping, divided into two groups, i.e. HPV16/18 vs. all other HR types, and extended genotyping, divided into three groups, i.e. HPV16/18, high oncogenic types (31, 33, 45, 52, and 58), and low oncogenic types (35, 39, 51, 56, 59, 66, 68).[31] The cases with negative BD Onclarity result were grouped together with the low oncogenic types. A sensitivity analysis has been conducted to compute the 24-month risk also excluding the BD Onclarity negative women. Cytology reports were also divided into three groups: the high-grade (HG) cytology, including carcinoma, AIS, H-SIL and ASC-H; the low-grade (LG) cytology, including ASC-US, L-SIL, and AGC; and the NILM cytology.

Using the combination of genotyping and cytology or DS, we defined different 2-level and 3-level triage strategies. In the 2-level strategies, women in the highest risk group were referred to immediate colposcopy, while intermediate and low risk groups were combined and referred to 1-year HPV retesting. In the 3-level strategies, women in the highest risk group were referred to immediate colposcopy, women in the intermediate risk group were referred to 1-year HPV retesting, and women in the lowest risk group were referred to a new screening round after three years. In all the strategies, women referred at 1-year HPV retesting were managed only according to Cobas/HC2 result: if positive, they were referred to colposcopy; if negative, to a new screening round after 5 years. Details for the algorithms used to compute the data, taking into account the study design, are reported in the Supplementary materials.

Table 1a summarizes the combinations of genotyping and cytology, listed from *a* to *i*, reporting the 24-month CIN3+ risk. The colours in Table 1 show the linked management options for each combination: red: immediate colposcopy; orange: immediate colposcopy or 1-year HPV retesting; yellow: 1-year or 3-year HPV retesting in partial genotyping strategies; green: 1-year HPV retesting or 3-year HPV retesting in both partial and extended genotyping. Similarly, Table 1b summarizes the combinations of genotyping and DS, listed from *a* to *f*. Based on these combinations we evaluated different triage strategies that are described in Table 2.

To compare the most representative strategies, we applied the screening parameters to a cohort of 100 HPV-positive women and described the tests and colposcopies they would receive, and the lesions detected at each step. We then described one 2-level protocol with partial genotyping, one 3-level protocol with partial genotyping, and one 3-level protocol with extended genotyping, for each morphological test, i.e. cytology or DS (Figs. 2 and 3). CIN3+ reported in these figures are those estimated to be prevalent at baseline, so the total number has been estimated from the observed ones adjusting for non-compliance to colposcopy, non-compliance to 1-year follow up, and regression for those referred at 1-year. To estimate the regression in a given group, i.e. CIN2+ that after 12 months regress to CIN1, normal tissue, or clear HPV, we compared the detection observed in women referred to immediate colposcopy to that observed in those referred to 1-year retesting, as previously described.[7] For details on the adjustments, see the Supplementary materials.

We report exact-binomial 95% confidence interval (95% CI) when both numerator and denominator are observed numbers. For modelled parameters, 95% CIs have been estimated running 50,000 Monte Carlo simulations, combining the binomial distributions of all the observed parameters used in the modelling as described in the formulas reported in the Supplementary materials.

Ethics

New Technologies for Cervical Cancer 2 (NTCC2) study is registered in Clinicaltrials.gov with number: NCT01837693. The NTCC2 study protocol was approved by the S. Giovanni Battista University Hospital, Turin, Italy, on 20 June 2012 (N. CEI513) and by the local committees of all recruiting centers. The present extension of the study protocol was approved by the Comitato Etico Centrale IRCCS Lazio, Fondazione G.B. Bietti, N 1153/18, on 20 November 2018. All recruited women provided a written informed consent to participate in the trial.

Role of funders

The funding sources did not have any role in the study design and conduct, data analysis, or the decision to submit data for publication.

Results

Of the 41,127 women participating in the NTCC2 study, 3180 were baseline HPV DNA-positive (7.7%). Among them, 3129 women, positive with Cobas 4800 (n = 1436) or with HC2 (n = 1693), were analysed for extended genotyping by Onclarity assay using biobanked material, and all of them gave a valid result (Fig. 1; Supplementary Table S1). Overall, we found 174 CIN2+, of which 95 CIN3 and one adenocarcinoma *in situ* (AIS). No cancers were reported in the whole cohort during the first 24 months of follow up. At cytology triage, 44 cases were inadequate or missing and only one CIN2, and none CIN3, was found among them. Of the 3085 women with a valid cytology, 790 had an ASC-US+ report and were referred to immediate colposcopy where 114 CIN2+ lesions, of which 69 CIN3, were found, mainly among the 184 women with a HG cytology (82 CIN2+ of which 52 CIN3, Supplementary Table S1). The majority of the women (n = 2295) had a NILM report and were randomized in two arms as per the NTCC2 protocol: 1072 women were submitted to immediate colposcopy where we found 34 CIN2+ (15 CIN3), while 1223 women were

a

Cytology	16/18	High oncogenic types*	Low oncogenic types#
High grade§	*a* 35.8% (N of women=95)	*b* 18.9% (N of women=53)	*c* 22.2% (N of women=36)
Low grade~	*d* 5.5% (N of women=145)	*e* 3.1% (N of women=193)	*f* 1.1% (N of women=268)
negative	*g* 3.7% (N of women=416)	*h* 2.6% (N of women=644)	*i* 0.4% (N of women=1235)

b

DS	16/18	High oncogenic type	Low oncogenic type
Positive	*a* 19% (N of women=289)	*b* 8.1% (N of women=297)	*c* 4.5% (N of women=235)
Negative	*d* 1.5% (N of women=331)	*e* 2.0% (N of women=537)	*f* 0.5% (N of women=1210)

* High oncogenic types include 31, 33, 45, 52, and 58 HPV types
# Low oncogenic types include 35, 39, 51, 56, 59, 66, 68 HPV types and the cases negative for BD Onclarity
§ High grade cytology includes carcinoma, AIS, H-SIL and ASC-H
~ Low grade cytology includes ASC-US, L-SIL, and AGC
Red cells: immediate colposcopy; orange cells: immediate colposcopy or 1-year HPV retesting; yellow cells: 1-year or 3-year HPV retesting in partial genotyping strategies; green cells: 1-year HPV retesting or 3-year HPV retesting in both partial and extended genotyping

Table 1: **Combinations of genotyping and cytology (a) and DS results (b).**

referred to 1-year HPV retesting; 1024 returned at 1 year and 547 (53.4%) were still positive. Among these 1024 women we found 25 CIN2+ of which 12 CIN3.

Based on the Onclarity genotyping results, the 96 CIN3+ were distributed in the three HPV groups as follows: 56 (58.3%) among the women positive for HPV16/18 (n = 667); 26 (27.1%) among the women positive for high oncogenic types (n = 901); 14 (14.6%) among the women positive for low oncogenic types (n = 797) or negative for Onclarity assay (n = 764).

Baseline HPV-positive women were also tested for DS. A total of 2899 cases had an evaluable result whereas 230 cases were inadequate or missing and, among them, we found 2 CIN3 both in the HPV 16/18-positive subgroup (Supplementary Table S1). In total 821 women resulted DS positive (Supplementary Table S1; Supplementary Figure S1a); among them 131 CIN2+, of which 80 CIN3, were detected, the majority of which in the HPV16/18-positive group (50/80, 62.5%), while 20 CIN3 were found in the high oncogenic (25.0%) and only 10 in the low oncogenic group (12.5%). In the 2087 DS-negative women, we detected 37 CIN2+, of which 14 CIN3 (Supplementary Table S1; Supplementary Figure S1b): 4 in the HPV16/18, 6 in the high oncogenic and 4 among the low oncogenic group.

Estimated performance of different triage strategies for HPV DNA-positive women

Table 3 reports the performance of each triage strategy described in Table 2. Referring to immediate colposcopy all the women with HPV16/18 positivity or ASC-US+ cytology (strategy Cyto-1, Fig. 2), the overall colposcopy referral would be 68.7%, with a 1-year HPV clearance of 51.4%, and an average PPV for CIN3+ of 5.2%. Conversely, if we refer to immediate colposcopy only women with HG cytology or with HPV16/18 positivity and simultaneous ASC-US+ cytology (strategy Cyto-2), we would have a slightly lower overall referral (58.9%) than in strategy Cyto-1, due to a reduced HPV clearance (45.9%); moreover, the average PPV for CIN3+ was slightly higher (6.1%). The combination of cytology and partial genotyping can also define a 3-level risk stratification, as in strategy Cyto-3 (Fig. 2). Cytology-negative women infected with non-16/18 HPV genotypes have the lowest 24-month CIN3+ risk, i.e. 1.1%. Referring to 3-year rescreening these women, and to immediate colposcopy only women HPV16/18 and cytology-positive, would require by far the lowest overall colposcopy referral (31.8%) with the highest average PPV (9.8), at the cost of low sensitivity, with 19.2% of the CIN3 missed in women referred at 3-year rescreening. Strategies Cyto-4, Cyto-5 and Cyto-6 illustrate three possible solutions to exploit extended genotyping with a 3-level risk stratification. In all these three strategies, women with Onclarity negative result or low oncogenic genotypes, and negative cytology, would be referred at 3-year rescreening. The 24-month CIN3+ risk of low oncogenic HPV-positive/cytology-negative women referred to a screening round after 3 years was 0.4%; if we exclude the BD-negative women, the

Short name	Morphological test	Genotyping	Risk-levels	Referral	Description of biomarker combination linked to the specific management
Cyto-1	Cytology	Partial	2-level	Colposcopy	Positive for HPV16/18 OR cytology positive (*a* + *b* + *c* + *d* + *e* + *f* + *g*)
				1-year HPV retesting	Positive for any other HR-types AND cytology negative (*h* + *i*)
Cyto-2	Cytology	Partial	2-level	Colposcopy	HG cytology independently of HPV status (*a*+*b* + *c*) Positive for HPV16/18 AND cytology positive (*d*)
				1-year HPV retesting	Positive for HPV16/18 AND cytology negative (*g*) Positive for any other HR types AND negative OR LG cytology (*e* + *f* + *h* + *i*)
Cyto-3	Cytology	Partial	3- level	Colposcopy	HG cytology independently of HPV status (*a*+*b* + *c*) Positive for HPV16/18 AND cytology positive (*d*)
				1-year HPV retesting	Positive for HPV16/18 AND cytology negative (*g*) Positive for any other HR types AND LG cytology (*e* + *f*)
				3-year HPV retesting	Positive for any other HR types AND cytology negative (*h* + *i*)
Cyto-4	Cytology	Extended	3- level	Colposcopy	HG cytology independently of HPV status (*a*+*b* + *c*) Positive for HPV16/18 AND cytology positive (*d*)
				1-year HPV retesting	Positive for any other HR types AND LG cytology (*e* + *f*) Positive for HPV16/18 OR high oncogenic HPV types AND cytology negative (*g* + *h*)
				3-year HPV retesting	Positive for low oncogenic HPV types AND cytology negative (*i*)
Cyto-5	Cytology	Extended	3- level	Colposcopy	Positive for HPV16/18 OR HG cytology (*a* + *b* + *c* + *d* + *g*) Positive for high oncogenic HPV types AND LG cytology (*e*)
				1-year HPV retesting	Positive for low oncogenic HPV types AND LG cytology (*f*) Positive for high oncogenic HPV types AND cytology negative (*h*)
				3-year HPV retesting	Positive for low oncogenic HPV types AND cytology negative (*i*)
Cyto-6	Cytology	Extended	3- level	Colposcopy	Positive for HPV16/18 OR cytology positive (*a* + *b* + *c* + *d* + *e* + *f* + *g*)
				1-year HPV retesting	Positive for high oncogenic HPV types AND cytology negative (*h*)
				3-year HPV retesting	Positive for low oncogenic HPV types AND cytology negative (*i*)
DS-1	p16/ki67 dual staining	Partial	2- level	Colposcopy	Positive for HPV16/18 OR DS positive (*a*+*b* + *c* + *d*)
				1-year HPV retesting	Positive for any other HR-types AND DS negative (*e* + *f*)
DS-2	p16/ki67 dual staining	Partial	2- level	Colposcopy	Positive for HPV16/18 AND DS positive (*a*)
				1-year HPV retesting	Positive for HPV16/18 AND DS negative (*d*) Positive for any other HR types independently from DS (*b* + *c* + *e* + *f*)
DS-3	p16/ki67 dual staining	Partial	3- level	Colposcopy	Positive for HPV16/18 AND DS positive (*a*)
				1-year HPV retesting	Positive for HPV16/18 AND DS negative (*d*) Positive for any other HR types AND DS positive (*b* + *c*)
				3-year HPV retesting	Positive for any other HR types AND DS negative (*e* + *f*)
DS-4	p16/ki67 dual staining	Extended	3- level	Colposcopy	Positive for HPV16/18 OR DS positive (*a* + *b* + *c* + *d*)
				1-year HPV retesting	Positive for high oncogenic HPV types AND DS negative (*e*)
				3-year HPV retesting	Positive for low oncogenic HPV types AND DS negative (*f*)
DS-5	p16/ki67 dual staining	Extended	3- level	Colposcopy	DS positive independently of HPV status (*a*+*b* + *c*)
				1-year HPV retesting	Positive for HPV16/18 or high oncogenic HPV types AND DS negative (*d* + *e*)
				3-year HPV retesting	Positive for low oncogenic HPV types AND DS negative (*f*)
DS-6	p16/ki67 dual staining	Extended	3- level	Colposcopy	Positive for HPV16/18 OR high oncogenic HPV types AND DS positive (*a*+*b*)
				1-year HPV retesting	Positive for HPV16/18 or high oncogenic HPV types AND DS negative (*d* + *e*) Positive for low oncogenic HPV types AND DS positive (*c*)
				3-year HPV retesting	Positive for low oncogenic HPV types AND DS negative (*f*)

In gray the strategies reported in Figs. 2 and 3.

Table 2: **Description of the triage strategies included in the evaluation.**

24-month CIN3+ risk increased to 0.5%. Referring to immediate colposcopy all the women with ASC-US+ cytology or with HPV16/18 positivity would have the highest proportion of CIN3+ identified at the immediate colposcopy (strategy Cyto-1, 72.4%). Referring to immediate colposcopy women with ASC-US+ cytology only when also positive for HPV16/18 or high oncogenic genotypes would have a slightly lower overall referral (strategy Cyto-5, 51.4%, Fig. 2).

In the strategies adopting DS instead of cytology (strategies from DS-1 to DS-6), the results are very similar to those described above for Cyto strategies (Table 3 and Fig. 3). In particular, applying a 3-level strategy (DS-3, DS-4, DS-5, and DS-6), we would obtain similar results to those shown with the corresponding strategies with cytology (Cyto-3, Cyto-4, Cyto-5, and Cyto-6) in terms of sensitivity and 24-month CIN3+ risk in women referred to a 3-year screening round. Comparing similar

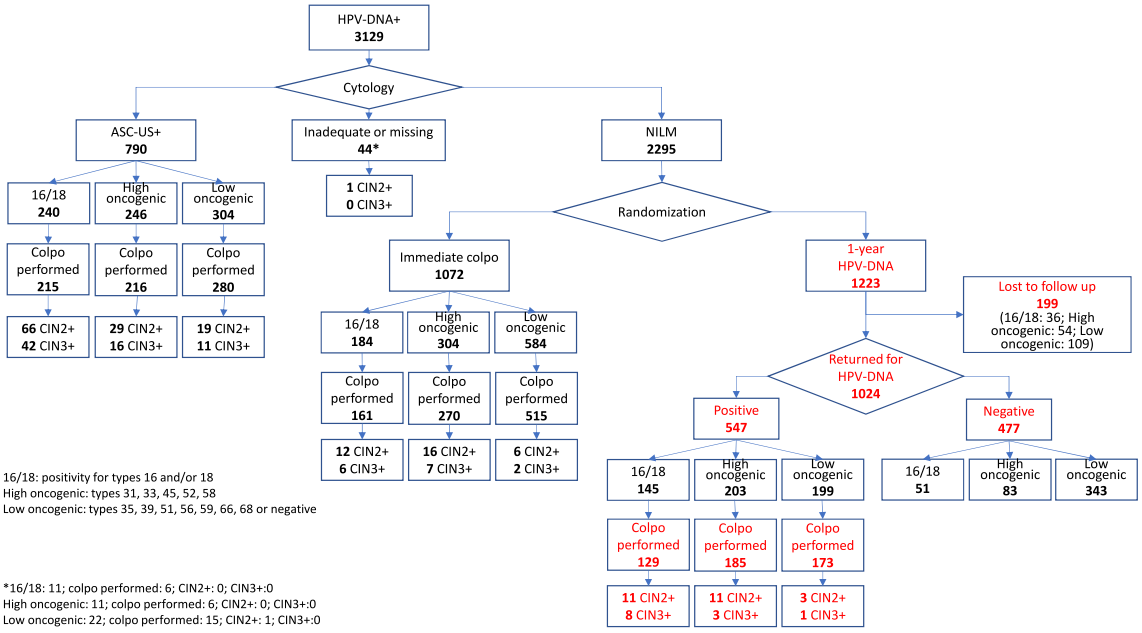


Fig. 1: Flowchart of the 3129 HPV DNA positive women triaged with cytology. All the baseline analyses are marked in black. All the analyses performed at 1 year are marked in red. As for the NTCC2 study design, cytology-positive women were referred to immediate colposcopy, while cytology-negative women were randomized to immediate colposcopy or to 1-year HPV DNA retesting. Those still HPV DNA positive were referred to colposcopy. The endpoints, CIN2+ and CIN3+ lesions, are reported according to the baseline genotyping results stratified based on the oncogenic potential of the genotype/s revealed.

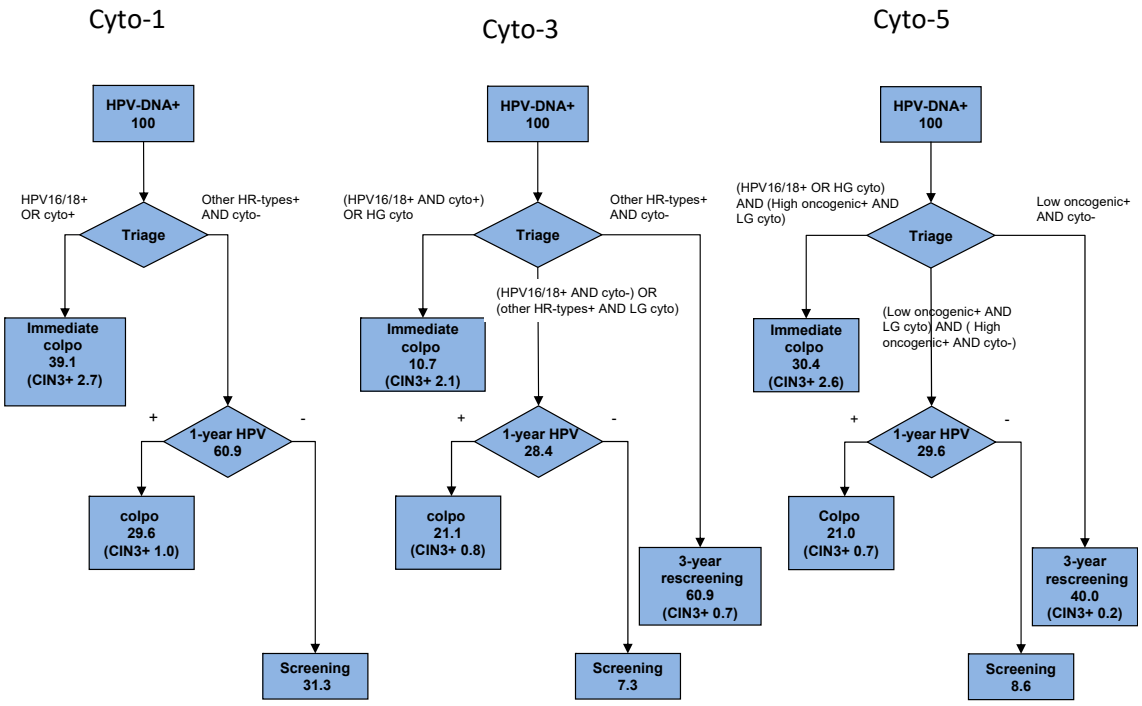


Fig. 2: Proportion of women referred to colposcopy, to 1-year retesting, or to 3-year retesting, and proportion of CIN3+ lesions, detected at each step, in a cohort of 100 HPV DNA positive women applying, as triage strategy, three most representative combinations of genotyping and cytology among those described in Table 2. Cyto-1: 2-level protocol with partial genotyping; Cyto-3: 3-level protocol with partial genotyping; Cyto-5: 3-level protocol with extended genotyping.

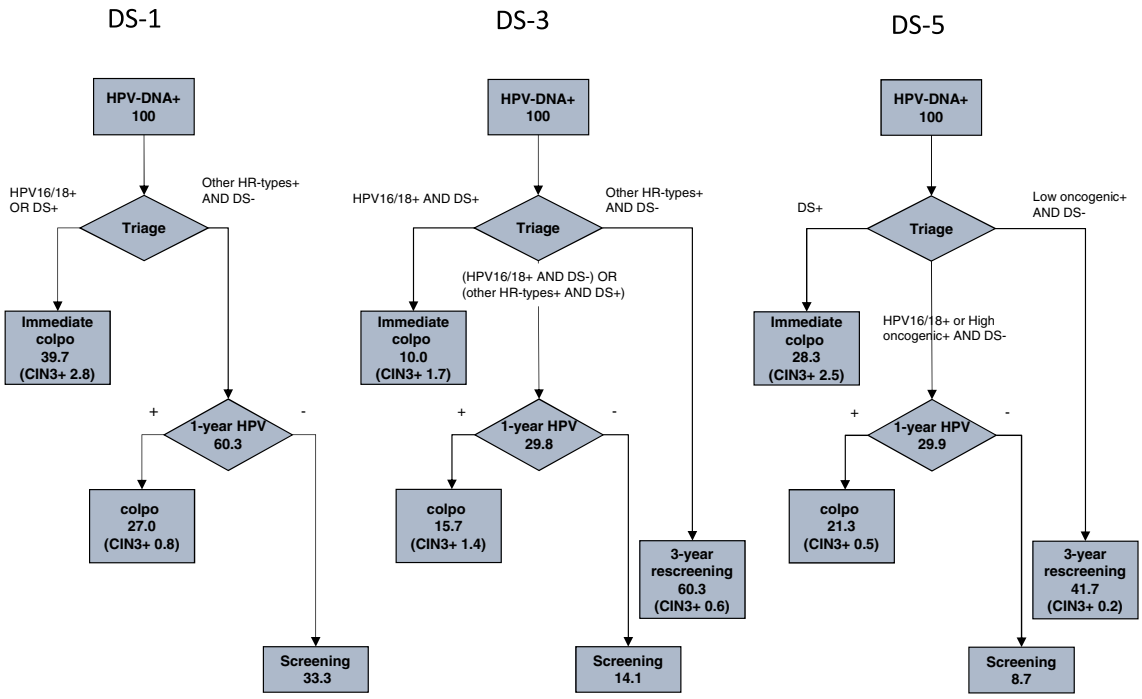


Fig. 3: Predicted outcomes, the proportion of women referred to colposcopy, to 1-year retesting, or to 3-year retesting, and proportion of CIN3+ lesions, detected at each step, in a cohort of 100 HPV DNA positive women applying, as triage strategy, three most representative combinations of genotyping and dual staining described in Table 2. DS-1: 2-level protocol with partial genotyping; DS-3: 3-level protocol with partial genotyping; DS-5: 3-level protocol with extended genotyping.

scenarios, the overall colposcopy referral was slightly lower with DS than with cytology, mostly due to the higher HPV clearance in DS-negative women when referred to 1-year HPV retesting.

Fig. 4 represents a summary graph of the overall colposcopy referral rate and 24-month CIN3+ risk for triage-negative women, comparing the different 3-level strategies that apply partial or extended genotyping. All the scenarios with extended genotyping showed, in women referred at 3-year retesting, a lower risk of CIN3+ and a higher colposcopy referral rate than those observed in strategies adopting partial genotyping.

Estimated regression of precancerous lesions

Comparing the CIN2+ detection in the two arms of randomization, we could estimate the regression of CIN2+ in HPV-positive/cytology-negative women. Overall, the detection in the immediate colposcopy arm was 3.6% (34 CIN2+ out of 946 women) and in the 1-year retesting arm was 2.6% (25 CIN2+ out of 964 women) with an estimated regression in one year of 28% (95% CI –20% to 57%) (Fig. 1, Table 4). Adopting partial genotyping, in women who are cytology-negative and positive for non-16/18 HPV types, the regression would be 29% (95% CI –38% to 63%). Adopting extended genotyping, in women cytology-negative and infected with the low oncogenic types or Onclarity negative, the regression would be 41% (95% CI –135% to 85%) (Fig. 1, Table 4). In women who are also DS-negative, the results are similar: adopting partial genotyping, in women positive for non-16/18 HPV, the estimated regression would be 23% (95% CI –74% to 66%) while adopting the extended genotyping, in the group with the low oncogenic types or Onclarity negative, the estimated regression would be 51% (95% CI –93% to 88%) (Supplementary Figure S1, Table 4). However, all the estimates are very imprecise, and differences could be due to chance. It is worth noting that regression in DS-negative women can be estimated only for those who are also cytology-negative, since the study design did not foresee randomization for cytology-positive women. Even with very sparse numbers, for all these low risk groups, also CIN3 detection was higher in the immediate colposcopy arm than in the 1-year retesting arm. For example, in women with negative cytology and non-16/18 HPV types, CIN3 detection was 1.1% (9/785) and 0.6% (4/703) in the two arms respectively (Fig. 1 and Supplementary Figure S1).

Discussion

Main findings

Three-level triage strategies combining extended genotyping and cytology or DS may define a group of HPV-

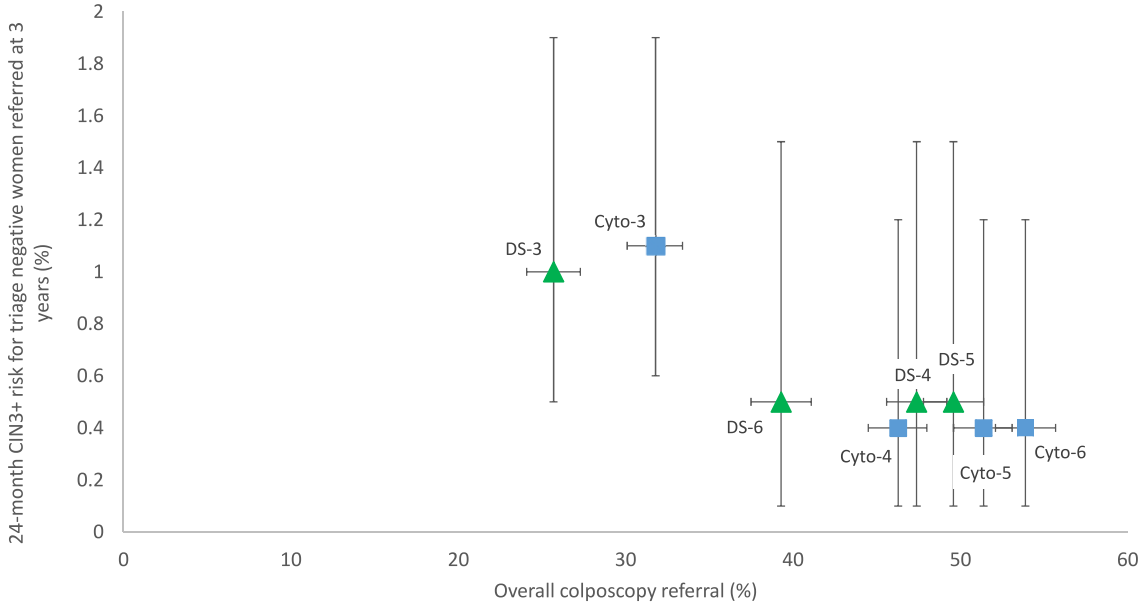
Triage strategies[a]	Overall colposcopy referral	PPV immediate	PPV 1 year retesting	HPV clearance after 1 year	Average PPV	CIN3 immediate	CIN3 at 3 years	24 month CIN3+ risk for triage negative women referred at 3 years
	% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)	% (95% CI)[b]	% (95% CI)[b]	% (95% CI)[b]	% (95% CI)
Cyto-1	68.7 (67.0–70.3)	7.8 (6.2–9.7)	1.8 (0.9–3.1)	51.4 (48.0–54.9)	5.2 (5.0–5.3)	72.4 (71.0–73.2)	–	–
Cyto-2	58.9 (56.5–60.0)	20.1 (15.7–25.1)	3.0 (2.1–4.1)	45.9 (43.5–49.7)	6.1 (5.9–6.4)	58.4 (56.9–61.1)	–	–
Cyto-3	31.8 (30.1–33.4)	20.1 (15.7–25.1)	4.5 (2.9–6.5)	25.7 (20.0–32.8)	9.8 (9.3–10.2)	58.4 (56.9–61.1)	19.2 (18.2–20.5)	1.1 (0.6–1.9)
Cyto-4	46.3 (44.5–48.0)	20.1 (15.7–25.1)	3.8 (2.6–5.3)	27.8 (23.8–32.0)	7.6 (7.2–7.9)	58.4 (56.9–61.1)	4.6 (4.2–4.9)	0.4 (0.1–1.2)
Cyto-5	51.4 (49.6–53.2)	9.7 (7.8–12.0)	2.5 (1.3–4.3)	29.0 (23.8–34.7)	6.8 (6.5–7.0)	76.2 (75.0–77.8)	4.6 (4.2–4.9)	0.4 (0.1–1.2)
Cyto-6	53.9 (52.1–55.7)	7.8 (6.2–9.7)	2.8 (1.4–5.1)	29.0 (23.8–34.7)	6.4 (6.2–6.5)	72.4 (71.0–73.2)	4.6 (4.2–4.9)	0.4 (0.1–1.2)
DS-1	66.7 (65.0–68.4)	8.2 (6.6–10.0)	1.6 (0.8–2.9)	55.2 (51.3–59.1)	5.5 (5.2–5.6)	82.3 (80.4–82.7)	–	–
DS-2	57.9 (54.8–58.4)	18.9 (14.4–24.4)	3.9 (2.8–5.2)	48.2 (44.9–51.5)	6.6 (6.2–6.9)	45.7 (41.0–47.8)	–	–
DS-3	25.7 (24.1–27.3)	18.9 (14.4–24.4)	7.0 (4.9–9.6)	30.3 (24.7–36.4)	10.9 (10.3–11.5)	45.7 (41.0–47.8)	15.9 (14.6–17.0)	1.0 (0.5–1.9)
DS-4	47.4 (45.6–49.2)	8.2 (6.6–10.0)	2.2 (0.7–5.2)	28.0 (21.6–35.0)	6.8 (6.4–7.0)	82.3 (80.4–82.7)	6.0 (5.5–6.6)	0.5 (0.1–1.5)
DS-5	49.6 (47.8–51.4)	10.7 (8.5–13.1)	2.0 (1.0–3.7)	28.9 (24.0–34.3)	7.0 (6.6–7.3)	78.0 (75.8–78.8)	6.0 (5.5–6.6)	0.5 (0.1–1.5)
DS-6	39.3 (37.5–41.1)	13.3 (10.5–16.6)	3.2 (1.8–5.2)	30.1 (25.4–35.1)	7.6 (7.3–8.0)	66.5 (62.8–67.5)	6.0 (5.5–6.6)	0.5 (0.1–1.5)

The same accuracy indicators for CIN2 are reported in Supplementary Table S2. [a]The description of the strategies is reported in Table 2. [b]95% CI estimated with Monte Carlo simulation.

Table 3: **Referral rate, positive predictive value (PPV), proportion of CIN3 detected, HPV clearance after one year and 24-month CIN3+ risk of different triage strategies.**

positive women who have a 24-month risk of CIN3+ low enough, i.e. 0.4%, to safely refer them at three years. Our data also suggest that a non-negligible part of CIN2, and possibly CIN3, found in women with low-risk patterns may regress in less than one year, further favouring non-intensive management. This approach could reduce the overall colposcopy workload to about 50% of HPV-positive women.

Strategies adopting cytology or DS in combination with extended genotyping gave similar performance.



The bars reports the 95% confidence intervals

Fig. 4: Summary graph of the overall colposcopy referral and 24-month CIN3+ risk, for triage negative women referred at 3-year retesting, considering all the 3-level triage strategies described in Table 2.

	Immediate colposcopy arm					1-year retesting arm					Estimated regression	
	N assessed	N of CIN2+	N of CIN3	% CIN2+ detected	% CIN3 detected	N assessed	N of CIN2+	N of CIN3	% CIN2+ detected	% CIN3 detected	%	95% CI
All cytology negatives	946	34	15	3.6	1.6	964	25	12	2.6	1.2	28	−20 to 57
HPV genotyping[a]												
16/18	161	12	6	7.5	3.7	180	11	8	6.1	4.4	18	−81 to 63
All non-16/18 types	785	22	9	2.8	1.1	703	14	4	2.0	0.6	29	−38 to 63
High oncogenic types	270	16	7	5.9	2.6	268	11	3	4.1	1.1	31	−46 to 67
Low oncogenic types	515	6	2	1.2	0.4	435	3	1	0.7	0.2	41	−135 to 85
DS												
Positive	169	17	7	10.1	4.1	165	13	9	7.9	5.5	22	−56 to 61
Negative	711	16	7	2.3	1.0	730	10	3	1.4	0.4	39	−33 to 72
HPV genotyping[a] **in DS-negative**												
16/18	101	3	1	3.0	1.0	119	1	1	0.8	0.8	72	−168 to 97
All non-16/18	610	13	6	2.1	1.0	611	10	2	1.6	0.3	23	−74 to 66
High oncogenic types	187	7	4	3.7	2.1	176	7	1	4.0	0.6	−6	−197 to 62
Low oncogenic types	423	6	2	1.4	0.5	435	3	1	0.7	0.2	51	−93 to 88

[a]Genotyping is reported both in two- and three-risk groups. In the two-risk group, i.e. partial genotyping, we distinguish 16/18 from all the other non-16/18 genotypes; in the three-risk group, i.e. extended genotyping, we further divided the non-16/18 genotypes into high oncogenic (31, 33, 45, 52, and 58) and low oncogenic (35, 39, 51, 56, 59, 66, 68, and the cases that resulted negative to BD Onclarity).

Table 4: **Estimated regression of CIN2+ and CIN3 in HPV-positive/cytology-negative women by baseline biomarker results.**

On the contrary, using partial genotyping only enabled the identification of a group with a 24-month CIN3+ risk higher than 1%, both with cytology and DS, which corresponded to about 20% of the CIN3 missed. Some of the current guidelines[5,6,38,39] would consider such a risk too high to refer to 3-year rescreening.

Among the 2-level strategies, we only considered those that referred to colposcopy both the HPV-positive women who are triage-positive and those who are triage-negative but still HPV-positive after one year, as currently recommended by several guidelines.[4,5] Our data confirm that, with such strategies, applying strict criteria for immediate colposcopy referral, i.e. HPV16/18 and cytology-positive, would lead to a small reduction of the overall colposcopy workload and would have a modest impact on overall PPV for CIN3+, compared to applying wider criteria, i.e. HPV16/18 or cytology-positive. Similar findings can be observed when cytology is substituted with DS.

Main limitations

The assays we used as primary HPV DNA screening tests, HC2 or Cobas, do not allow extended genotyping. Therefore, we retrospectively assessed extended genotyping on HC2 or Cobas HPV-positive samples stored in the study biobank. Because of this two-step testing, we found a high proportion of unconfirmed baseline HC2 positive tests (578/1693, 34.1%) and some unconfirmed Cobas positive tests (186/1436, 13%). Compared with using only the Onclarity assay, which simultaneously allows screening and typing, this two-step testing led to an increase in the specificity of the whole process due to the identification of the discordant cases, HC2 or Cobas-positive and Onclarity-negative. In fact, these discordant cases had a very low 24-month risk of CIN3+; on the contrary, in our study, we miss the Onclarity-positive and Cobas or HC2-negative women, which also probably had very low 24-month CIN3+ risk, but would be classified as HPV positive using Onclarity as the only test. In a random sample of women who tested HPV-negative with HC2 or Cobas, we found 5/333 (1.5%) Onclarity-positive samples. Thus, we can expect an absolute decrease in the specificity of this magnitude by using Onclarity as the first level test. These findings are consistent with those from previous studies showing that the concordance between HPV DNA tests is far from perfect, even if all of the assays reach extremely high sensitivity.[40] Moreover, our data suggest that there is room for optimization of the commercially available tests, focusing on increasing their specificity. However, the 2021 Global HPV LabNet HPV DNA proficiency showed an overall improvement in comparability and reliability of HR-HPV assays, and panels with screening-relevant concentrations of HPV genotypes have been developed.[41]

The new approach of "equal management for equal risk" proposed by the US guidelines, shifted from result-based to risk-based management recommendations. In the recent management guidelines,[5,42] the decision about the management of women who have such a low prevalence of CIN3+ that do not require an immediate colposcopy, is based on the prospective 5-year risk of detecting a CIN3+.[5,42] To the end of allowing the use of the risk-based approach for new technologies

that have less than 5 years of follow-up, 3-year correlates of the 5-year risk management thresholds were recently developed and they will be used in the US Guidelines.[43] In our study, we have a limited follow-up period, i.e. 24-months. Therefore, we cannot make recommendations based on the established 5-year and 3-year risk thresholds. Nevertheless, other studies confirmed a very low 5-year CIN3+ risk in cytology-negative women who were positive for those HR-HPV genotypes that had the lowest oncogenic potential.[25,44]

It is worth noting that our findings refer to an unvaccinated population. In the screening of vaccinated cohorts, the accuracy of triage biomarkers will change since both specificity and PPV depend on the probability of having a CIN2+ lesion when a woman is HPV infected, which in turn determines the prevalence of CIN2+.[45] This probability is type-specific, and a change in the circulating types will change the accuracy of triage tests. Nevertheless, the risk within the groups characterized by extended genotyping should not change in the vaccinated cohorts.

Implications for practice

Delaying to 3 years the HPV retesting of a subset of HPV-positive women requires a very low risk of CIN3+, as substantial delays in the detection of CIN3+ can occur, with a risk of progression to cancer. For rescreening after 3 years the threshold proposed by the USA guidelines is a 5-year cumulative CIN3+ risk of 0.55% (which is what was observed after a negative Pap test). In the 3-level strategies that combine cytology or DS with extended genotyping we estimated, although with a large confidence interval, a low 24-month CIN3+ risk and only a small proportion of CIN3 that would be detected with a delay ≥3 years. In addition, our data suggest important regression of the high-grade CIN occurring in the women who would be retested after 3 years, even if the study is underpowered to compare regression across groups.

In the 3-level strategies adopting extended genotyping, we observed colposcopy referral rates below 50% of the HPV-positive women, which represent a reduction of about 20% compared to current protocols. Such a reduction would be attractive even considering that with these strategies the interval between screening rounds would be reduced from 5 to 3 years for about 30% of the population, and that women who will be still HPV positive at the new round would likely need more aggressive management than those positive for the first time. Longer follow-up from this and other ongoing studies is needed to assess the 5-year CIN3+ risk of women in the low-risk group.[16,46–48] These studies will also help quantify the actual clearance after three years, the HPV infection prevalence, and the colposcopy referral rate, thus allowing a complete quantification of the desirable (reducing colposcopies and unneeded treatments) and undesirable (delaying treatment of persistent CIN3, thus increasing the risk of cancer) effects of these less intensive triage strategies.

Cytology-based triage strategies provide small benefit in terms of reduction of colposcopies by delaying colposcopies for one year because the HPV clearance after one year in cytology-negative women is only 45%. Thus, in strategies that foresee the rescreening for part of cytology-negative women, the reduction in colposcopies due to 1-year referral of the intermediate risk group ranges from 0 to 9% of the overall colposcopy burden. With DS, the proportion of avoided colposcopies with 1-year referral of the intermediate risk group, ranges from 5 to 11%, the difference due to the DS's ability to predict HPV persistence.[7] Nevertheless, in this study an unbiased comparison between the HPV clearance in cytology-negative and DS-negative women is not possible, since all cytology-positive women have been referred to immediate colposcopy even if DS-negative. Therefore, we had to assume the HPV clearance in the cytology-positive/DS-negative women. This assumption may overestimate the advantage of DS in predicting HPV clearance compared to cytology.

Conclusions

The effects on both efficiency and protection, of screening strategies including short-term retesting are not always intuitive. The present work provides some hints. We showed that by classifying genotypes into three groups and combining them with cytology or DS results, we could stratify women according to their 24-month risk of CIN3+. Women in the highest risk group, with a PPV for CIN3+ ranging from 7.8 to 20.1%, should be referred to immediate colposcopy. An intermediate risk group should be referred to 1-year retesting, and the PPV in those HPV-positive at retesting ranges from 2.2 to 3.8%. Finally, we can identify a relatively large group of women, about 40%, that have such a low 24-month CIN3+ risk that could be referred to a new screening round after three years, without further assessment.

Contributors

MB: Conceptualization; Funding acquisition; Project administration; Supervision; Writing—original draft, review, editing. **GR:** Conceptualization; Methodology; Supervision; Validation; Writing—original draft, review, editing. **PM**: Data curation; Formal analysis; Software. **FC**: Conceptualization; Investigation; Methodology; Resources; Supervision; Validation; Writing—review, editing. **LDM**: Data curation; Investigation; Resources; Supervision; Validation; Writing—review, editing. **EA**: Data curation; Investigation; Validation. **SB**: Data curation; Investigation; Validation. **RR**: Data curation; Project administration; Validation. **DG**: Investigation; Supervision; Validation. **ADM**: Data curation; Investigation; Methodology; Supervision; Validation; Writing—review, editing. **HF**: Data curation; Investigation; Validation. **MC**: Investigation; Methodology; Validation. **JV**: Investigation; Methodology. **AI**: Data curation; Investigation; Supervision; Validation. **EC**: Data curation; Investigation. **SB**: Data curation; Investigation. **BP**: Funding acquisition; Project administration; Resources; Supervision. **SG**: Data curation; Investigation. **LT**: Data curation; Project administration; Supervision. **LB**: Data curation; Formal analysis. **FV**: Data curation; Formal analysis. **NW**: Supervision; Validation; Writing—review, editing. **PGR**:

Conceptualization; Methodology; Funding acquisition; Resources; Supervision; Validation; Writing—original draft, review, editing.

All authors read and approved the final version of the manuscript. Maria Benevolo, Paolo Giorgi Rossi and Pamela Mancuso have accessed and verified the underlying data.

The members of the New Technologies for Cervical Cancer 2 Working Group contributed to the project administration, resources and investigation.

Data sharing statement

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. 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.

Declaration of interests

Maria Benevolo and Paolo Giorgi Rossi as principal investigator and former PI of the NTCC2 study reports nonfinancial support from Roche Diagnostics and Hologic S. r.l., which provided part of the reagents for free or at reduced price. Moreover, Maria Benevolo, Paolo Giorgi Rossi, Simonetta Bisanzi, and Laura De Marco obtained financial and nonfinancial support from Becton & Dickinson. Maria Benevolo also reports financial and nonfinancial support from Arrow S. r.l. for works outside this project. All other authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105149.

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