Review paper

p16/Ki-67 dual-stain immunocytochemistry in precision cervical cancer prevention: immunoprofile and clinical decision-making

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Cervical cancer prevention has undergone a major shift with the implementation of human papillomavirus (HPV)-based screening, which offers high sensitivity but limited specificity due to the detection of transient infections. This has created a need for triage methods capable of identifying infections associated with clinically meaningful disease. Primary HPV testing provides a high level of reassurance when negative; however, individuals with a positive result require additional evaluation using triage methods that effectively identify precancerous lesions while avoiding unnecessary colposcopy referrals. This review addresses the role of p16/Ki-67 dual-stain (DS) immunocytochemistry as a biomarker of transforming HPV infection, with a focus on HPV-based screening settings. We discuss its biological basis and diagnostic performance in comparison with cytology, with particular attention to the principles and practical aspects of DS interpretation in routine cytopathological practice. We also summarize current evidence, including data from national studies and routine clinical practice, demonstrating that DS can be effectively integrated into screening pathways and support more informed clinical decision-making. Its increasing use reflects the broader transition toward biologically informed cervical cancer prevention.

Key words: cervical cancer screening, dual-stain cytology, HPV, cervical precancer.

Introduction: from HPV detection to clinically meaningful disease

Persistent infection with oncogenic human papillomavirus (HPV) is the central and well-established etiological factor in cervical carcinogenesis, and cervical cancer continues to be a significant global health challenge [1, 2]. The implementation of HPV-based screening has substantially increased the sensitivity of detection in randomized trials and has been associated with improved long-term protection against invasive disease. Randomized trials have demonstrated that HPV-based screening substantially increases sensitivity for detecting cervical intraepithelial neoplasia grade 3 or worse (CIN3+), with long-term follow-up showing improved protection against invasive disease [3, 4]. However, this increased sensitivity is accompanied by reduced specificity, largely due to the high frequency of transient HPV infections that do not lead to clinically significant pathology [5, 6].

As a result, the focus of cervical cancer screening has evolved. Rather than concentrating solely on the identification of cytological abnormalities, current approaches aim to distinguish infections that are biologically relevant from those that are self-limiting. In this context, HPV positivity reflects

the presence of viral infection but does not in itself indicate the presence of disease. The critical clinical issue is therefore whether HPV infection has initiated a transforming process that may lead to the development of cervical intraepithelial neoplasia grade 2 or worse (CIN2+). Modern clinical management strategies reflect this shift in perspective. Current guidelines emphasize the need to move beyond isolated test results and instead incorporate information that supports more informed clinical decisions. This is particularly important in HPV-positive populations, where outcomes vary widely and are influenced by viral genotype, patient-related factors, and the biological effects of infection [7, 8]. Within this framework, triage plays a central role by helping to identify those individuals who are most likely to benefit from further diagnostic evaluation.

p16/Ki-67 dual-stain (DS) immunocytochemistry has become established as a biomarker that directly addresses this clinical need. By capturing HPV-induced cell cycle dysregulation and proliferative activity, DS provides evidence of transforming infection rather than mere viral presence [9]. This places DS between molecular detection and clinical interpretation. In line with this, recent international and European recommendations support the use of biomarker-informed triage approaches in HPV-positive women as part of contemporary cervical cancer prevention strategies [10–13].

Dual-stain cytology in HPV-based screening

For decades, cytology has played a central role in cervical cancer screening; however, its diagnostic limitations are well established. Its performance is influenced by multiple factors, including specimen adequacy, staining quality, and interobserver variability in morphological interpretation, resulting in detection of clinically relevant lesions in the range of 50–70% of underlying CIN2+ cases. In the context of highly sensitive and less subjective HPV-based screening, the role of cytology has shifted toward use as a triage method for HPV-positive women rather than as a standalone screening tool [12].

Dual-stain cytology represents a fundamentally different approach. Rather than relying on morphological features alone, it detects a specific biological phenomenon – the simultaneous presence of p16 overexpression and Ki-67 positivity within the same epithelial cell. This co-expression reflects disruption of cell cycle regulation driven by oncogenic HPV infection and distinguishes DS from morphology-based triage, underlying its improved clinical performance. The differences between cytology and dual-stain cytology extend beyond diagnostic performance and reflect distinct biological and clinical principles. While cytology relies on cytomorphological assessment, DS directly identifies transformation-associated cellular changes. A structured comparison of these approaches, including real-world data from routine clinical practice, is presented in Table I. As shown, DS offers improved sensitivity, more consistent interpretation, and closer alignment with clinical management, while maintaining clinically acceptable specificity across different screening settings.

Clinical studies have consistently shown improved performance of DS compared with cytology. Across multiple large clinical studies and validation cohorts,

Table I. Comparative characteristics of cytology and p16/Ki-67 dual-stain as triage strategies in HPV-based cervical cancer screening, including real-world evidence

Parameter	Cytology	DS	Real-world evidence (DS)
Biological basis	Morphologic-based assessment	Biomarker of transforming HPV infection	Consistent with DS biology
Target signal	Cytomorphological abnormalities	Cell cycle dysregulation (E7-driven)	Transformation-associated signal
Sensitivity (CIN2+) (%)	Moderate, with variability (~50–70)	High (~85–90)	> 85 (opportunistic settings)
Specificity	Moderate	Comparable to or slightly higher than cytology	Clinically acceptable
Interpretation	Morphology-based, subjective	Binary (≥ 1 dual-positive epithelial cell); biomarker-based	Feasible in routine practice
Role in HPV-based screening	Primary screening test in younger women, triage in HPV-based strategies	Preferred or alternative triage strategy	Effective in opportunistic screening
Performance in younger women	Established primary screening approach	Emerging evidence in selected populations	Promising results in younger women (limited data)

CIN2+ – cervical intraepithelial neoplasia grade 2 or worse, DS – p16/Ki-67 dual-stain, HPV – human papillomavirus
Performance estimates are approximate and may vary depending on population characteristics, screening setting, and clinical endpoint definition (CIN2+ vs. CIN3+).

DS has demonstrated higher sensitivity for the detection of CIN2+ and CIN3+ while maintaining clinically acceptable specificity. These findings have been reproduced in both screening and referral populations, supporting the robustness of DS across different clinical settings [14–16]. The advantage of DS is particularly evident in clinically challenging groups. In HPV-positive, cytology-negative women, DS identifies a substantial proportion of underlying CIN2+ lesions that would otherwise remain undetected [17]. Longitudinal analyses further indicate that DS provides meaningful separation between women with a low likelihood of disease following a negative result and those requiring closer clinical attention when DS is positive [18]. p16/Ki-67 dual-stain also improves triage in women with minor cytological abnormalities, where cytology alone has limited predictive value. Studies have demonstrated that DS improves identification of clinically relevant disease in atypical squamous cells of undetermined significance and low-grade squamous intraepithelial lesion populations and performs favorably compared with other triage approaches [19–21]. The evidence base has expanded considerably in recent years. Large cohort studies and real-world screening programs have confirmed the robustness of DS across different populations and healthcare systems [22, 23] and provided additional support for its clinical utility [12, 24, 25]. Particular attention has been given to women with HPV HR12-positive infections (with high-risk non-16 and non-18 HPV genotypes), a heterogeneous group in which cytology often fails to adequately distinguish between clinically relevant and less significant disease. p16/Ki-67 dual-stain appears especially useful in this context, as it reflects transformation-associated biology rather than genotype alone. Studies have shown that DS improves detection of clinically relevant lesions in this group and provides additional discrimination beyond genotype-based classification [24, 25].

The role of dual-stain cytology is best understood within the broader context of HPV-based screening and current clinical management. Rather than functioning as an isolated diagnostic test, DS integrates molecular detection with biologically informed triage, enabling more precise identification of clinically relevant disease in HPV-positive women. As illustrated in Figure 1, DS serves as a central decision point within the screening pathway, distinguishing between individuals who require colposcopic evaluation and those who may be safely managed with surveillance. This approach reflects the transition from morphology-based assessment to biologically driven decision-making

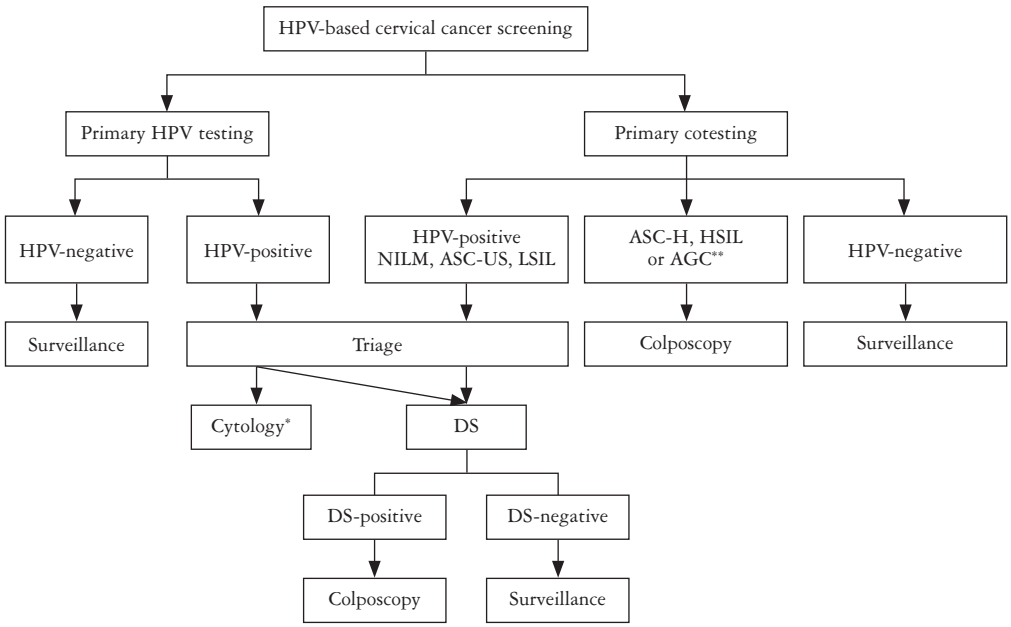

Figure 1. Conceptual framework for integrating p16/Ki-67 dual-stain into HPV-based cervical cancer screening without genotyping

AGC – atypical glandular cells, ASC-H – atypical squamous cells – cannot exclude HSIL, ASC-US – atypical squamous cells of undetermined significance, DS – p16/Ki-67 dual-stain, DS-negative – negative dual-stain result, DS-positive – positive dual-stain result, HPV – human papillomavirus, HPV-negative – negative high-risk HPV test result, HPV-positive – positive high-risk HPV test result, HSIL – high-grade squamous intraepithelial lesion, LSIL – low-grade squamous intraepithelial lesion, NILM – negative for intraepithelial lesion or malignancy

Following primary HPV testing, p16/Ki-67 dual-stain (DS) is used as a triage method in HPV-positive individuals. In cotesting setting, DS may be applied in individuals with NILM, atypical squamous cells of undetermined significance, or low-grade squamous intraepithelial lesion cytology. As a biomarker of transforming infection, DS supports clinical decision-making by identifying individuals who should be referred to colposcopy and those who may be managed with surveillance. Cytology represents an alternative triage approach within HPV-based screening strategies. When limited genotyping is available, DS is applied in HPV HR12-positive individuals, whereas those positive for HPV 16 or HPV 18 are typically referred directly to colposcopy.

* *Further management according to current guideline recommendations*

** *Major cytology abnormalities in cotesting requiring direct referral to colposcopy regardless of HPV status*

and highlights the practical value of DS in contemporary cervical cancer prevention strategies.

Biological basis and practical interpretation of dual-stain cytology

A major strength of DS lies in its biological foundation. Oncogenic HPV types exert their effect primarily through the viral proteins E6 and E7, which disrupt normal cell cycle control. One of the central consequences of E7 activity is functional inactivation of the retinoblastoma pathway, leading to upregulation of p16 as a compensatory response. At the same time, Ki-67 expression reflects ongoing cellular proliferation. The coexistence of these signals within a single epithelial cell therefore represents an abnormal biological state consistent with transformation rather than physiological cell cycle regulation [5, 6].

p16/Ki-67 dual-stain interpretation is based on a clear and standardized criterion. A test is classified as positive when at least one epithelial cell shows simultaneous expression of p16 and Ki-67, independent of cytomorphological appearance [9]. This approach improves reproducibility compared with cytology. However, interpretation still requires experience, particularly in cases with artefacts, low cellularity, inflammation, atrophy, or immature metaplasia. In routine cytopathological practice, certain patterns may present diagnostic uncertainty. Weak or focal p16 staining, scattered Ki-67 positivity in inflamed samples, and overlapping cell groups can complicate evaluation. In such cases, correlation with cytology, HPV status, and clinical context remains essential, as does assessment of the overall cellular and extracellular staining pattern. Careful verification that the red and brown signals are co-localized within the same microscopic plane of focus is also critical. Particular attention should be paid to immature metaplastic cells and reparative changes, which may show increased proliferative activity. Although true dual-positive staining requires clear co-expression within the same cell, borderline patterns may occasionally create diagnostic uncertainty. Awareness of these scenarios is essential to avoid overinterpretation. Representative examples of DS interpretation in primary HPV-based screening and cotesting are presented in Figure 2.

Interpretation of DS slides extends beyond identification of isolated positive cells or cell clusters and includes evaluation of staining quality, cellular preservation, and distribution of positive elements. True dual-positive cells typically display a characteristic pattern with strong cytoplasmic p16 expression and distinct nuclear Ki-67 staining. The criteria for identifying a dual-stained epithelial cell also encompass variability in staining intensity, ranging from weak to strong expression. The Ki-67 signal may appear as a red nuclear staining pattern that is either uniformly distributed or partial, including speckled or granular patterns. In DS slides, p16 expression is typically observed as a brown cytoplasmic signal, although nuclear staining may also be present. In true dual-positive epithelial cells, the signals corresponding to p16 and Ki-67 may overlap within the same cell, particularly in areas of strong expression. Care should be taken to distinguish these features from nonspecific background staining.

Reference cells include mature superficial and intermediate squamous epithelial cells lacking expression of p16 and/or Ki-67. These cells serve as an internal control for interpretation. Nonspecific background staining may be encountered as brown cytoplasmic coloration in a proportion of reference cells. In such cases, assessment of staining intensity becomes critical. The cytoplasmic p16 signal in a true dual-positive cell should be clearly stronger than the background staining observed in surrounding reference cells, allowing reliable identification of specific p16 expression. The key practical principles of DS interpretation are summarized in Figure 3.

From a practical perspective, the strength of DS lies not only in its biological basis but also in its operational simplicity, supported by interpretation algorithms that facilitate consistent and reproducible evaluation in routine practice. However, consistent application of this approach requires appropriate training and experience. In this regard, DS represents a method that is easier to standardize than cytology, yet still dependent on careful assessment of immunocytochemical signals. Although DS interpretation is generally more reproducible than cytology, it is not exempt from the learning curve, particularly in the assessment of subtle or borderline staining patterns. Interobserver variability may occur in cases with low cellularity or complex background. Consistent and reliable interpretation therefore requires dedicated training, awareness of potential pitfalls, and familiarity with characteristic staining patterns. Structured training and experience remain essential to ensure reproducible results in routine practice.

Practical considerations and quality assurance

Implementation of DS in routine practice requires an appropriate quality assurance framework. Although DS interpretation is less subjective than cytology, it may still be influenced by both technical factors and observer-dependent variability. Adequate training and diagnostic experience are therefore essential. Quality control should include correlation with histopathological findings, review of discordant cases, and monitoring of positivity rates, including in HPV-positive populations. Very low DS positivity rates may indicate undercalling, whereas excessively high DS positivity rates may be associated with reduced specificity.

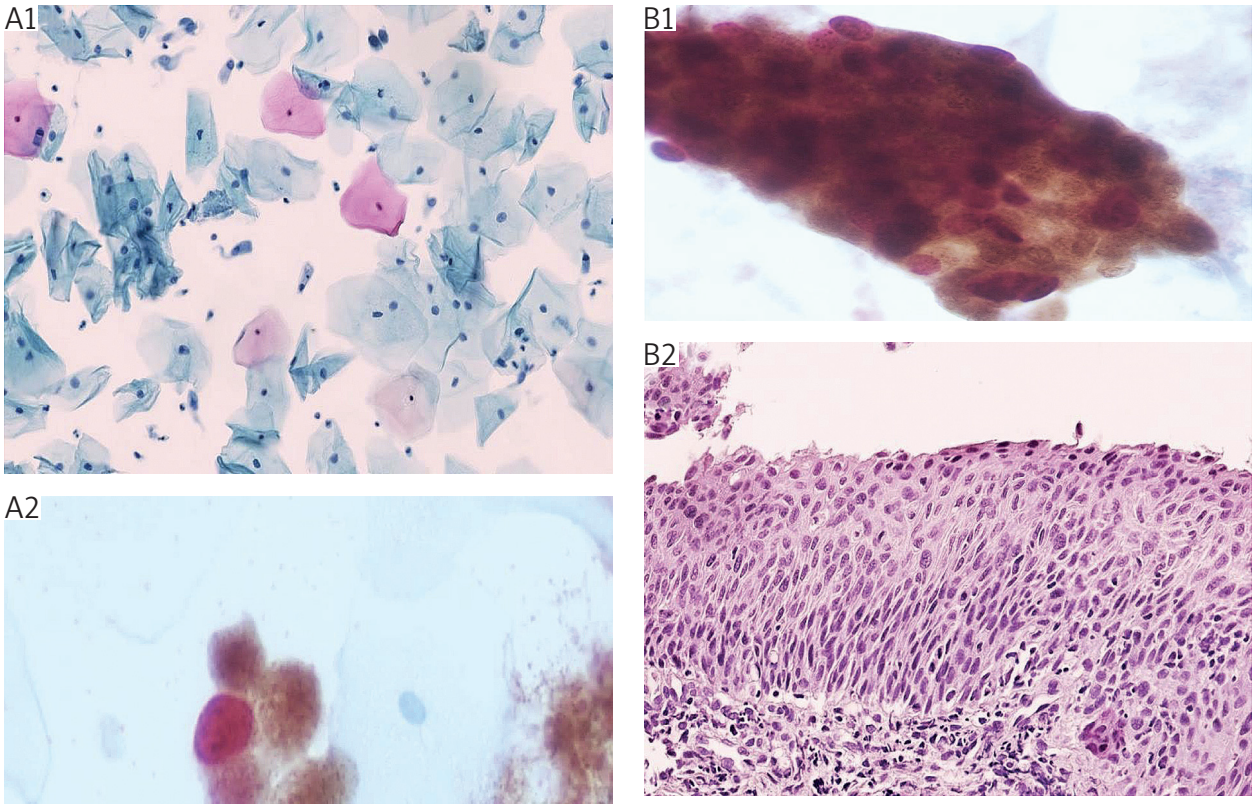

Figure 2. Representative cytopathological contexts illustrating the role of p16/Ki-67 dual-stain in HPV-based cervical cancer screening with histopathological correlation in HPV-positive women

Panel A (Primary cotesting). A1: negative for intraepithelial lesion or malignancy cytology (10×). A2 p16/Ki-67 dual-stain (DS) positive result with a single dual-positive epithelial cell identified within a small cell group; the very thin rim of p16-positive cytoplasm may be easily overlooked. Nonspecific background staining represents a potential diagnostic pitfall, particularly when it obscures cellular details (40×). The positive DS result does not alter the previous cytological diagnosis. A3: HSIL/CIN2 on colposcopic biopsy with koilocytosis in the superficial epithelial layers and mid-epithelial mitotic activity (HE, 20×); confirmed by diffuse block-type p16 expression (IHC p16, 20×).
Panel B (Primary HPV testing). B1: DS-positive result showing a typical cohesive epithelial cluster with strong, diffuse p16 expression; true dual-positive cells seen at the edge of the cluster should be carefully distinguished from overlapping Ki-67-positive red nuclei (40×). B2: HSIL/CIN3 on colposcopic biopsy (HE, 20×), no clinical and/or histomorphological indications for additional p16 testing. Images are representative and derived from routine diagnostic material and do not correspond to a single patient.

Integration of DS into structured screening programs, together with participation in external quality assurance systems, is recommended [13].

Clinical relevance in real-world screening practice

The clinical relevance of dual-stain cytology is increasingly supported by evidence from routine screening practice. While early investigations were predominantly conducted in controlled research settings, more recent data provide insight into the performance of DS in everyday clinical environments.

Observations from European cohorts, including studies conducted in Poland, indicate that DS maintains its diagnostic performance under such conditions. These findings also emphasize the importance of assessing DS across diverse healthcare settings, including opportunistic screening environments. In HPV-based screening with limited genotyping, DS consistently demonstrates higher sensitivity than cytology for detecting clinically relevant lesions. At the same time, it enables more precise identification of patients with clinically significant disease, particularly among women with HPV HR12 infections – a group in which management decisions remain challenging due to heterogeneous clinical profiles [24, 25]. Experience from routine clinical practice further shows

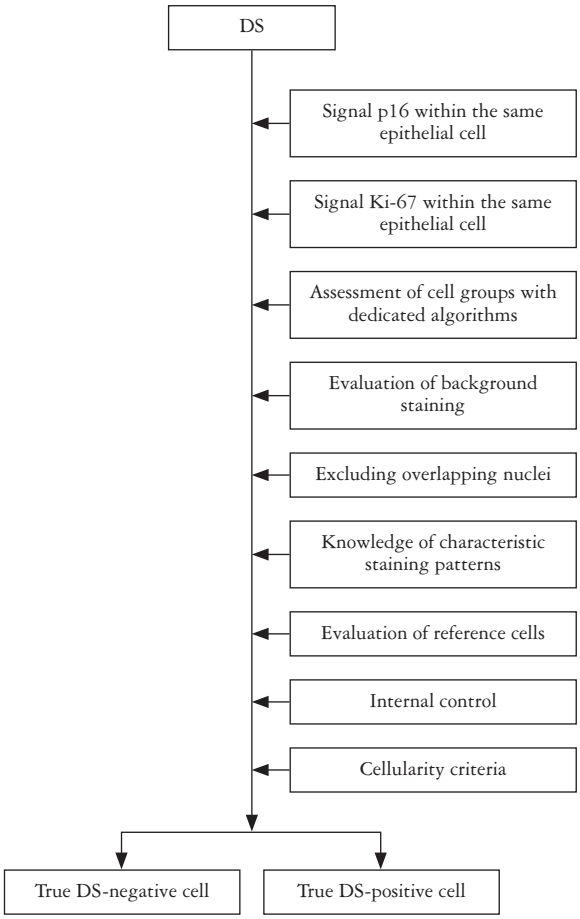

Figure 3. Practical principles of dual-stain cytology interpretation

A dual-stain positive cell is defined as an epithelial cell showing co-expression of p16 and Ki-67 within the same cell. Interpretation requires dedicated training to ensure accurate evaluation of specific immunocytochemical signals and to avoid diagnostic pitfalls related to nonspecific staining patterns.
DS – p16/Ki-67 dual-stain

that DS can be effectively incorporated into standard diagnostic workflows. When HPV HR12 are detected, additional triage tests are required to guide clinical management and avoid unnecessary procedures. In contrast, individuals positive for HPV16 or HPV18 are typically referred directly to colposcopy; however, emerging data, including findings from real-world analyses, indicate that the application of DS in HPV16/18-positive cases may identify a subset of individuals without evidence of transforming infection and thereby support more individualized clinical decision-making in selected scenarios [26]. The integration of DS into HPV-based screening pathways, including its established role in HPV HR12-positive cases and its potential application in HPV16/18-positive individuals, is illustrated in Figure 4.

The combined evaluation of HPV testing, cytology, and DS supports more individualized clinical decision-making and improves the alignment between test results and patient management. In this context, DS contributes to improved detection of cervical precancers and cancer while reducing colposcopy referrals. Data from women under 30 years of age suggest that DS may also have a role in populations traditionally managed with cytology-based screening. In this setting, primary HPV testing with DS triage was able to identify clinically relevant lesions, supporting a more biologically oriented approach to patient assessment [27].

The Polish experience is particularly informative, as it not only confirms findings from other settings but also demonstrates the applicability of DS in real clinical conditions. Early European adoption was reflected in clinical guidance introduced in 2020, supporting primary HPV-based screening with optional self-sampling in opportunistic settings, followed by the incorporation of DS into national interim pandemic guidelines issued jointly by professional gynecological and colposcopic societies in 2021 [28, 29].

The growing body of evidence from routine practice aligns with broader European perspectives on cervical cancer prevention, which emphasize the integration of validated biomarkers into contemporary screening pathways [13, 30]. Taken together, these findings indicate that the clinical utility of DS extends beyond controlled study settings and is maintained in real-world screening practice conditions.

Conclusions

p16/Ki-67 dual-stain immunocytochemistry represents an important development in HPV-based cervical cancer prevention. By identifying the cellular consequences of oncogenic HPV infection, DS bridges molecular detection and clinical decision-making. Compared with cytology, it provides higher sensitivity for clinically meaningful disease while maintaining acceptable specificity. Evidence from international studies and real-world clinical practice indicates that DS is an effective triage tool within current screening strategies. Its role is particularly relevant in HPV-positive populations with heterogeneous clinical profiles.

Automation and artificial intelligence have recently been explored in the evaluation of DS. Because DS identifies a distinct and biologically defined signal, it is well suited to computational image analysis. Early studies have demonstrated promising results for AI-assisted interpretation of DS, suggesting potential for improved standardization and scalability in large-scale screening settings [31].

As cervical cancer prevention continues to evolve, DS is likely to remain an important component of modern screening pathways. Its integration into clinical workflows reflects the broader transition toward biologically informed cervical cancer prevention.

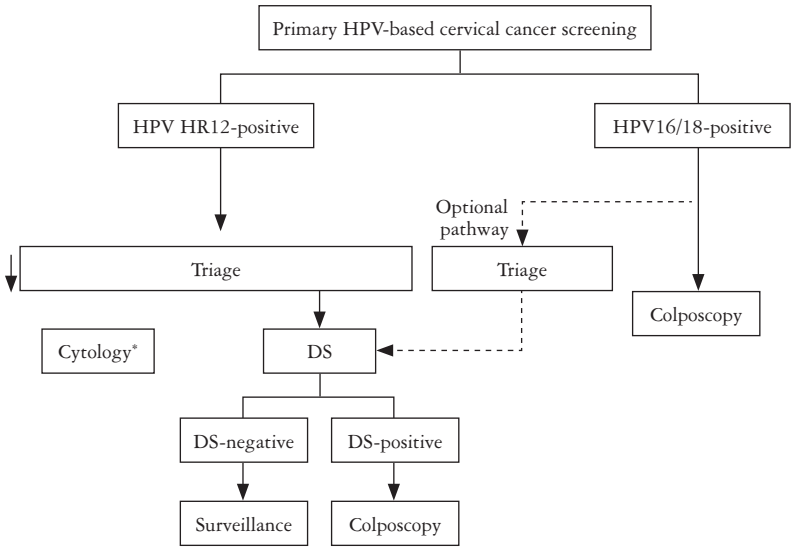

Figure 4. p16/Ki-67 dual-stain triage in primary HPV-based cervical cancer screening with genotyping: approach for HR12 and optional consideration for HPV16/18-positive women

For HPV HR12-positive individuals p16/Ki-67 dual-stain (DS) is used as a triage method to guide clinical management following a positive HPV test result. Individuals with a positive DS result are referred for colposcopy, whereas those with a negative DS result may be managed with surveillance. Individuals positive for HPV16 or HPV18 are typically referred directly for colposcopy. The dashed line indicates an optional triage pathway using DS in HPV16/18-positive cases, as suggested by emerging evidence.
DS – p16/Ki-67 dual-stain, DS-negative – negative dual-stain result, DS-positive – positive dual-stain result, HPV – human papillomavirus, HPV16/18 – human papillomavirus types 16 and 18, HPV HR12 – high-risk human papillomavirus types other than HPV16/18, HPV-negative – negative high-risk HPV test result, HPV-positive – positive high-risk HPV test result.
** Further management according to current guideline recommendations*

Disclosures

1. Institutional review board statement: Not applicable.
2. Assistance with the article: None.
3. Financial support and sponsorship: None.
4. Conflicts of interest: M.T. has given lectures sponsored by Hologic and reimbursement for travel and accommodation costs. R.J. has given a lecture sponsored by Roche.

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