ADVANCING THE DIFFERENTIAL DIAGNOSIS OF HSIL AND LSIL IN PERIMENOPAUSE: A REVIEW OF MODERN OBJECTIVE CRITERIA

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Abstract: Cervical cancer remains a significant global health burden, ranking as the fourth most common malignancy among women worldwide, with approximately 660,000 new cases and 350,000 deaths reported in 2022. Despite advances in vaccination and screening, the disease disproportionately affects low- and middle-income countries, where incidence and mortality rates remain alarmingly high. Persistent infection with high-risk human papillomavirus (HPV) types, predominantly HPV 16 and 18, drives cervical carcinogenesis through the oncogenic actions of E6 and E7 proteins, which inactivate tumor suppressors p53 and pRb. The premenopausal period represents a particularly vulnerable window, during which hormonal fluctuations, immunological changes, and increased HPV susceptibility converge to elevate the risk of cervical intraepithelial neoplasia progression. This review synthesizes recent evidence on innovative diagnostic approaches for precancerous cervical lesions, with emphasis on integrated morphological, immunohistochemical, and nanoscale analytical methods. We examine the diagnostic utility of dual p16/Ki-67 immunostaining as a triage strategy for HPV-positive women, highlighting its superior sensitivity and specificity compared to conventional cytology. Emerging technologies including atomic force microscopy, scanning electron microscopy, and elemental tissue analysis are discussed as complementary tools for characterizing the biophysical and biochemical landscape of cervical dysplasia. The review further considers the role of DNA methylation markers, artificial intelligence-assisted diagnostics, and the incorporation of these modalities into risk stratification algorithms. Special attention is devoted to the clinical implications of these innovations for women in the premenopausal age group, where early detection and individualized management are critical for cancer prevention and fertility preservation.

1. Introduction

Cervical cancer is a preventable disease that continues to exact a heavy toll on women's health globally. The World Health Organization estimates that in 2022, approximately 660,000 women were diagnosed with cervical cancer and 350,000 died from the disease, with over 90% of these deaths occurring in low- and middle-income countries (1,2). In Uzbekistan, cervical cancer ranks as the second most common malignancy among women, accounting for 12.4% of all female cancer cases, with a standardized incidence rate of 5.5 per 100,000 population (3). These figures underscore the urgent need for improved screening and early detection strategies that can be

implemented across diverse healthcare settings.

The natural history of cervical carcinogenesis provides a substantial window of opportunity for intervention. High-risk HPV infection is necessary for the development of cervical cancer, yet the majority of HPV infections are transient and resolve spontaneously (4). It is the persistent infection with oncogenic HPV types, particularly HPV 16 and HPV 18, that drives the progression from low-grade squamous intraepithelial lesions (LSIL) to high-grade squamous intraepithelial lesions (HSIL) and ultimately to invasive carcinoma (5). The E6 and E7 oncoproteins of high-risk HPV types mediate malignant transformation by degrading p53 and inactivating the retinoblastoma protein pRb, respectively, thereby disrupting cell cycle regulation, apoptosis, and DNA repair mechanisms (6,7).

The premenopausal period represents a particularly critical window for cervical carcinogenesis. Epidemiological data indicate that HSIL is diagnosed with disproportionate frequency in women under 35 years of age, and cervical cancer incidence rises steeply from the early reproductive years through the perimenopausal transition (8). Hormonal fluctuations characteristic of the premenopausal state, including alterations in estrogen and progesterone levels, may influence HPV persistence and lesion progression through modulation of the cervical immune microenvironment and epithelial differentiation patterns (9,10). Despite this elevated risk, screening participation often declines during the perimenopausal transition, creating a gap in preventive care (11).

Traditional screening methods including Papanicolaou cytology and HPV DNA testing have limitations in sensitivity and specificity, particularly in premenopausal women where inflammatory changes and reactive cellular alterations can mimic dysplastic features (12,13). Liquid-based cytology has improved specimen adequacy and cellular preservation, but its diagnostic accuracy for high-grade lesions remains suboptimal (14). Colposcopy with biopsy serves as the gold standard for diagnosis, yet its accuracy is operator-dependent and the procedure is resource-intensive (15).

Recent advances in molecular pathology and high-resolution imaging have expanded the diagnostic armamentarium for cervical precancer detection. Dual immunostaining for p16INK4a and Ki-67 has emerged as a highly effective triage tool for HPV-positive women, demonstrating superior sensitivity and specificity compared to reflex cytology (16,17). The simultaneous detection of p16INK4a, a surrogate marker of HPV E7-mediated pRb inactivation, and Ki-67, a proliferation-associated nuclear antigen, in the same cell indicates cell cycle dysregulation and identifies transforming HPV infections with high precision (Figure 1).

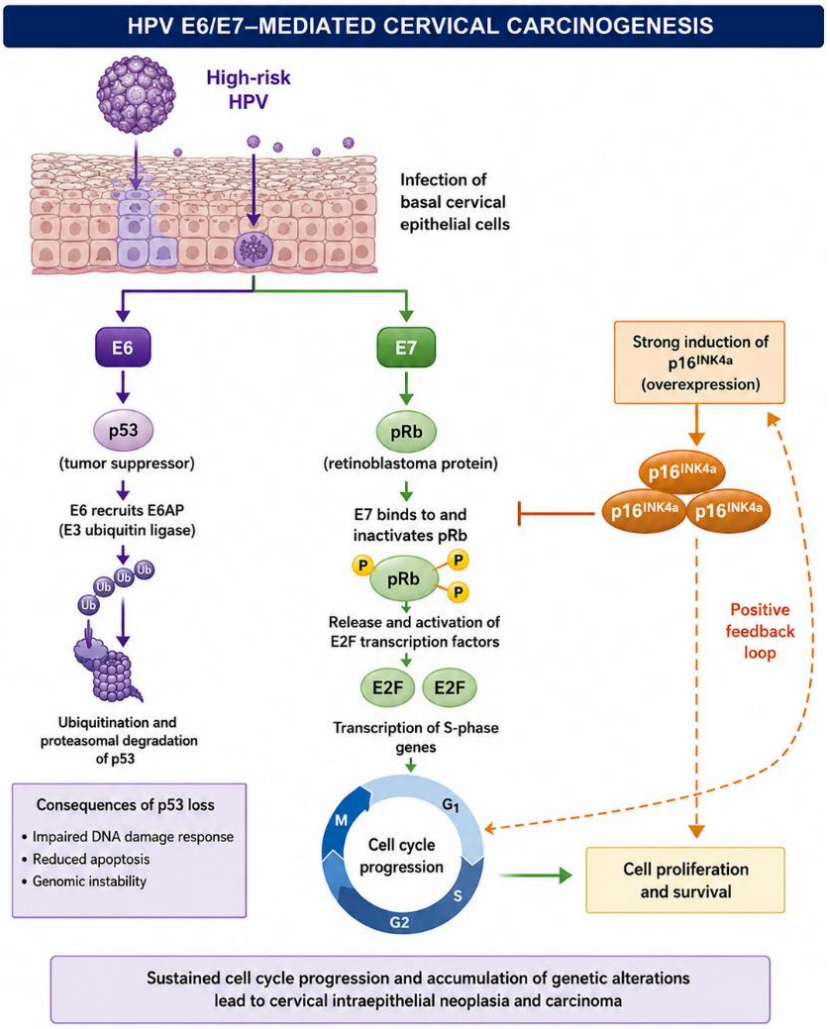

Figure 1. HPV E6/E7-mediated cervical carcinogenesis. High-risk HPV E6 promotes p53 degradation, impairing DNA damage response and apoptosis, while E7 inactivates pRb, releasing E2F transcription factors and driving p16INK4a overexpression and sustained cell cycle progression.

Beyond immunohistochemical biomarkers, nanoscale analytical techniques including atomic force microscopy (AFM) and scanning electron microscopy (SEM) have opened new frontiers in the characterization of cervical epithelial ultrastructure (18). These methods enable the visualization of surface topography, cytoskeletal organization, and extracellular matrix remodeling at resolutions unattainable by conventional light microscopy. Elemental microanalysis of cervical tissue has further revealed alterations in the concentration of oxygen, phosphorus, sulfur, and calcium that correlate with neoplastic progression (19).

Cervical cancer develops through a well-characterized sequence of precursor lesions classified according to the proportion of epithelial thickness occupied by dysplastic cells. The current World Health Organization classification and the Lower Anogenital Squamous Terminology standardization project have harmonized the

reporting of these lesions, adopting the Bethesda System terminology of LSIL, which encompasses CIN grade 1, and HSIL, which encompasses CIN grades 2 and 3 (20,21). This unified reporting system facilitates clear communication between cytopathologists, histopathologists, and clinicians.

The molecular basis of HPV-driven carcinogenesis centers on the sustained expression of the viral E6 and E7 oncoproteins. E6 promotes the ubiquitin-mediated degradation of p53, abrogating the G1/S cell cycle checkpoint and impairing apoptotic responses to DNA damage (22). E7 binds to and degrades pRb, leading to the release of E2F transcription factors and the constitutive activation of genes required for S-phase entry (23). The resulting proliferative signal induces cell cycle dysregulation detectable through the overexpression of p16INK4a, a cyclin-dependent kinase inhibitor transcriptionally activated through a negative feedback loop in response to pRb inactivation (Figure 2) (24).

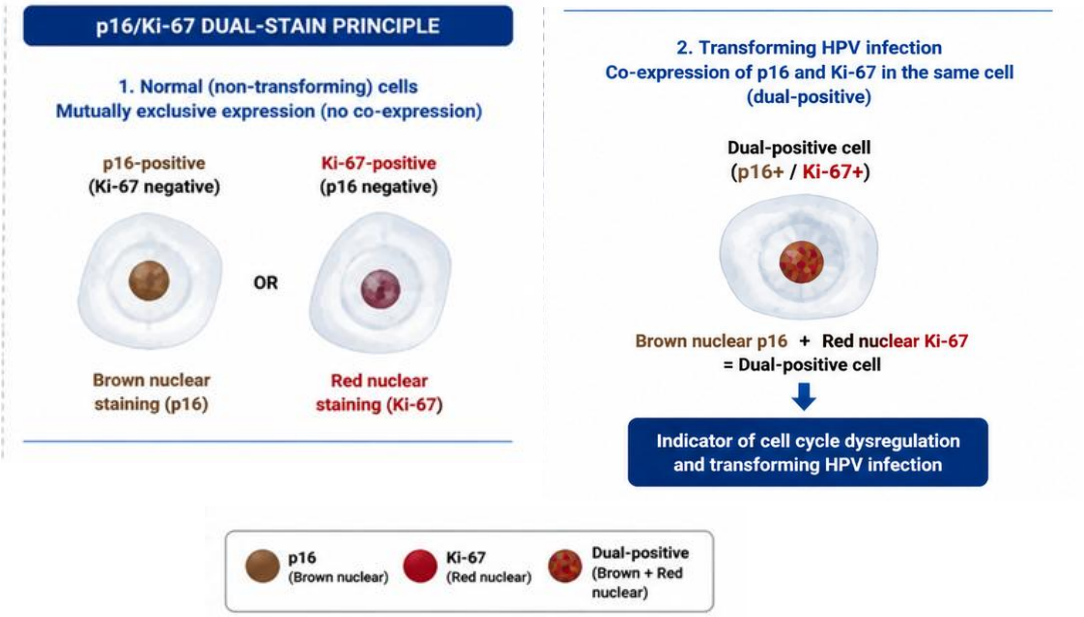

Figure 2. The p16/Ki-67 dual-stain principle. In normal (non-transforming) cervical epithelium, p16 and Ki-67 expression are mutually exclusive -- a cell stains positive for either p16 (brown nuclear) or Ki-67 (red nuclear), but not both. In transforming HPV infection, cell cycle dysregulation leads to co-expression of both markers within the same nucleus (dual-positive, p16+/Ki-67+), which serves as a specific indicator of transforming HPV infection and identifies cells at risk of progression to high-grade lesions.

High-risk HPV types, most notably HPV 16 and HPV 18, are responsible for approximately 70% of all cervical cancers worldwide (4). Additional high-risk types including HPV 31, 33, 35, 45, 52, and 58 contribute to the remaining cases, with

regional variations in genotype distribution that influence screening and vaccination strategies (25). Viral load, physical state of the viral genome (episomal versus integrated), and host immune factors collectively determine the likelihood of progression from infection to high-grade neoplasia (26).

LSIL typically reflects a productive, often transient HPV infection characterized by viral replication in differentiated epithelial cells. Kollocytosis, the cytopathic hallmark of HPV infection, is most prominent in LSIL and reflects the accumulation of virions in the superficial epithelial layers (27). In contrast, HSIL represents a transforming infection in which viral oncogene expression drives abnormal proliferation throughout a greater thickness of the epithelium. The probability of regression decreases with increasing lesion grade: LSIL regresses in approximately 60% of cases, whereas the regression rate for CIN 2 is approximately 40%, and spontaneous regression of CIN 3 is uncommon (28,29). Figure 2 illustrates the progressive morphological and molecular alterations across the spectrum of cervical carcinogenesis.

The premenopausal hormonal milieu modulates several aspects of HPV infection and lesion progression. Estrogen receptor expression in cervical epithelium varies with the menstrual cycle and menopausal transition, and estrogen has been shown to promote HPV transcription and viral persistence through interaction with HPV upstream regulatory regions (30). Progesterone and its receptors also influence the cervical immune environment, potentially affecting the clearance of HPV-infected cells (31). These hormonal interactions are particularly relevant to the design of screening protocols for premenopausal women, as both false-positive and false-negative results may be influenced by hormonal status at the time of sampling (9).

p16/Ki-67 Dual Immunostaining: A Cornerstone of Modern Triage

The p16INK4a/Ki-67 dual-stain assay has emerged as one of the most clinically impactful biomarkers in cervical cancer screening. p16INK4a is overexpressed in cells with HPV-mediated pRb inactivation, serving as a surrogate marker for transforming HPV infection (16). Ki-67 is expressed in proliferating cells throughout all active phases of the cell cycle. Because p16INK4a and Ki-67 are mutually exclusive in normal cervical epithelium due to the negative feedback between cell cycle arrest and proliferation, their co-expression in the same cell provides a highly specific indicator of cell cycle dysregulation induced by HPV oncoproteins (32). The dual-stain principle, whereby a dual-positive cell signals cell cycle dysregulation, while single-positive or double-negative cells reflect normal biology, is illustrated schematically in Figure 1.

The clinical performance of the dual-stain assay has been extensively validated. A landmark study involving over 30,000 screening tests demonstrated that p16/Ki-67 triage of HPV-positive women achieved significantly higher specificity than reflex

cytology for detecting CIN grade 2 or worse, with comparable sensitivity (33). In HPV 16/18-positive women, dual-stain triage yielded a specificity of 53.1% versus 16.8% for cytology, while maintaining a sensitivity of 95.7% for CIN2+. The negative predictive value for CIN3+ was 100% in HPV 16/18-positive women with negative dual-stain results, indicating that colposcopy could be safely deferred in this group (33).

Harper et al. confirmed that the dual-stain test identifies oncogenic transformation with higher sensitivity than cytology using the same samples collected for HPV testing and liquid-based cytology (34). The test received US Food and Drug Administration approval for triage of

HPV-positive women and has been incorporated into the American Society for Colposcopy and Cervical Pathology risk-based management guidelines (35).

The 2024 Enduring Consensus Guidelines recommend colposcopy for dual-stain-positive individuals and 12-month HPV-based follow-up for those testing negative, with the exception of HPV 16/18-positive or high-grade cytology results, where immediate colposcopy is still advised (35).

Evidence from the IMPACT clinical validation trial demonstrated that incorporation of dual-stain testing into screening algorithms projected to reduce unnecessary colposcopy referrals while maintaining detection of clinically significant lesions (36). The economic modeling showed favorable incremental cost-effectiveness ratios when dual-stain reflex was added to HPV and cytology co-testing strategies (36). A meta-analysis by Ouh et al. reported a pooled sensitivity of 87.7% and specificity of 76.7% for detecting CIN2+, with corresponding values of 89.7% and 79.6% for CIN3+ (16).

Mazurec et al. demonstrated that dual-stain positivity was significantly higher in HSIL compared to LSIL and negative histology, with no significant difference between negative and LSIL groups, confirming the test's ability to identify clinically relevant disease (37). The distribution of HPV genotypes across diagnostic categories revealed the predominant role of HPV 16 in high-grade lesions, reinforcing the importance of genotyping in risk stratification (37).

Zhou et al. evaluated screening strategies in 6,085 premenopausal and 4,766 postmenopausal women, finding that HPV testing combined with cytology achieved high sensitivity for CIN2+ in both groups, but the dual-stain approach offered particular advantages in premenopausal women where the higher prevalence of inflammatory changes can confound cytologic interpretation (9). Luo et al. demonstrated that p16/Ki-67 immunoscore combined with PAX1 and ZNF582 methylation status improved risk stratification in HPV-positive women (29). Liu et al. found that p16 paired with minichromosome maintenance protein 2 achieved the highest Youden index among single-marker methods, and when combined with liquid-based cytology, yielded 96.3% sensitivity for HSIL detection with no missed cancers (38).

4. Immunohistochemical Profiling: Beyond p16/Ki-67

The p53 tumor suppressor protein plays a central role in the cellular response to genotoxic stress, mediating cell cycle arrest, DNA repair, and apoptosis. In HPV-associated cervical carcinogenesis, the E6 oncoprotein targets p53 for ubiquitin-mediated degradation, resulting in loss of p53 function without mutation of the TP53 gene (22). As lesions progress to HSIL, p53 expression may increase, indicating either stabilization of wild-type p53 in response to oncogenic stress or, in a subset of HPV-independent lesions, TP53 mutation with consequent protein accumulation (39,40). Increased nuclear p53 expression has been associated with higher CIN grade and greater risk of progression, although interpretation requires integration with p16 status to distinguish HPV-associated from HPV-independent disease (40,41).

The proliferation marker Ki-67, when assessed as a continuous variable rather than dichotomized in dual-stain protocols, provides quantitative information about the extent of cell cycle activation (42). Ki-67 labeling index increases progressively from normal epithelium through LSIL to HSIL, reflecting the expansion of the proliferative compartment. In normal cervical epithelium, Ki-67 expression is confined to the basal and parabasal layers. In LSIL, positive cells extend into the lower one-third of the epithelium, while in HSIL, Ki-67 positivity may involve the full epithelial thickness (Figure 2) (43). Quantitative assessment has been shown to distinguish HSIL from mimics including immature squamous metaplasia and reparative changes with high accuracy (42,43).

Histologic grading of cervical intraepithelial neoplasia remains subject to interobserver variability, particularly at the CIN 2 threshold (44). Immunohistochemistry with p16 and Ki-67 reduces this variability and improves diagnostic reproducibility. Triple-marker panels incorporating p16, Ki-67, and p53 have been proposed as comprehensive tools for cervical precancer characterization (45). Combined assessment demonstrates progressive increases in staining intensity and distribution corresponding to lesion severity: p16 expression shifts from negative or focal in LSIL to diffuse full-thickness positivity in HSIL, while Ki-67 labeling increases from less than 10% in LSIL to involvement of the full epithelial thickness in CIN 3. The integration of these markers into a composite scoring system may enhance the accuracy of histologic grading and improve the prediction of lesion behavior (45).

5. Nanoscale Imaging: Atomic Force and Scanning Electron Microscopy in Cervical Precancer Diagnosis

AFM and SEM provide spatial resolution at the nanometer scale, enabling the visualization of surface topography, cytoskeletal architecture, and extracellular matrix organization not discernible by conventional light microscopy (18). AFM operates by raster-scanning a sharp probe across the tissue surface while measuring interaction

forces, generating three-dimensional topographic maps with vertical resolution on the order of angstroms (46). In cervical tissue, AFM has revealed progressive alterations in surface morphology correlating with the grade of intraepithelial neoplasia. Normal cervical epithelium exhibits regular surface architecture with well-defined cellular boundaries. LSIL samples show surface irregularities with focal loss of intercellular junctions. HSIL specimens demonstrate marked topographic disorganization, loss of regular cell borders, and pronounced nuclear enlargement (47,48).

Beyond topographic imaging, AFM quantifies mechanical properties through force spectroscopy. The Young's modulus of cervical epithelial cells decreases with neoplastic progression, reflecting cytoskeletal reorganization that accompanies malignant transformation (46). This mechanical softening has been attributed to reorganization of the actin cytoskeleton, reduced cell-cell adhesion, and changes in nuclear stiffness (48,49).

Proietti et al. combined AFM with SEM and Raman spectroscopy to characterize cervical squamous cell carcinoma, demonstrating the complementary nature of these techniques (49). SEM revealed the loss of regular microvilli, disruption of intercellular bridges, and exposure of the underlying basement membrane in areas of high-grade dysplasia (49). Ozbey and Aytac developed a multimodal classification approach utilizing both SEM and AFM images for early cervical cancer detection, achieving a classification accuracy of 95.9%, substantially outperforming single-modality approaches (18).

Feng et al. developed an adaptive harmonic AFM probe capable of generating stable signals in both atmospheric and liquid environments, enabling high-resolution cellular imaging of cervical epithelial cells and cancer cell lines with a 52.17% improvement in amplitude response sensitivity and 60% enhancement in imaging clarity (50). O'Dowling et al. reviewed the potential of machine learning to translate AFM-based biomarkers into clinical diagnostic tools, highlighting that the combination of nanoscale morphologic and biomechanical features with machine learning classification holds promise for automated screening platforms (51).

6. Elemental Microanalysis of Cervical Tissue

Elemental composition of cervical tissue undergoes characteristic alterations during neoplastic progression detectable by energy-dispersive X-ray spectroscopy. These changes reflect metabolic reprogramming, including alterations in oxygen metabolism, phosphate metabolism, and sulfur-containing compound synthesis (19,52). Oxygen content increases progressively from normal cervical epithelium through increasing grades of dysplasia, likely reflecting mitochondrial proliferation associated with the Warburg effect (19). Phosphorus levels also increase with lesion severity, correlating with increased nucleic acid synthesis and membrane phospholipid

turnover (52).

Calcium and sulfur concentrations show more complex patterns. Intracellular calcium plays critical roles in cell signaling, differentiation, and apoptosis, and its dysregulation has been implicated in HPV-mediated carcinogenesis (53). Calcium levels increase in LSIL compared to normal epithelium but may decrease in HSIL and invasive cancer, potentially reflecting disruption of calcium-dependent apoptotic pathways (19,53). Sulfur, incorporated into proteins through cysteine and methionine, shows increased levels in dysplastic epithelium correlating with the degree of cellular proliferation (52).

Iron content in cervical tissue has been investigated as a potential biomarker, with elevated iron levels reported in dysplastic and malignant cervical epithelium compared to normal tissue (54). This increase may reflect enhanced iron uptake by proliferating cells to support DNA synthesis and mitochondrial respiration. The combination of elemental profiling with morphological and immunohistochemical assessment provides multidimensional characterization of cervical precancer that may improve diagnostic accuracy (49).

7. Emerging Biomarkers and Artificial Intelligence in Cervical Cancer Screening

DNA methylation markers have emerged as promising triage tools offering high specificity for high-grade lesion detection (55). The PAX1 and JAM3 methylation test, evaluated in a prospective Chinese cohort of 4,394 HPV-positive women, demonstrated a specificity of 89.6% for detecting CIN3+, substantially higher than HPV 16/18 genotyping (66.4%) and cytology (23.9%), with an odds ratio of 18.5 for CIN3+ detection (56). The FAM19A4/miR124-2 methylation panel has been extensively validated and included in European screening guidelines as an alternative triage strategy (55). The WID-qCIN test, analyzing methylation of DPP6, RALYL, and GSX1 genes, achieved 93.4% sensitivity for CIN3 detection when combined with HPV 16/18 genotyping in a large Swedish prospective study (57).

MicroRNAs represent another emerging class of biomarkers. Circulating and cervical microRNAs have been investigated as minimally invasive markers for lesion detection and risk stratification (58). Studies have identified panels of microRNAs that distinguish HSIL from LSIL and normal cytology with high accuracy, and microRNA expression profiles may provide information about the likelihood of lesion regression or progression (59). Liquid biopsy approaches, including analysis of circulating tumor DNA and cell-free RNA from blood samples, offer the potential for non-invasive screening (60).

An et al. developed a deep learning-based model for recognition of cervical SILs achieving an accuracy of 91.4% for HSIL detection, using p16 immunohistochemistry to guide the training process (27). The model achieved a sensitivity of 92.2% and

specificity of 82.9% for HSIL at the patch level (27). Chang et al. evaluated artificial intelligence-assisted colposcopy in a multicenter study of 825 women and found that it achieved 100% sensitivity, outperforming conventional colposcopy (85.9%) at the cost of lower specificity (15.8% versus 57.7%) (15). Figure 3 presents an integrated diagnostic algorithm incorporating these emerging technologies.

8. Clinical Implications and Risk Stratification in Premenopausal Women

Premenopausal women face distinct challenges in cervical cancer prevention, including higher rates of transient HPV infection, greater likelihood of regression of low-grade lesions, and the imperative to avoid overtreatment that could compromise future fertility (9,61). The risk-based management framework developed by the American Society for Colposcopy and Cervical Pathology provides clinical action thresholds for colposcopy referral based on the estimated risk of CIN3+ (35). Evidence from the Kaiser Permanente Northern California cohort and the STudying Risk to Improve DisparitiES study confirmed that risk estimates for p16/Ki-67 dual-stain testing are portable across diverse populations (35).

HPV E6/E7 mRNA testing represents a promising primary screening modality for premenopausal women. Liu et al. evaluated the Aptima HPV assay in 16,917 women and demonstrated that in premenopausal women, the assay achieved a sensitivity of 93.5% and negative predictive value of 97.3% for LSIL+ detection, significantly outperforming its performance in postmenopausal women (12). The integrated screening algorithm shown in Figure 3 incorporates HPV testing, biomarker triage, colposcopy, and risk-stratified management tailored to the premenopausal population.

Li et al. reviewed biomarkers differentiating regression from progression in untreated CIN 2 lesions, identifying HPV genotype, viral methylation status, p16/Ki-67 status, host gene methylation, and immune microenvironment characteristics as promising predictors (28). The integration of these biomarkers into clinical decision algorithms enables a nuanced approach, reducing overtreatment in women whose lesions are likely to regress while ensuring timely intervention for those at highest risk of progression (28).

9. Conclusions and Future Perspectives

The convergence of molecular pathology, nanoscale imaging, and computational analysis is transforming the diagnostic approach to cervical precancer in premenopausal women. The p16/Ki-67 dual-stain assay has emerged as a validated triage tool incorporated into international screening guidelines that reduces unnecessary colposcopies while maintaining high sensitivity for high-grade lesions. AFM and SEM have opened new dimensions in the structural and biomechanical characterization of cervical epithelium, revealing nanoscale alterations that precede neoplastic progression. The integration of these imaging modalities with machine

learning analysis promises automated, objective classification of cervical lesions. Elemental microanalysis identifies characteristic metabolic alterations associated with carcinogenesis, offering a complementary dimension to morphological and immunohistochemical assessment.

Future research should focus on prospective validation of integrated diagnostic algorithms in diverse populations, including premenopausal women from low- and middle-income countries where the burden of cervical cancer is highest. The development of point-of-care platforms combining biomarker testing, high-resolution imaging, and artificial intelligence interpretation could dramatically expand access to advanced diagnostic capabilities in resource-limited settings. For premenopausal women, precision diagnostics that accurately identify lesions requiring intervention while allowing safe surveillance of those likely to regress offer the promise of cancer prevention without unnecessary compromise of fertility and quality of life.

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