RESEARCH ARTICLE

JOURNAL OF
MEDICAL VIROLOGY WILEY

Criteria for second generation comparator tests in validation of novel HPV DNA tests for use in cervical cancer screening

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Abstract

While HC2 and GP5+/6+ PCR-EIA were pivotal in test validation of new HPV assays, they represent the first generation of comparator tests based upon technologies that are not in widespread use anymore. In the current guideline, criteria for second-generation

comparator tests are presented that include more detailed resolution of HPV genotypes. Second-generation comparator tests should preferentially target only the 12 genotypes classified as carcinogenic (IARC-group I), and show consistent non-inferior sensitivity for CIN2+ and CIN3+ and specificity for ≤CIN1 compared to one of the first-generations comparators, in at least three validation studies using benchmarks of 0.95 for relative sensitivity and 0.98 for relative specificity. Validation should take into account used storage media and other sample handling procedures. Meta-analyses were conducted to identify the assays that fulfill these stringent criteria. Four tests fulfilled the new criteria: (1) RealTime High-Risk HPV Test (Abbott), (2) Cobas-4800 HPV test (Roche Molecular System), (3) Onclarity HPV Assay (BD Diagnostics), and (4) Anyplex II HPV HR Detection (Seegene), each evaluated in three to six studies. Whereas the four assays target 14 carcinogenic genotypes, the first two identify separately HPV16 and 18, the third assay identifies five types separately and the fourth identifies all the types separately.

KEYWORDS
cervical cancer screening, HPV, diagnostic test accuracy, HPV-based screening, test validation

1 | INTRODUCTION

Cervical cancer screening using human papillomavirus (HPV) testing is more effective in preventing cervical cancer than cytology-based screening or visual inspection after application of acetic acid (VIA).[1–4] Many countries and regions have already moved from cytology-based screening to HPV screening or are currently in a process of doing so. Numerous HPV tests are commercially available, but only few of these tests have been rigorously validated or have received regulatory approval for use in screening.[5] International consensus guidelines require that HPV screening is undertaken with clinically validated assays.[4] Clinical HPV test validation includes evaluation of the accuracy for detection of a histologically defined endpoint (cervical precancer or worse). Given that the prevalence of cervical precancer in a screening population is very low, primary evaluation of HPV tests for cervical screening has previously required conducting large prospective randomized trials or cohort studies screened with two or more tests.[1,2,6,7] A more efficient and affordable strategy to validate novel HPV screening assays is to compare performance against a standard HPV comparator test to demonstrate noninferior clinical accuracy in addition to sufficient intra- and interlaboratory reproducibility as originally proposed by Meijer et al.[8] Two assays, Hybrid Capture 2 HPV DNA Test (HC2) (Qiagen)[9,10] and GP5+/6+ PCR-EIA (Diassay),[11,12] have long served as standard comparator tests[8] since randomized controlled trials demonstrated that screening with these high-risk (hr)HPV DNA tests resulted in better detection of cervical intraepithelial neoplasia Grade 3 or above (CIN3+) and lower cumulative incidence rates of invasive cervical cancer compared to screening with conventional or liquid-based cytology among baseline screen-negative women.[1,13]

More precisely, an emerging HPV test can be considered validated for use in primary HPV screening if the new assay fulfills the following conditions[8]:

1) detects viral DNA of carcinogenic HPV genotypes as defined by the International Agency for Research on Cancer (IARC) under the World Health Organization (WHO);

2) had demonstrated accuracy in terms of relative clinical cross-sectional sensitivity and specificity to either of the above "first generation" standard comparator tests (accepting the benchmark of 0.90 and 0.98 for relative sensitivity and relative specificity, respectively) to detect CIN2+, applying the non-inferiority statistics proposed by Tang et al. for comparison of matched proportions.[14] By approximation, this practically means that a lower bound of the paired 90% confidence interval (CI) is ≥0.90 for relative sensitivity and ≥0.98 for relative specificity. In addition, the novel HPV test should demonstrate good intra- and inter-laboratory reproducibility as defined in the international HPV test validation criteria.[8] These criteria currently apply to HPV DNA assays. HPV tests targeting other molecules than DNA require additional longitudinal safety data, by demonstrating at least similar longitudinal sensitivity and/ or a nonsuperior cumulative CIN3+ risk after negative baseline testing for a new marker versus after a negative HPV DNA comparator as shown for HPV mRNA testing.[15]

Other methods to conduct regulatory validation of new HPV assays for use in cervical screening involve evaluation in large clinical trials with cervical precancer endpoints against cytology as comparator test, as was previously required by the Food and Drug Administration of the USA (FDA).[16–18]

While HC2 and GP5+/6+ PCR-EIA were pivotal in test validation of new HPV assays, they represent the first generation of comparator tests based upon technologies and platforms that are not in widespread use anymore as most laboratories have moved to newer generation HPV tests. HPV detection technology has advanced from aggregated detection toward HPV tests with more detailed resolution including

individual detection of HPV genotypes. Together with the rapid increase in the number of commercial molecular HPV assays over the last decade,[5] there is a pressing need to ensure robust validation of novel assays that have an application for screening, and for that, a contemporary international consensus on second-generation comparator tests is needed that reflects the developments described above.[19]

In this review, authors propose criteria for second-generation comparator tests, assess which currently available assays fulfill the criteria already and anticipate to more assays, yet to be developed or in the process of validation, that may reach these criteria in the future.

2 | METHODS

Criteria for second-generation comparator tests for use in the validation of novel HPV assays for cervical cancer screening expand on the original Meijer criteria and are based on published systematic reviews that are continuously updated.[19,20] The criteria that an HPV test must fulfill to be recognized as a second-generation standard comparator test are summarized in Table 1 and are further detailed below.

2.1 | HPV genotype coverage

a. By design, a comparator test must provide targeted detection of viral nucleic acids from all 12 IARC group I carcinogenic HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59).

b. A comparator test should preferentially not target HPV genotypes not classified as carcinogenic. Inclusion of genotypes classified as *probably* (IARC group IIA) or *possibly* carcinogenic (IARC group IIA) do not contribute meaningfully to detection of cervical precancerous lesions that have the potential to evolve into

cancer. Instead, their inclusion can result in a substantial loss in test specificity leading to unnecessary follow-up and over-treatment in screening.[13,21,22] Both first generation comparator assays (GP5+/6+ PCR-EIA) target HPV68, whereas GP5+/6+ also targets HPV66 and HC2 is known to cross-react with HPV66.[8] Therefore inclusion of these two genotypes does not disqualify use as a comparator test. An assay that targets more *possibly* carcinogenic HPV genotypes (IARC group IIB) or non-oncogenic genotypes can only be accepted as a comparator test when these other genotypes are identifiable separately.

2.2 | Clinical validation criteria

A comparator test must fully and consistently be validated according to the international validation criteria for non-inferior sensitivity and non-inferior specificity for detection of both CIN2+ and for CIN3+ compared to HC2 or GP5+/6+ PCR-EIA in at least three independent studies published in peer-reviewed journals to appreciate consistency. Of these, at least one study needs to address intra- and inter-laboratory reproducibility of the test[8] and demonstrate sufficient reproducibility where it is evaluated. The validation studies should be of good quality assessed according to established study design checklists such as QUADAS for diagnostic accuracy studies[23] and the Cochrane and ROBINS-I tools for assessing bias in randomized[24] or non-randomized trials,[25] respectively, as applied in previous published lists of validated HPV assays.[19,26] Collaboration between academia and industry is instrumental to advance HPV testing research, but contractual independency of researchers conducting validation studies and autonomy of publication of outcomes enhance scientific credibility. Thus, the assessment of conflict of interest is an important element when reviewing evidence.[27]

Whereas in the 2009 validation criteria,[5] CIN2+ was the only endpoint recommended to define a test's sensitivity for precancer,

TABLE 1 Criteria for second generation standard comparator tests that can used in the validation of novel HPV assays which can be used in cervical cancer screening.

	Criteria	Explanation
1.	HPV genotype coverage	Target the HPV genotypes with recognised carcinogenic potential (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol 100B, 2012).
2.	Clinical validation criteria	
	Consistent prior validation	Consistent and full validation according the international criteria published in *Int J Cancer* 2009[8] in at least 3 studies using a first generation standard comparator test (HC2 or GP5+/6+ pCR-EIA).
	Relative sensitivity	Benchmark for non-inferior sensitivity for CIN2+ and for CIN3+ compared to a first generation comparator test: $p \geq 0.95$.
	Relative specificity	Benchmark for non-inferior specificity for <CIN2 compared to a first generation comparator test: $p \geq 0.98$.
3.	Storage medium	The validation is restricted to the storage media applied in the validation studies (used to store cells scraped by a health care worker from the cervical surface; or to conserve molecules targeted by the test).
4.	Future second generation standard comparators	New second generation standard comparator tests can be recognised as such by fulfilling above criteria. The comparators can forthwith be first or second generation standard comparator tests.

Note: CIN2: cervical intraepithelial neoplasia of grade II; CIN3: cervical intraepithelial neoplasia of grade III; CIN2+: CIN2 or worse disease; CIN3+: CIN3 or worse disease; <CIN2: CIN1 or no abnormality of the cervical epithelium.

the CIN3+ endpoint is additionally needed since it is a more robust precursor of cervical cancer than CIN2. In contrast to CIN3, CIN2 is less reproducible, has a lower potential to progress to cancer, and is more likely to be associated with HPV types which are only possibly or are not carcinogenic.[28–31]

The 90% CI around the pooled relative clinical sensitivity and relative clinical specificity includes unity or is at the right side of unity. Using this criterion avoids accepting a second-generation comparator test with a lower left CI bound exceeding the benchmark but with an upper CI bound lower than unity. In addition, the benchmark for relative clinical sensitivity for CIN2+ and for CIN3+ is forthwith defined at 0.95 to establish second-generation comparator tests instead of the 0.90 benchmark for test validation.

2.3 | Storage medium and other sample handling procedures

Validation studies must diligently report the storage media and procedures to collect and handle collected cervical samples. Validation on one storage medium does not automatically confer validation on other storage media. A new HPV test applied on cervical samples stored in a given transport medium can be evaluated against a second-generation standard comparator test if the comparator was validated before (considering the accuracy criteria outlined in point 2) on specimens stored in that specific medium.

Additional second-generation standard comparator tests can be recognized using a previously recognized first or a second-generation comparator.

3 | RESULTS

An update of the meta-analyses,[19] conducted to establish previously published lists of HPV tests qualifying for cervical cancer screening, enabled to identify five hrHPV DNA assays that consistently fulfilled the clinical accuracy criteria stated above. Table 2 and Figure 1 summarize to what extent these five tests match the criteria outlined above. Non-inferior clinical sensitivity and clinical specificity to detect CIN2+ compared to the first-generation standard comparator

TABLE 2 Characteristics of candidates for second generation comparator HPV tests that could be used for validation of emerging HPV tests for use in cervical cancer screening. The last two columns contain the pooled relative sensitivity and specificity of second versus first generation comparator HPV tests to detect CIN2+.

Assay	Nb of validation studies:		Group I carcino-genic HPV types targeted[h]	Group II carcino-genic HPV types targeted[i]	Collection and storage media[j]	Pooled relative clinical sensitivity (90% CI)[k]	Pooled relative clinical specificity (90% CI)[k]
	Accuracy[f]	Reprodu-cibility[g]					
Anyplex[a]	3	2	all 12	66, 68	PC, HP	1.007 (0.979-1.037)	1.004 (0.991-1.018)
Cobas 4800[b]	5	2	all 12	66, 68	UCM, PC, SP	1.002 (0.979-1.025)	1.003 (0.992-1.013)
HPV-risk[c]	3	1	all 12	66, 67, 68	PC, SP	0.993 (0.962-1.024)	1.018 (1.002-1.035)
Onclarity[d]	4	2	all 12	66, 68	PC, SP	1.001 (0.974-1.029)	0.998 (0.981-1.014)
RealTime[e]	6	3	all 12	66, 68	STM, PC	0.992 (0.971-1.014)	1.015 (1.009-1.022)

[a]Anyplex: Anyplex II HPV HR Detection (Seegene, Seoul, South Korea); full genotyping capacity identifying separately 14 HPV types 16, 18, 31, 33, 35, 38, 45, 51, 52, 56, 58, 59, 66 and 68.

[b]Cobas 4800: Cobas 4800 HPV test (Roche Molecular System, Pleasanton, CA, USA); partial genotyping capacity; HPV16, HPV18 and the aggregate of 12 other types (HPV31/33/35/38/45/51/52/56/58/59/66/68).

[c]HPV-Risk: HPV-Risk Assay (Self-Screen BV, Amsterdam, The Netherlands): partial genotyping capacity: HPV16, HPV18 and the aggregate of 12 other types (HPV31/33/35/38/45/51/52/56/58/59/66/67/68).

[d]Onclarity: BD Onclarity HPV Assay (BD Diagnostics, Sparks, MD, USA); extended genotyping capacity: 5 types identified separately (HPV16, HPV18, HPV31, HPV45, HPV52) and 3 groups of types aggregated (HPV33/58, 35/39/68, and 56/59/66).

[e]Realtime: Abbott RealTime High Risk HPV Test (Abbott, Wiesbaden, Germany); partial genotyping capacity; HPV16, HPV18 and the aggregate of 12 other types (HPV31/33/35/38/45/51/52/56/58/59/66/68).

[f]Number of studies where non-inferior sensitivity and specificity to detect cervical intraepithelial neoplasia of grade 2 or worse compared to a standard comparator HPV DNA test is demonstrated as defined in international validation criteria[8] as documented in Arbyn et al.[19] and Dhillon et al.[32]

[g]Number of studies where good intra- and inter-reproducibility of hrHPV-positivity is demonstrated (left bound of the confidence interval around the reproducibility for presence of absence of high-risk HPV ≥87% & kappa ≥0.5) as defined in international validation criteria.[8]

[h]IARC Group I carcinogenic types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59.[33]

[i]Group IIA carcinogenic type (=probably carcinogenic): HPV68; Group IIB carcinogenic types (=possibly carcinogenic): HPV26, 53, 66, 67, 70, 73, 82.[33]

[j]PC: ThinPrep-PreservCyt medium (Hologic, Bedford, MA, USA), HP: Huro Path medium (CelltraZone Co., Seoul, Korea); UCM: Universal Collection Medium (Hardy Diagnostics, Santa Maria, CA, USA); SP: SurePath medium (BD Diagnostics, Sparks, MD, USA); STM: Specimen Transport Medium (Qiagen, Gaithersburg, MD, USA).

[k]In order to fulfil the current international validation criteria, the sensitivity and specificity to detect CIN2+ of the index HPV test should be non-inferior to the sensitivity and specificity of the standard comparator tests (HC2 or GP5+/6+ PCR-EIA], which means that left 90% confidence interval [CI] bound around the relative sensitivity and relative specificity should be ≥0.90 and ≥0.98, respectively. For the second generation comparator tests, the benchmarks are: ≥0.95 for sensitivity to detect CIN2+ or CIN3+ and ≥0.98 for specificity to detect <CIN2.
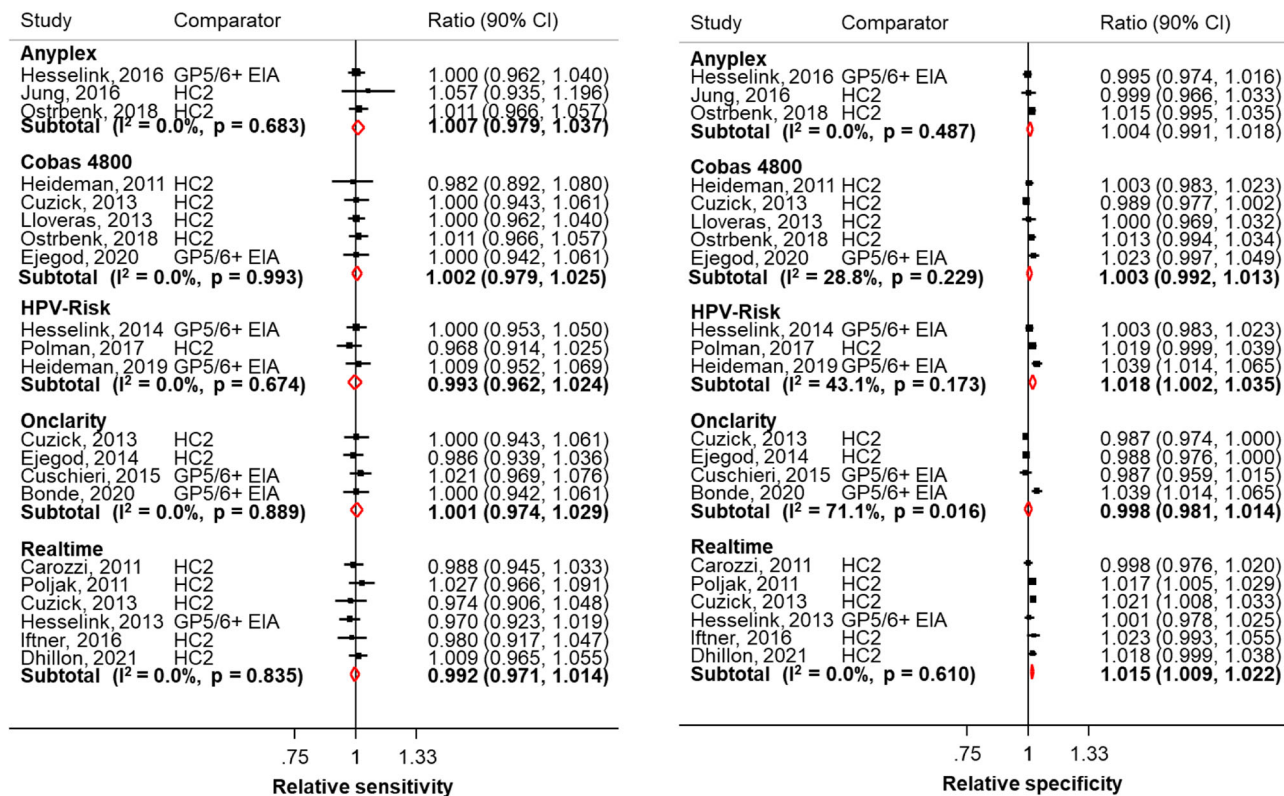
FIGURE 1 Meta-analysis of the relative sensitivity and specificity to detect CIN2+ of second-generation versus first-generation comparator tests. CI, confidence interval.
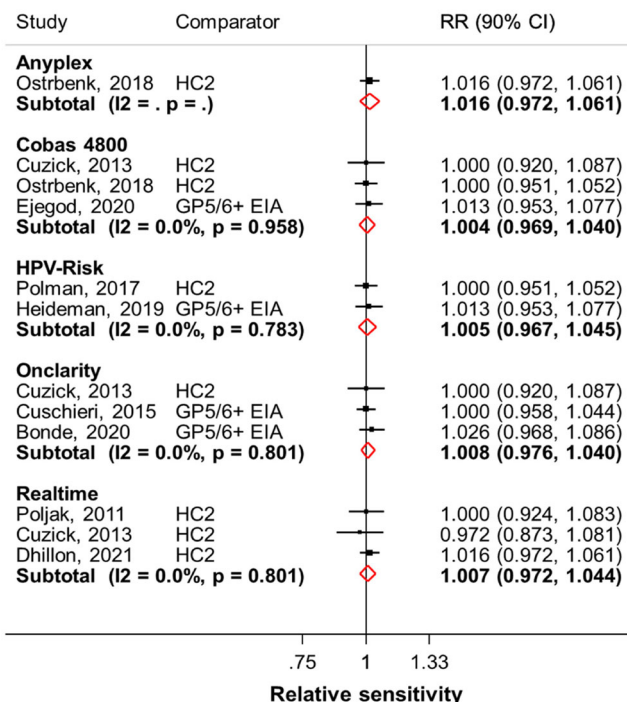
TABLE 3 Relative sensitivity for detection of CIN3+ of five hrHPV DNA tests compared to the standard comparator tests.

Assay	Number of studies	Relative sensitivity (90% CI)[1]
Anyplex[a]	1	1.016 (0.972–1.061)
Cobas 4800[b]	3	1.004 (0.969–1.040)
HPV-Risk[c]	2	1.005 (0.967–1.045)
Onclarity[d]	3	1.008 (0.976–1.040)
RealTime[e]	3	1.007 (0.972–1.061)

[1]For the second generation comparator tests, the benchmark for relative senitivity to detect CIN3+ compared to first generation comparator tests is ≥0.95.

tests (HC2 or GP5+/6+ PCR EIA) was demonstrated in the following studies that documented how candidate comparator tests fulfill the conditions as second-generation comparator assays:

- In six studies: RealTime High Risk HPV Test ([RealTime]; Abbott)[19,32];
- in five studies: Cobas 4800 HPV test ([Cobas 4800]; Roche Molecular System)[19];
- in four studies: BD Onclarity HPV Assay ([Onclarity]; BD Diagnostics)[19];

FIGURE 2 Meta-analysis of the relative sensitivity to detect CIN3+ of second-generation versus first-generation comparator tests. CI, confidence interval.

- in three studies: Anyplex II HPV HR Detection ([Anyplex] (Seegene) and HPV-Risk Assay (Self-Screen BV).[19]

All the five HPV assays showed also a clinical sensitivity for CIN3+ that was non-inferior to the sensitivity of a standard comparator test (Table 3 and Figure 2). Sufficient intra- and inter-laboratory reproducibility was shown for all the five assays (Table 2). All five assays detect the 12 carcinogenic HPV genotypes and two additional HPV genotypes (HPV68 and HPV66) belonging to group II carcinogenic genotypes. HPV-Risk Assay detects also HPV67 in a group of aggregated HPV genotypes.

The above five assays are formally validated on cervical samples stored in ThinPrep-PreservCyt medium (Hologic) and three assays (Cobas 4800, HPV-Risk, and Onclarity) are also formally validated for use on cervical samples stored in SurePath medium (BD Diagnostics).

Three other collection media: Huro Path LBC medium (CelltraZone Co.), Universal Collection Medium (UCM) (Hardy Diagnostics), and Specimen Transport Medium (STM) (Qiagen) were used in one study each, evaluating Anyplex, Cobas 4800 and RealTime, respectively. For all the tests, the left 90% CI bound around the pooled relative clinical sensitivity (index vs. standard comparator test) exceeded the proposed new benchmark of 0.95.

Four hrHPV DNA assays fulfill all the outlined criteria (Anyplex, Cobas 4800, Onclarity, and Realtime) and can be accepted as second-generation comparator tests in studies aiming to validate novel hrHPV DNA tests for use in cervical cancer screening on clinician-taken cervical specimens.

Although performing well in three validation studies, the HPV-Risk Assay is excluded since it also targets HPV67 (IARC group IIB, possibly carcinogenic genotype) in aggregate with other hrHPV genotypes which from a public health perspective is less relevant for cervical cancer screening.

4 | DISCUSSION

In this review, an international group of experts in cervical cancer screening epidemiology, molecular microbiology, clinical virology, and test validation methodology, formulated criteria that HPV tests must fulfill to be accepted as second-generation comparator tests. These comparator tests need to be widely evaluated and will serve as benchmarks to enable the validation of emerging HPV tests for use in cervical cancer screening on cervical clinician-taken specimens. The definition of the criteria for second-generation comparators is the first of 14 items to be addressed in the new comprehensive validation guidelines which are currently being developed.[19] Consensus about the new comparator tests will facilitate evaluation of new HPV assays in the pipeline. A major priority in public health is to increase the number of appropriately clinically validated assays to encourage competition and market-shaping, which will encourage the availability of test technologies that are robust, affordable, and easy to use in field conditions and are urgently needed to reach the desired 70% global cervical cancer screening coverage recommended by the WHO, besides the other goals for elimination of cervical cancer (to vaccinate at least 90% of girls before the age of 15 years; to treat at least 90% of women with cervical (pre-)cancer).[34]

The author group wants to emphasize that future HPV tests used in primary cervical cancer screening should not target HPV66. In 2005, HPV66 was classified by IARC[35,36] as *carcinogenic* and therefore included in many commercial assays. After additional evidence became available, HPV66 was downgraded to *possibly carcinogenic* (IARC class IIB) in 2009.[33,37] In 2022, HPV66 was further downgraded to *not attributable to cancer* based on growing understanding from updated meta-analyses.[13,34] HPV68 was in Volume 100B of the IARC Monograph on the Evaluation of Carcinogenic Risks to Humans classified as *probably carcinogenic* (IARC class IIA) because of mechanistic evidence though there was limited epidemiologic evidence.[33,37] Among the four HPV tests fulfilling the criteria of second generation comparators, only one (Anyplex) can identify the 12 class I carcinogenic genotypes separately without HPV66 and HPV 68 because this assay has full genotyping capacity which means that it individually reports all targeted genotypes. The other second generation comparator assays (Cobas 4800, Onclarity and RealTime) have partial or extended genotyping capacity and cannot report individually neither HPV66 nor HPV68.

The current review focuses on the qualitative interpretation of HPV tests (positive, negative, or invalid) and does not address the quantitative signal such as Ct (cycle threshold) value, viral concentration, viral load. HPV-specific quantitative PCRS and droplet digital PCR approaches are broadly employed in the preclinical research settings or may be used in the future as prognostic markers or in triage of HPV-positive women.[38–41] Current HPV assays, considered for use in cervical cancer screening, are used qualitatively, with HPV test positivity defined according to pre-specified cutoffs. For tests targeting other molecules than HPV DNA, longitudinal safety evidence needs to be shown. Currently, only one non-DNA HPV test, the APTIMA HPV Assay (Hologic) targeting, in aggregate, mRNA of 14 HPV genotypes, has demonstrated non-inferior cross-sectional performance and longitudinal effectiveness in primary cervical cancer screening.[42,43] Recommendations and guidance on how to evaluate screening methods targeting other molecular targets, as well as assay validation for use with self-collected samples are scheduled to be addressed in a comprehensive future update of validation guidelines. Finally, the author group will continuously survey the literature and periodically extend the list of comparator HPV tests that can be used in future validation studies.

5 | CONCLUSION

This review highlights the criteria that standard comparator HPV tests should fulfill to enable their use for the validation of emerging HPV tests for cervical cancer screening. Four HPV assays, the Cobas 4800, Realtime, Onclarity and Anyplex currently fulfill all these criteria.

AUTHOR CONTRIBUTIONS

Marc Arbyn, Kate Cuschieri, Jesper Bonde, Joakim Dillner, Maribel Almonte, Nicolas Wentzensen, and Mario Poljak conceived the concept of second generation comparator tests and contributed actively in writing the paper. Marc Arbyn conducted the statistical analyses and wrote the first draft of the manuscript. All authors critically reviewed the first draft, provided feedback, and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

M. Arbyn, M. Poljak, M. Gultekin, J. Dillner and J. Berkhof were supported by the Horizon 2020 Framework Programme for Research and Innovation of the European Commission, through the Risk-based screening for Cervical Cancer. Sciensano, the employer of M. Arbyn received funding from the European Society of Gynaecological Oncology (ESGO), German Guideline Program in Oncology (German Cancer Aid project), World Health Organization (Geneva, Switzerland, via Agreement for Performance of Work for guidelines on Screening and Treatment of Pre-Invasive Cervical Disease); the European Commission Initiative on Cervical Cancer (EC-CvC) and the VALGENT and VALHUDES projects.[44,45] J. Bonde reports speaking engagements or advisory board participation with Roche Molecular Systems, BD Integrated Diagnostic Systems, Copan, and MSD. JBOs institution has received funding through the VALGENT and VALHUDES projects.[44,45] K. Cuschieri's institution has received research funding or gratis consumables to support research from the following commercial entities in the last 3 years: Cepheid, Euroimmun, GeneFirst, SelfScreen, Hiantis, Seegene, Roche, Hologic, Barinthus Biotherapeutics PLC & Daye. KC has attended advisory board meetings for Hologic and Barinthus Biotherapeutics PLC. C. Cocuzza's institution has received research funding and/or free-of-charge consumables from BD Diagnostics, Seegene, Arrows Diagnostics, Copan, Novosanis, Genefirst, Hiantis, Vitro and Bioperfectus. C Cocuzza has participated in the last 3 years to congress symposia sponsored by BD, Seegene and Copan. K. Canfell and M. Saville are coprincipal investigator (PI) of an investigator-initiated trial of HPV screening in Australia (Compass), which is conducted and funded by the Australian Centre for the Prevention of Cervical Cancer (ACPCC), a government-funded health promotion charity. The ACPCC has previously received equipment and a funding contribution for the Compass trial from Roche Molecular Systems USA. They are also co-PIs on a major implementation program, "Elimination of Cervical Cancer in the Western Pacific", which receives support from the Minderoo Foundation and equipment donations from Cepheid Inc. K Canfell receives salary funding from the National Health and Medical Research Council Australia (NHMRC Leadership Fellowship APP1194679). D. Hawkes's institution has received research funding or gratis consumables to support research from the following commercial entities in the last 3 years: Abbott, AusDiagnostics, BD, Cepheid, Copan, Qiagen, Roche, Rovers, and Seegene. He has been an investigator on the Compass and SCoPE2(VALHUDES) clinical trials. P. Hillemanns declares no personal conflict. His institution receives funding from the German Guideline Program in Oncology (German Cancer Aid project). M. Poljak declares no personal conflict of interest; his institution received research funding, free-of-charge reagents and consumables to support research in the last 3 years from Qiagen, Seegene, Abbott, and Roche, all paid to his employer. He has been a coinvestigator on the VALGENT projects. R. Schuurman, F-H. Zhao, R. Rezhake, M. Almonte, and N. Wentzensen declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new original data were created or analyzed in this study.

DISCLAIMER

Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the official position, decisions, policy, or views of the International Agency for Research on Cancer/World Health Organization.

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How to cite this article: Arbyn M, Cuschieri K, Bonde J, et al. Criteria for second generation comparator tests in validation of novel HPV DNA tests for use in cervical cancer screening. *J Med Virol*. 2024;96:e29881. doi:10.1002/jmv.29881