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Triage options for positive high-risk HPV results from HPV-based cervical cancer screening: a review of the potential alternatives to Papanicolaou test cytology

Michael J. Thrall, MD[a,*], Erin McCarthy, MPH, CT (ASCP)[b], Jeffrey K. Mito, MD, PhD[c], Jianyu Rao, MD[d] On behalf of the Clinical Practice Committee of the American Society of Cytopathology

[a] *Houston Methodist Hospital, Houston, Texas*
[b] *Independent Cytologist, Oakland, California*
[c] *Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts*
[d] *UCLA Health, Los Angeles, California*

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The American Cancer Society has recommended high-risk human papillomavirus (HPV) testing as the primary screening method for cervical cancer since 2020. Up to this point, the transition from Pap test cytology-based screening or co-testing with cytology and HPV testing has been slow and limited. However, more health systems in the United States are in the process of implementing this change. The transition to HPV-based screening requires a triage strategy for positive results. Genotyping to specifically detect HPV types 16 and 18 in conjunction with reflex cytology for the remaining high-risk HPV genotypes has been the recommended method. Testing options including Dual Stain for p16/Ki-67 and extended HPV genotyping are currently being incorporated into treatment algorithms as alternatives. Methylation testing is another promising method extensively investigated around the world. This review, performed by members of the Clinical Practice Committee of the American Society of Cytopathology, examines the rationale behind the switch away from reliance on Pap test cytology in the cervical cancer screening algorithm and the opportunities and problems associated with the most promising alternative approaches. Published studies that give insight into the performance characteristics of these newer tests are reviewed. At the present time, Pap

*Corresponding author: Michael J. Thrall, MD; Houston Methodist Hospital, 6565 Fannin M227, Houston, TX 77030.
E-mail address: mjthrall@houstonmethodist.org (M.J. Thrall).

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test cytology remains a viable triage option for positive HPV screening results, but alternative tests have significant appeal and should be considered in tandem with the decision to offer primary HPV screening.

Contents

Introduction

Since the introduction of human papillomavirus (HPV) testing in the early 2000s, there has been much interest in its utility as a primary screening tool for cervical cancer because of its high sensitivity, relative objectivity, and potential for alternative self-collection methods to improve screening participation. In 2020, the American Cancer Society began recommending primary HPV testing as the preferred screening modality for cervical cancer.[1] Although the transition to primary HPV screening has been slow in the United States, more health care systems are beginning to make the transition from Papanicolaou (Pap) test cytology, which has been the most prevalent screening method since the beginning of cervical cancer screening.

The International Agency for Research on Cancer classifies 12 high-risk HPV genotypes (HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) as Group I carcinogens.[2] These types are the underlying cause of almost all cervical cancers, and many molecular testing platforms have been developed to test for them. Three have been approved by the Food and Drug Administration (FDA) for primary screening in the United States using both ThinPrep and SurePath vials: Roche cobas, BD Onclarity, and Abbott Alinity m. Onclarity and cobas have also recently been FDA approved, under limited circumstances, for use on vaginal self-collected specimens. The existence of multiple robust, readily available, high-throughput platforms for HPV testing makes a transition from morphology-based to molecular-based screening technically feasible.

The need for transition to HPV-based screening is driven by vaccination against HPV. Starting in 2006, highly effective vaccines against HPV have been available in the US. Early vaccines covered 2 types (16 and 18) or 4 types (16, 18, 6, and 11). The most current vaccine, in use since 2016, covers 9 types (low-risk types 6 and 11 and high-risk types 16, 18, 31, 33, 45, 52, and 58). Adoption of the vaccine has been slower than hoped in the United States but has risen steadily.[3] By 2021, adolescent coverage had increased to 76.9% for one or more dose(s) of HPV vaccine, and 61.7% were up to date with all recommended doses.[3] Access to the vaccine has also expanded over time, from the initial recommendation to vaccinate girls aged 11-13 to the current recommendation to vaccinate both genders as early as age 9 and as late as age 26,[4] with vaccination also available to older individuals on an individualized basis.[5]

Reconsideration of screening approaches makes sense as increasing numbers of vaccinated individuals enter the highest-risk age ranges for cervical precancer and cancer. Lower prevalence of HPV infection in the target population makes primary HPV-based screening more attractive. HPV is the cause of the great majority of cervical cancers, but most HPV infections are transitory and never integrate into the human genome, a necessary step in carcinogenesis. Therefore, HPV testing is highly sensitive, but the specificity for lesions requiring treatment is low. This is especially the case in younger women with a higher rate of recent infections that are likely to spontaneously clear. Highly vaccinated populations; however, have a much lower prevalence of HPV infection, resulting in fewer

positive results that require follow-up. Vaccination would also be expected to make cytology screening less effective. With lower HPV prevalence the percentage of cytology screens that are false positives due to reactive changes would presumably increase. In essence, as the disease becomes rarer, the more sensitive test (HPV) becomes increasingly preferable to the more specific test (cytology).

Since 2015 the recommended algorithm for primary HPV screening in the US has been colposcopy for women testing positive for types 16 or 18 with reflex cytology for the "other" high-risk types.[6] Type 16 is by far the most virulent HPV type, and type 18 is also a common cause of squamous carcinoma as well as highly associated with cervical adenocarcinoma. Therefore, the risk profile is high enough to justify colposcopy and biopsy regardless of additional testing. The "other" high-risk types are less virulent and less common than type 16, but nevertheless cause a significant percentage of cancers when taken together. Traditionally, it has been widely accepted that the lower risk profile of these types means that reflex colposcopy for an HPV result positive for non-16/non-18 "other" types would result in excessive testing. Triage of these types thus has become an important issue in designing an optimal screening program. Cytology has served as the standard triage test, but there has been persistent and increasing interest in alternatives. One method known as extended HPV genotyping uses more detailed information about which "other" types are positive to determine optimal follow-up based purely on HPV test results. Other methods, most prominently p16/Ki-67 Dual Stain and DNA methylation assays, involve a separate reflex test for the "other" types. Ideally, any alternative separate triage test would have both higher sensitivity and specificity than the Pap test it would replace. If the performance characteristics were good enough, triage of types 16 and 18 could potentially be considered as well.

Despite decades of tremendous success, the limitations of Pap testing are well known. It is a labor-intensive test relying on a skilled workforce of screeners using specialized laboratory equipment, based on subjective morphologic assessments with variability across laboratories, compounded by sampling issues that limit sensitivity. Given these multiple suboptimal features of Pap testing, it might not seem difficult to find a significantly better reflex test for women testing HPV positive, but this has not proven to be the case. A growing body of literature describes the strengths and weaknesses of alternative approaches. Dual Stain and methylation testing show promise, with numerous published studies, including some that strongly support these alternate methods, but others that are not as clear-cut. Extended genotyping of HPV has also emerged recently as an option, but published studies of its utility are limited. This review aims to help US laboratory directors make informed choices about whether they want to adopt cytology or some other method as triage when making the transition to primary HPV screening.

International experience with primary HPV screening

Despite having multiple automated testing platforms commercially available for more than a decade, adoption of primary HPV testing has been slow globally. In recent years, several countries have begun shifting national and regional screening programs away from cytology-based methods and toward HPV primary screening, utilizing cytology as a triage test for certain abnormal HPV results. In Europe, this shift has largely been driven by projections of improved clinical and cost effectiveness of screening programs in the context of increasingly vaccinated populations.[7-9]

The Netherlands and Australia led the shift to nationwide systematic primary HPV screening in 2017.[8-11] Australia has continued the use of Pap test cytology for triage of positive HPV screen results but with a threshold for colposcopy set to the equivalent of atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H), to reduce the colposcopy rate to a manageable level.[8,9] Sweden also revised its national screening program in 2017-2018 to introduce primary HPV testing, with the addition of a one-time co-test (cytology and HPV) for the first screening encounter after age 40 to mitigate the potential of HPV-negative cervical dysplasia that can be missed with primary HPV screening.[7,12,13] However, the utility of the single cytology screen has been questioned.[12,13] Additionally, Finland, Italy, Norway, and the United Kingdom have also incorporated primary HPV testing regionally or are exploring it as an option.[10,14]

No optimal strategy has emerged for the management of HPV positive results. Several countries that have been more successful in vaccinating their populations are, however, further along than the United States in the transition and have pioneered some of the new methods currently only beginning to be widely considered in this country.

Self-collection of samples for primary HPV testing

An additional motivation for HPV-based screening in many countries is the facilitation of self-collection methods using vaginal or urine sampling techniques.[15] Self-collection could overcome barriers to screening including a lack of gynecologists or a reluctance to undergo pelvic exams. Although the largest potential benefits of self-collection would accrue to developing countries, it could also facilitate screening in underserved populations in the United

States as well.[16] The current FDA approvals for Onclarity and cobas self-collected vaginal HPV tests in the United States still require supervision and guidance in a medical setting to increase the likelihood of adequate samples, though this does open increased opportunities for testing in conjunction with sexually transmitted infection treatment, in emergency rooms, and in other unconventional locations such as pharmacies.[17] Approval is likely to be expanded to home testing using mailed kits in the future, with US clinical trials currently underway. The necessity of follow-up colposcopy for positive results for type 16 or 18 or follow-up clinician-collected cervical samples for cytology or Dual Stain for the "other" types also limits the potential scope of self-collection. Thus, appropriate care for HPV-positive patients deterred by cost, lack of physician access, or by fear due to such issues as lack of previous gynecologic exams or a history of sexual trauma will remain difficult. In general, molecular methods are more suited to these less cellular specimens than cytology or Dual Stain and would require fewer clinic visits. The simpler follow-up algorithms in an all-molecular screening protocol would also facilitate the process of educating providers and increasing their acceptance of HPV-based screening using self-collected samples. Molecular triage tests such as methylation or extended HPV genotyping may therefore be more appealing if large scale self-collection is envisioned.

The effect of knowledge of HPV results on cytology interpretations

In the context of cytology triage of positive HPV tests, the perspective of the screeners changes relative to the traditional situation in which HPV status was unknown. It is apparent that the results of HPV testing may affect the interpretation of Pap tests by cytologists (cytotechnologists) and cytopathologists. Positive HPV results increase the likelihood that cytologic abnormalities correspond to virus-induced changes rather than nonspecific reactive changes. This may lead to an increase in the rate at which Pap tests are interpreted as positive, especially by causing an increase in the rate of atypical squamous cells of undetermined significance (ASC-US) interpretations. This jeopardizes the utility of cytology as an independent test since screeners would always know that each case had already tested positive for HPV, making it more psychologically difficult to render a negative result.

Indeed, findings from several studies performed in the context of co-testing seemed to support such an argument. One early study selected forty HPV-positive liquid-based Pap test slides initially interpreted as negative for squamous intraepithelial lesion or malignancy (NILM) to be reviewed by 22 members of the College of American Pathologists Cytopathology Committee. The pathologists were divided into two groups, each reviewing 20 slides without knowledge of HPV status. Then cases were crossed to another group of pathologists who were then told the patients had positive HPV status. The findings showed that there was a statistically significant decrease in NILM results and an increase in the diagnosis of other categories of epithelial cell abnormalities when viewed in the HPV-positive context.[18] In a retrospective study of 250 liquid-based (ThinPrep) cytology slides, knowledge of the HPV status of cases significantly increased the chances of negative cases being upgraded to ASC-US or worse. This resulted in an increase in post-screening referral to a pathologist, which did not result in increased detection of disease.[19] An international study involving retrospective review of 1767 Pap tests from Canada, Democratic Republic of the Congo, and Brazil showed significant drops in specificity in 2 of the 3 cohorts with a corresponding gain in sensitivity in only 1 of the 3 cohorts.[20]

Although such shifts in the interpretation of Pap tests decrease the independence of the 2 results, the resulting increase in sensitivity could also be considered a positive.[21] In a prospective colposcopy-controlled study, a total of 2905 women were enrolled to identify cases with cervical intraepithelial neoplasia grade 2 or higher (CIN 2+) by histology, within a 24-month follow-up period. Liquid-based cytology (SurePath) was performed twice on every sample, with and without knowledge of HPV DNA test results. The results showed that prior knowledge of HPV status resulted in a significantly higher detection rate of high-grade squamous intraepithelial lesions (HSIL), corresponding to follow-up histologic CIN 2+ compared with screening blinded to HPV status, with limited loss of specificity.[22] Likewise, a retrospective study of a subset of 428 cases from the ATHENA study showed that unblinded re-interpretation of Pap tests with positive HPV would have significantly increased sensitivity with only a small drop in specificity.[23] Recently, a multi-center randomized trial involving 15 cytopathologists evaluated 71 digitalized ThinPrep slides including 31 ASC-US, 21 NILM, and 19 low-grade squamous intraepithelial lesion (LSIL) cases in 2 rounds, one without and one with the knowledge of the HPV finding. The results showed that cytopathologists are generally unbiased by the knowledge of HPV data, but that being informed of the HPV status leads to a better intra-observer agreement.[24] In summary, while there is evidence showing that the knowledge of HPV positivity may result in an increased abnormal cytological interpretation, especially ASC-US diagnosis, there are also potential benefits including improved detection of significant lesions (CIN 2+) and better intra-observer agreement.

Another issue that arises in the context of cytology reflex is the traditional reluctance to allow cytologists to sign out high-risk HPV positive cases as negative without additional review. Many laboratories in the United States currently use HPV positivity as a criterion for reflex to second review by a senior cytologist in the event of an initial negative interpretation.[25] In the context of reflex cytology following a positive HPV result, every case would potentially require

multiple reviewers, reducing cytologist autonomy. This would also tend to further increase the ASC-US rate, resulting in more follow-up testing with a relatively small increase in the frequency of finding significant lesions.[26]

If a laboratory finds that a switch to primary HPV screening with knowledge of a positive result has an undesirable effect on the performance of Pap testing, cytologists and cytopathologists do have the ability to adjust their practice in response to feedback about their performance. Systematic threshold changes can also be employed if specificity becomes unacceptably low on a population basis. The example of Australia can serve as a model, where the colposcopy threshold was shifted to the equivalent of ASC-H instead of the equivalent of ASC-US. This approach leads to a significant decrease in sensitivity, which may be undesirable in countries like the United States where screening is more opportunistic, and vaccination is less prevalent, than in the highly organized and optimized Australian program. The United States has, however, implemented a similar though less dramatic shift successfully in the past, when the original Bethesda category of ASC-US, favor reactive was eliminated in the Bethesda 2001 system. Another reconsideration of the criteria for the ASC-US category could be undertaken in the context of reflex Pap testing if that became the predominant mode of gynecologic cytology practice.

Dual Stain with p16/Ki-67 as a triage test

Immunocytochemistry directed against p16, which is markedly upregulated secondary to the activity of HPV oncogenic proteins E6 and E7, has been extensively evaluated as a biomarker for triage of ASC-US and LSIL Pap test results.[27,28] A "Dual Stain" that also incorporates simultaneous staining for the proliferation marker Ki-67 to increase specificity has been studied in Europe since 2011[29,30] and received FDA approval in the United States in 2020. However, it has yet to gain wide acceptance. In part this is due to the difficulty and expense of using the test. The test includes a brown chromogen that identifies p16 in the cytoplasm and nucleus, with a red nuclear chromogen highlighting Ki-67. Staining the cells in the sample in this manner requires specialized and proprietary staining equipment provided by Roche, the manufacturer, and runs on the ThinPrep platform. Staff capable of performing this complex assay that combines cytologic and immunochemical processing techniques are also needed. Billing may be problematic, with a high rate of rejected claims,[31] though presumably this would improve with more widespread implementation. Although various thresholds have been evaluated,[32] a single cell with brown cytoplasmic and red nuclear staining is usually considered to be enough for a positive result. Thus, staining and screening must be carefully optimized. High background staining and thick cell clusters cause interpretive difficulties in some samples.[31] Interpretation may also be problematic in older women due to lower cellularity and atrophic changes.[33,34] Although cytologists have training that prepares them to excel in the meticulous screening needed for these specimens, it nevertheless requires greater effort per patient than Pap-stained cytology. Unfortunately, existing regulations do not allow cytologists to sign out negative cases independently, meaning that their labor is not directly compensated. Furthermore, the very low threshold for positive, at least in theory, creates increased medicolegal risk that makes it difficult for cytopathologists to commit to a negative result.

In addition to these problems, the specificity of the Dual Stain is not as high as might be imagined. In histologic sections strong and diffusely positive p16 (also known as CINtec), at least at the base, is considered "block positive" and supports a diagnosis of HSIL.[35] However, LSIL also has weak or focal "patchy" staining in many cases,[36,37] and similar staining can also be seen in glandular cells, especially tubal metaplasia.[38,39] Disaggregated cells in cytology preparations eliminate the possibility of easily distinguishing between "block positive" and "patchy" staining in the same way that is routinely and usefully employed when evaluating biopsies. Ki-67 is also nonspecific and will be present in proliferating cells regardless of the underlying cause. Thus, Dual Stain of cytologic specimens can be expected to produce false positive results. The key question is whether Dual Stain outperforms available alternatives, most notably Pap test cytology.

Many published studies have compared Dual Stain and cytology triage for positive high-risk HPV results. All published studies reporting the relative sensitivities and specificities for Dual Stain and cytology have been compared in Table 1.[40-50] The results of these studies are variable, but clearly support Dual Stain as a viable triage option. Two studies performed on the population served by Kaiser Permanente of Northern California (KPNC) showed both superior sensitivity and specificity for Dual Stain as compared with cytology.[43,45] The high utilization of the KPNC data set in numerous other studies of cervical cancer screening make these results especially noteworthy. Recently, the largest study yet published, performed in China,[40] as well as studies in Poland[51] and Spain,[52] also show superior performance characteristics for Dual Stain with higher sensitivity and specificity. On the contrary, 3 smaller studies performed in China[49] and the Netherlands[47,48] show higher sensitivity for Pap testing relative to Dual Stain. Yet other studies show that increased sensitivity for Dual Stain is offset to varying degrees by lower specificity relative to Pap tests.[41,42,44,46,50,53,54]

Another factor to consider is the role of HPV genotyping in the triage algorithm. Many of the published studies provide a separate evaluation of the performance of Dual Stain and cytology in the subset positive for non-16/18 "other" HPV types, as shown in Table 2.[40-42,46,51] In general, these studies find a similar pattern of relative sensitivity and specificity in this subset that excludes types 16 and 18. Dual Stain could be used as triage for all high-risk HPV results,

Table 1 Studies comparing sensitivity and specificity of Dual Stain (1 positive cell) and Pap test cytology (ASC-US+) as a triage test for all high-risk HPV types with a histologic end-point of CIN 2+.

First author and year	Geographic location	Number of HPV + women	HPV testing platform	Dual Stain sensitivity	Dual Stain specificity	Pap test type	Pap test sensitivity	Pap test specificity
Chen 2022[40]	China	10,500	PCR Dot Blot	82.8	51.6	ThinPrep	66.7	44.4
Wright 2021[41]	USA	5250	Cobas	86.5	57.5	ThinPrep	65.9	66.8
Wright 2017[42]	USA	3467	Cobas	70.3	75.6	ThinPrep	51.8	76.1
Wentzen-sen 2019[43]	USA	3416	HC2	82.8	55.7	SurePath	81.1	44.6
Giorgi Rossi 2021[44]	Italy	3147	Cobas/HC2	75.2	74.8	ThinPrep	61	76.6
Wentzen-sen 2015[45]	USA	2363	HC2	83.4	58.9	SurePath	76.6	49.6
Ovestad 2023[46]	Norway	1415	Cobas	82.7	65.9	Liquid-Based	62.4	74.5
Ebisch 2017[47]	Netherlands	834	GP5+/6+	86	73	Liquid-Based	94	62
Luttmer 2016[48]	Netherlands	535	GP5+/6+	85.5	60	ThinPrep	86.7	54.3
Yu 2016[49]	China	463	Cobas	92.7	52.7	ThinPrep	94.5	53.5
Stanczuk 2017[50]	UK	340	Cobas	77.7	74.2	Liquid-Based	62.7	82.4

CIN 2+, cervical intraepithelial neoplasia grade 2 or higher; PCR, polymerase chain reaction; HPV, human papillomavirus.

including types 16 and 18, or for only the "other" types.[43] Reflexing all HPV positive results to Dual Stain regardless of subtype would simplify the algorithm and reduce the colposcopy rate. However, this would come at the expense of lower overall sensitivity.

The Enduring Consensus Cervical Cancer Screening and Management Guidelines Committee in association with the American Society for Colposcopy and Cervical Pathology have recently issued updated guidance on the use of Dual Stain results in screening algorithms.[55] The guidance evaluates the Dual Stain by the same methodology as HPV and Pap testing, on the basis of risk thresholds. A 4% immediate risk of cervical intraepithelial neoplasia grade 3 or higher (CIN 3+) is the threshold for colposcopy. The American Society for Colposcopy and Cervical Pathology has chosen to use a CIN 3+ standard due to greater specificity for risk of invasive cancer, though CIN 2+ is typically used in clinical practice and in most older studies of screening methods and their follow-up. When applied to Dual Stain as a reflex test for HPV using a 4% risk of CIN 3+, high-risk HPV positive patients who are also Dual Stain positive should proceed to colposcopy. Data from KPNC and the STRIDES cohort in Mississippi was used to determine the risk thresholds. Interestingly, even though women with HPV types 16 or 18 and negative Dual Stain have a less than 4% immediate risk in the cited data set, immediate colposcopy is still recommended, illustrating the reluctance to forego colposcopy in this group. This recommendation may change in the future, however, if justified by additional data.[31]

One recent study has led to more widespread consideration of Dual Stain as a triage option in the United States. A large prospective study using 32 clinical sites across 16 states, the IMPACT trial, found that use of Dual Stain for triage of HPV results positive for the 12 "other" types had much higher baseline sensitivity for follow-up CIN 3+ than Pap test cytology (86.0% versus 66.7%), with a relatively small reduction in specificity (53.7% versus 63.8%).[41] The increased sensitivity resulted in a higher colposcopy rate at baseline (63.3% for Dual Stain versus 56.0% for cytology), but the number of colposcopies needed to detect each HSIL was about the same.[41] Although use of Dual Stain and the increased colposcopy rate would increase the cost of screening, many would consider the gain in sensitivity to be worth the added expense.

In general, like cytology, Dual Stain is usually considered to be not a viable option on self-collected samples. The published literature examining Dual Stain in this context

Table 2 Studies comparing sensitivity and specificity of Dual Stain (1 positive cell) and Pap Test Cytology (ASC-US+) as a triage test for "other" non-16/non-18 HPV types with a histologic end-point of CIN 2+.

First author and year	Geographic location	Number of HPV + women	HPV testing platform	Dual Stain sensitivity	Dual Stain specificity	Pap test type	Pap test sensitivity	Pap test specificity
Chen 2022[40]	China	8842	PCR Dot Blot	83.8	49	ThinPrep	71.7	34.4
Wright 2021[41]	USA	3468	Cobas	83	56.8	ThinPrep	58.8	65.5
Wright 2017[42]	USA	3467	Cobas	64.2	78.2	ThinPrep	46.6	78.1
Trzeszcz 2023[51]	Poland	1086	RealTime	92.3	51.3	SurePath	74.4	14.3
Ovestad 2023[46]	Norway	1034	Cobas	76.7	67	Liquid-Based	61	73.9

PCR, polymerase chain reaction; HPV, human papillomavirus.

includes only a few small studies.[56,57] These show that Dual Stain is technically possible with vaginal self-collection kits, though with decreased sensitivity. Dual Stain can be performed on a follow-up specimen collected in clinic for patients who screen positive by self-collection, but this requires an additional round of testing.

The use of a deep learning algorithm to potentially automate Dual Stain screening has also been evaluated. This approach showed promise in a large retrospective study, increasing the specificity of Dual Stain results without much loss of sensitivity using a threshold of 2 positive cells detected by the algorithmic method.[58] If similar results can be demonstrated in prospective studies, artificial intelligence-based Dual Stain results may become an alternate method of triage to consider. This could transform Dual Staining into a more automated and objective test with less or no need for human evaluation of morphology.

Dual Stain has a long track record, and numerous studies illustrate that it has promise as a triage test following positive HPV test results. Whether Dual Stain should be adopted in any given laboratory or patient population depends on a thorough evaluation of the tradeoffs of sensitivity and specificity as well as the challenges of implementing the test.

Methylation testing as a triage test

Molecular testing that detects methylation of target genes has emerged as a promising method of triaging positive HPV results without the use of morphologic assessment. Methylation assays have been widely studied with extensive literature from around the world. Much of this work has been led by researchers in the Netherlands who are seeking a more objective and automated method for screening that will also facilitate self-collection.

Methylation is an epigenetic process that involves attachment of methyl groups to areas of the genome known as "CpG islands" that consist of repeated cysteine-guanosine pairs. This change is linked to the regulation of downstream genes. Methylation is a normal process that goes awry in malignant cells. Aberrant hypermethylation in malignant cells occurs frequently in promoters and enhancers of tumor suppressor genes, resulting in silencing of these key regulators of the cell cycle, contributing to oncogenesis.[59,60]

Methylation occurs commonly in many different types of cancer and is not specific to cervical carcinogenesis or HPV-driven processes. Although the significance of methylation has been known for some time, the relative difficulty of testing for these epigenetic changes has slowed the development of commercial high-throughput platforms. Simple polymerase chain reaction will not detect methylated sequences of DNA. Additional processing steps are needed, with many different methods in use to facilitate detection of hypermethylated regions of the genome.[61] With improved technology, highly automated methylation assays are now commercially available. The most familiar use of a methylation assay in the US is the FDA-approved Cologuard colon cancer screening test for self-collected stool samples.

While methylation assays have excellent sensitivity for invasive cancer, their ability to reliably detect noninvasive precursors is more limited. Hypermethylation of key genes increases in frequency during the process of accumulating genetic errors that ultimately result in invasive cancer, meaning that the earliest precursors have less frequent hypermethylation at any given genetic locus. To achieve acceptable sensitivity for histologic HSIL (CIN 2+), the readily curable precursor of most cancers, methylation assays must be carefully designed and calibrated. Assays targeting many different genes, either singly or in combination, have been evaluated in published studies. Two recent meta-analyses of this vast literature have been published. One that included 43 studies found a pooled sensitivity and specificity for CIN 2+ of 63.2% and 75.9% and a pooled sensitivity and specificity for CIN 3+ of 70.5% and 74.7%.[62] Analysis of the subset of studies that compared methylation assays with cytology found that methylation was less sensitive (relative sensitivity 81%), but more specific (relative specificity 125%). The other meta-analysis, which used different criteria and included 23 studies, reported pooled sensitivity and specificity of 68% and 75% for CIN 2+ and 78% and 74% for CIN 3+.[63]

A few specific assays have been the subject of multiple high-quality recent studies and are worthy of more detailed review. One of these, known as S5, targets the human gene *EPB41L3* as well as the late gene regions of HPV types 16, 18, 31, and 33.[64] Of note, a study of this assay has been conducted in the US, in the state of New Mexico.[65] In the study, 798 women with liquid-based Pap tests and positive high-risk HPV results were tested with S5. The S5 results were broken down by cutoff value, with the lower cutoff of 0.8 having a sensitivity for CIN 2+ of 71.84% and specificity of 60.06%, whereas the higher cutoff of 1.4 showed sensitivity of 62.64% and specificity of 72.44%. Performance for CIN 3+ showed better sensitivity of 83.33% at the lower cutoff and 77.82% at the higher cutoff, with similar specificity. The authors concluded that S5 positive results in HPV positive women would correspond to high enough risk of CIN 3+ to trigger colposcopy, making it a reasonable alternative triage method. A study performed in Canada also found that S5 positivity in HPV positive women could identify a population with high enough risk of CIN 3+ to justify colposcopy.[66] Furthermore, they found that S5 had overall sensitivity and specificity for CIN 3+ comparable to the existing algorithm of combined type 16/18 and cytology triage. A

study using samples from many different nations showed that S5 performs well in both developed and developing countries for detection of CIN 3+,[67] a finding supported by additional studies that have been performed in Mexico,[68] Colombia,[69] and China.[70]

Another assay extensively studied in Europe, especially the Netherlands, analyzes the methylation of *FAM19A4* and *miR124-2* (QIAsure). A multicenter European study of 2384 HPV-positive cervical samples showed that this test had sensitivity of 46.8% and specificity of 78.3% with a CIN 2+ threshold, with higher sensitivity for CIN 3+ of 77.2%.[71] A study performed in the Netherlands showed that in a cohort of 979 women, the sensitivity and specificity of QIAsure were both lower than cytology at the CIN 3+ threshold, 71.3% versus 76.0% for sensitivity and 78.3% versus 87.0% for specificity.[72] These results derived from a retrospective review of samples from a long-standing clinical trial. Due to this, the authors were able to examine the negative predictive value of the methylation test and cytology for invasive cancer over a 14-year period, concluding that HPV-positive, QIAsure-negative women over age 30 had only a 1.7% cancer risk, versus a 2.4% risk if cytology was negative.[73] Thus, despite worse performance characteristics for all CIN 3+ lesions, the methylation assay was better able to identify the women truly at highest risk of developing invasive cancer. In essence, one could argue that methylation status is a better indicator of risk for invasive carcinoma than not only cytomorphology, but also histomorphology as well, potentially undermining the assumption that the histology-based CIN 3+ threshold should serve as the "gold standard" when evaluating triage methods. Additional recent studies using both QIAsure[74] and S5[75] provide further support for this contention. However, additional larger studies will likely be needed before there will be widespread acceptance of methylation positive status as a better indicator of invasive cancer risk than CIN 3 histology. In the meantime, cytology appears to be at least comparable to methylation, if not better, as a reflex test in HPV-positive women.

Further improvements in methylation assays may allow for development of tests with significantly better performance characteristics than Pap testing or Dual Stain. The main advantages of methylation testing are the ability to fully automate cytology screening in the molecular laboratory, facilitation of self-collection, and simplification of the follow-up algorithm. At present, however, no methylation-based assays are FDA-approved and readily available for use in the United States. Presumably, if primary HPV screening became more commonplace, methylation assays would enter the US market. However, this would not happen immediately, necessitating an interim strategy for any US labs seeking a short-term shift to primary HPV screening with a long-term vision of using methylation as the reflex test.

HPV extended subtyping and viral load as alternatives to reflex testing

Although Dual Stain and methylation are the most frequently considered alternatives to cytology as reflex tests for positive HPV results, many studies have also considered trying to extract more information from the HPV test itself to reduce or eliminate the need for reflex testing at all. Extended genotyping beyond types 16 and 18 is one possible strategy. HPV viral load analysis has also been extensively studied.

Three FDA-approved HPV tests available in the US offer typing information beyond types 16 and 18, but all have significant limitations. The Hologic Aptima test reports the presence of type 16 and the 2 related types 18 and 45 as a combined unit, without further specifying other types. This test is therefore usually considered to have only "limited genotyping." The recently approved Abbott Alinity m reports types 16, 18, and 45 separately as well as 2 groupings of HPV types, 31/33/52/58 and 35/39/51/56/59/66/68.[76,77] This test gives more information about the "other" types than Aptima, but the 2 large groupings may limit its usefulness.

The only HPV test that is FDA approved that offers "extended genotyping" with published data on the utility of the additional genotyping information is the BD Onclarity test. This assay specifically identifies types 16, 18, 31, 45, 51, and 52, as well as the related-type groupings of 33/58, 35/39/68, and 56/59/66. Retrospective review of data from the Onclarity trial that involved 29,513 women from the west coast of the United States (KPNC and Portland, Oregon) showed that type 31 had higher CIN 3+ risk than type 18, though lower than type 16.[78] The type 33 of 58 grouping also had a risk of CIN 3+ above the 4% threshold for colposcopy. Furthermore, the study showed that persistence of the same high-risk genotype at a 3-year interval also had an associated CIN 3+ risk above 4%, though there were not enough women with the less common high-risk types in the study to evaluate the risk associated with persistence for every individual type or grouping. Nevertheless, this study indicates a possible HPV-only screening paradigm if repeat testing after 3 years is considered acceptable for the high-risk types with lower immediate risk of CIN 3+.

Another study, also using data from KPNC, used the BD Linear Array HPV test to retrospectively identify HPV types in a cohort of 54,133 women.[79] This study showed that type 33 had an immediate risk of CIN 3+ intermediate between type 16 and 18. Furthermore, types 31, 35, 45, 52, and 58 all

had immediate risk greater than the 4% threshold. These results differ slightly from those derived from the BD Onclarity test in a similar cohort. The relative rarity of some of these types limits the availability of data and makes precise estimates of immediate CIN 3+ risk more difficult. This problem is exacerbated by the paucity of published data regarding extended genotyping, particularly in US populations.

Early adopters looking to make a fast transition to purely molecular-based testing may find extended genotyping an appealing option, since the BD Onclarity platform is now FDA-approved for both self-collection and primary screening. However, making a gradual transition using morphology-based triage, whether cytology or Dual Stain, would make sense for labs not wishing to make a commitment to a less well-studied approach.

Studies of same-type HPV persistence consistently show that this increases the long-term risk for CIN 3+.[80] Unfortunately, the currently FDA approved tests do not allow for precise identification of all types, limiting the potential use of a re-test after an interval as an alternate means of reflex. Many HPV tests that offer extended genotyping, including some that individually identify all high-risk types, are commercially available outside the US.[81] Such tests would be more amenable to repeat testing as a strategy. Some countries with current access to such tests have started to include extended genotyping in their screening algorithms.[80] Despite the limitations of the Onclarity platform, the Enduring Consensus Cervical Cancer Screening and Management Guidelines Committee has also made substantial progress toward updating its algorithms to allow for entry of extended genotyping results as a means of refining risk estimates for patients who have this information available.

HPV viral load has also been extensively studied, mostly outside the United States.[82] Although intuitively appealing, the link between the amount of HPV in a sample and the likelihood of histologic HSIL or invasive cancer is less robust than one might expect. Viral loads for type 16 correlate reasonably well with disease severity, but the relationship is weaker and less consistent for other types, including 18, as shown in a study performed in the United States in New Mexico.[83] As a result, the performance characteristics of viral load are suboptimal and inferior to cytology, especially if type 16 automatically reflexes to colposcopy regardless of the amount of virus detected. Accordingly, use of HPV viral load from a single HPV test as a basis for triage is mostly of interest only in parts of the world without a robust cytology screening infrastructure, such as China.[84-86] Settings where higher sensitivity and specificity are expected have evaluated longitudinal analysis of HPV viral loads after an interval and shown a relationship between increasing viral loads of specific types over time and risk of HSIL.[87-90] However, these studies are limited by small numbers and the acceptability of a multiple-round screening protocol is questionable.

Conclusions

Primary HPV screening is increasingly being considered by laboratories in the United States. If the decision to switch is made, the next question becomes how to triage the women who test positive for high-risk HPV other than types 16 and 18. Cytology has been the recommended triage test for many years, but there has been an ongoing search for superior alternatives. Dual Stain with p16/Ki-67 appears to perform at least as well as cytology, and some large studies show both superior sensitivity and specificity. However, the data remain limited, and at the present time the most that can be said with certainty is that Dual Stain is a reasonable alternative to Pap testing. Dual Stain has the disadvantage of being a proprietary platform and, as with any new test being considered by a laboratory, the associated expense and technical issues must be weighed along with performance characteristics when deciding about implementation. Methylation testing is a promising molecular method of triage that offers automated and objective results with no reliance on morphology. However, no FDA-approved platforms are currently available in the United States. Labs seeking to expand self-collection to enable greater access to cervical cancer screening would not be able to immediately implement methylation testing. Presumably, if sufficient demand existed, methylation products would be forthcoming, but this process could take many years. Extended HPV genotyping using the BD Onclarity platform offers the possibility of immediate movement toward expanded access via self-collection, but issues remain regarding how to triage some of the less common and virulent types.

Considering all possibilities, cytology triage remains a viable choice. Dual Stain offers an alternative morphology-based approach but requires a major investment in terms of money, time, and training. The available literature is not entirely conclusive as to the superiority of Dual Stain in terms of sensitivity and specificity. Even if the studies showing slightly better performance characteristics are accurate, whether this outweighs the increased costs to the laboratory, patients, and health system is also an open question. In the long run, the desire to move cervical cancer screening to entirely molecular platforms to facilitate self-collection will likely also necessitate a second switch for those who adopt Dual Stain now. Although the conversion to primary HPV screening may seem like the optimal time to move beyond cytology, alternative methods remain problematic. Despite its limitations, cytology triage may be the best approach for laboratories in the interim period of uncertainty when molecular triage options are limited and largely untested in the United States. The long-term trend of

an increasingly vaccinated population necessitates change, but the optimal course remains unclear.

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Conflict of interest disclosures

Erin McCarthy was formerly an employee of Hologic, Inc. during the drafting of this manuscript. Hologic is the manufacturer of ThinPrep, a product mentioned in this review. The other authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Michael J. Thrall: Writing − review & editing, Writing − original draft, Conceptualization. **Erin McCarthy:** Writing − review & editing, Writing − original draft. **Jeffrey K. Mito:** Writing − review & editing, Writing − original draft. **Jianyu Rao:** Writing − review & editing, Writing − original draft.

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