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Evaluating *PAX1* methylation for cervical cancer screening triage in non-16/18 hrHPV-positive women

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Abstract

Background In China, the national cervical cancer screening protocol involves initial testing for high-risk human papillomavirus (hrHPV), followed by cytology for hrHPV-positive cases. This study evaluates the effectiveness of *PAX1* methylation (*PAX1*^{m}) analysis in identifying precancerous or cancerous lesions in cervical samples from Chinese women positive for non-16/18 hrHPV strains.

Methods Between February 2022 and March 2023, 281 cervical samples from non-16/18 hrHPV-positive women underwent cytological examination and *PAX1*^{m} analysis. The study assessed the statistical relationship between *PAX1*^{m} levels and the presence of cervical lesions, comparing the diagnostic performance of *PAX1*^{m} to conventional cytology.

Results A significant association was found between *PAX1* methylation levels and the risk of CIN2 + and CIN3 + lesions, with 47 instances of CIN2 + detected. Odds ratios (ORs) for moderate and high *PAX1*^{m} levels were 8.86 (95% CI: 2.24–42.17) and 166.32 (95% CI: 47.09-784.97), respectively. The area under the ROC curve for *PAX1*^{m} in identifying CIN2 + lesions was 0.948 (95% CI: 0.895–0.99). *PAX1*^{m} demonstrated similar sensitivity and negative predictive value (NPV) to cytology but reduced the colposcopy referral rate from 47.7% with cytology alone to 25.6% with *PAX1*^{m}, showing superior specificity and positive predictive value across age groups.

Conclusions *PAX1* methylation is a strong indicator of CIN2 + and CIN3 + risk, offering diagnostic performance comparable to cytology with the added benefit of reduced unnecessary colposcopy referrals. These findings support the use of *PAX1*^{m} analysis as a reliable tool for triaging non-16/18 hrHPV-positive women in outpatient settings.

Keywords Cervical cancer, hrHPV, PAX1 methylation, CIN, Colposcopy referral

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Introduction

Cervical cancer represents a significant public health challenge worldwide, with persistent human papillomavirus (HPV) infection identified as a key etiological factor. In 2022, China witnessed 150,659 new cervical cancer cases and 55,694 related deaths [1], reflecting its status as a major concern within the national public health agenda. These figures underscore the pressing need for effective screening and triage strategies to manage the burden of this disease. Given China's population comprises approximately 17.7% of the global total, the proportion of cervical cancer cases and mortalities closely aligns with its demographic footprint, highlighting the disease's global relevance and the importance of targeted prevention efforts. A multicenter randomized controlled clinical study in China showed that HPV testing outperformed cytological examination in detecting a greater number of high-grade squamous intraepithelial lesions (CIN2+), underscoring the effectiveness of an HPV-based primary screening approach for cervical cancer [2]. Consequently, the 'Chinese Cervical Cancer Screening Guidelines (Part I)' now expressly advocate for hrHPV testing as the primary screening method for cervical cancer in China [3].

While 90% of high-risk HPV (hrHPV) infections naturally resolve within two years, a positive HPV test can still lead to significant patient anxiety, unnecessary follow-ups, colposcopies, and the risk of overtreatment. [4]. At present, cervical cytology following a positive HPV test is the standard for triaging women. Yet, the low specificity of this method, starting from the ASC-US + threshold, leads to an elevated rate of colposcopy referrals [5]. Cytology is also highly subjective given that it is based on morphological examination, contributing to findings that suffer from poor reproducibility [6]. For cytology findings categorized as ASC-US, the subsequent risk of a CIN2 + diagnosis in cervical biopsies is below 10%, with the risk of progressing to invasive cancer being notably low (between 0.1% and 0.2%) [7]. In January 2023, the National Health Commission and 10 other departments jointly issued the "Action Plan to Accelerate the Elimination of Cervical Cancer (2023–2030)," urging for the expansion of free cervical cancer screening coverage. However, cytological examination requires the services of specialized cytopathologists with appropriate professional qualifications, adversely affecting the expansion of screening in China.

The methylation of host tumor suppressor genes (TSGs) is a common epigenetic finding associated with cervical cancer in patients persistently infected with HPV [8–10]. High levels of CpG island methylation within the promoter regions upstream of TSGs often result in the silencing of these genes, thereby modulating the odds of cancer onset and progression [11]. Many studies have observed increased methylation of the PAX1 gene in precancerous lesions and invasive cervical cancer cases, suggesting that the methylation status of this gene may offer utility as a biomarker for the early diagnosis of this cancer type [12–14]. $PAX1^m$ can also predict cervical lesion progression [15, 16], and has been shown to exhibit favorable clinical performance when used for the triage of hrHPV-positive cases, with a sensitivity level of ~ 85% and a specificity level of ~ 80% [17–19]. At present, however, there remains a lack of comparative analyses of the use of $PAX1^m$ and cytology tests in an outpatient setting.

The present study was conducted as an evaluation of the clinical performance of $PAX1^m$ relative to cytology for women positive for non-16/18 hrHPV in a hospital outpatient setting. The aim of these analyses is to provide an evidence-based foundation in support of the potential replacement of cytology with $PAX1^m$ as a triage tool.

Materials and methods

Study design and population

This cross-sectional analysis was conducted in a hospital outpatient clinic and was designed to assess the utility of $PAX1^m$ testing analyses performed using exfoliated cervical cells from non-16/18 hrHPV-positive women. Between February 2022 and March 2023, all non-16/18 hrHPV-positive participants in this study underwent cytologic examination and were referred for colposcopy, with the residual cytological specimens then being used to test for *PAX1* methylation status. The Trial Ethics Committee of Zhuzhou Hospital Affiliated to Xiangya School of Medicine, Central South University approved this study (No: ZD2022001-01), and all participants provided written informed consent.

For cases where cytological examination results were classified as "Negative for Intraepithelial Lesion or Malignancy" (NILM) and colposcopy revealed normal findings, the pathological findings for these samples were classified as cervicitis. Any patients exhibiting abnormal cytology or colposcopy findings who failed to undergo biopsy were excluded from this study. Pathological results were used to determine the diagnostic performance of $PAX1^m$ testing and cytology.

To be eligible for study inclusion, subjects had to be women from among the outpatient population who were positive for non-16/18 hrHPV. Inclusion criteria were (1) non-16/18 hrHPV positive and (2) 18 years of age or older. Women were excluded if they were: (1) pregnant, (2) not willing to participate, (3) lacking sufficient cytology specimens, (4), or (5) declined to undergo colposcopy-biopsy when colposcopy was indicated. The criteria of exclusion were (1) previous diagnosis of ICC, (2) diagnosed with other genital tract tumors or after LEEP or CKC, (3) current pregnancy, (4) insufficient material for cytology and methylation analysis, (5) declined

to undergo colposcopy-biopsy when colposcopy was indicated.

High-risk HPV genotyping

In total, hrHPV DNA genotyping was performed for 21 HPV genotypes, including 14 hrHPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), 1 suspected hrHPV genotype (HPV53), and 5 low-risk genotypes (HPV6, 11, 42, 43, 44, and 81). Genotyping was performed with the HPV gene array detection kit (HybriBio Ltd, Guangzhou, China).

Cytology and histology

Gynecologists harvested exfoliated cervical cells from patients for cytological evaluation. Cytological analyses were performed via liquid-based cytology (LBC) tests, as per the ThinPrep Papanicolaou test and imaging system manual (Hologic, Inc., MA, USA). LBC results were categorized by trained cytologists in accordance with the 2014 Bethesda system guidelines.

Biopsy specimens were stained with hematoxylin and eosin (H&E), after which slides were initially reviewed by a pathologist, followed by an independent secondary review by another pathologist. In cases where these first two evaluations were inconsistent, a third pathologist was consulted to make a final determination.

Quantitative methylation-specific PCR (qMSP) analysis of PAX1 methylation

The remaining cytological specimens were sent to the pathology department, where DNA was extracted and treated with bisulfite using the DNA Methylation Pretreatment Kit (Hoomya, Changsha, China) as instructed. The resultant bisulfite-converted DNA (B-DNA) was used as a template for *PAX1* gene methylation analyses that were performed with the *PAX1* Methylation kit (Hoomya, Changsha, China) and a real-time PCR instrument (LC4800 II).

Collagen type II alpha 1 chain (*COL2A1*) served as a reference to confirm that bisulfite conversion was successful, evaluate sample quality, and enable normalization. The ΔCp value was used to measure the degree of $PAX1^m$, where $\Delta Cp_{PAX1} = Cp_{PAX1} - Cp_{COL2A1}$. Smaller ΔCp values were considered indicative of a higher degree of $PAX1^m$, whereas larger ΔCp values were indicative of lower methylation levels.

Statistical analysis

R v 4.3.1 was used for all statistical analyses, using packages including pROC, gmodels, DescTools, and DTComPair. Clinical characteristics were summarized using descriptive statistics. The performance of *PAX1* methylation as a tool to detect CIN2+and CIN3+lesions was assessed using receiver operating characteristic (ROC) curves, establishing the two most optimal cut-off values based on maximum Youden index values. The degree of $PAX1^m$ (ΔCp) was categorized into three levels: low (ΔCp>15), medium (7.2<ΔCp≤15.0), and high (ΔCp≤7.2) using the cut-off values for CIN3+and ΔCp=15. Fisher's exact test was used to calculate odds ratios (ORs) when evaluating associations, while trends were analyzed using the Cochran-Armitage test. A $PAX1^m$ cut-off value of ΔCp=10.1 was established for the triage of non-16/18 hrHPV positive women. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the detection of CIN2+and CIN3+were calculated with corresponding 95% Wilson score confidence intervals (95% CIs). The Clopper-Pearson method was used to compute the 95% CI for the colposcopy referral proportion. The McNemar test was employed to assess differences in sensitivity and specificity, while relative predictive values were used to explore differences in PPV and NPV. The clinical performance of triage strategies was also assessed by stratifying patients into age groups. A two-sided $P<0.05$ was used to assess significant difference.

Results

Case series

This study enrolled 558 total subjects positive for non-16/18 high-risk HPV, with these participants being infected with types including HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV53, HPV56, HPV58, HPV59, HPV66, and HPV68. Of these cases, 227 were excluded, while 281 cases were retained for statistical analyses (Fig. 1). The age of participants spanned from 19 to 75 years, with a median age of 42. The median $PAX1^m$ level was 19.2 (IQR: 9.7–21.0). The proportions of cytological and histological results are presented in Table 1. Cytology results included ASC-US in 31.7% of cases ($n=89$), LSIL in 11.4% of cases ($n=32$), ASC-H in 3.2% of cases ($n=9$), and HSIL in 1.4% of cases ($n=4$). In this patient group, cervicitis was diagnosed in 208 individuals (74.0%), followed by 26 cases (9.3%) of CIN1, 23 cases (8.2%) of CIN2, 21 cases (7.4%) of CIN3, and 3 cases (1.1%) of cervical cancer. Overall, 47 patients (16.7%) had a diagnosis of CIN2 or more severe.

The relationship between the methylation of *PAX1* and high-grade cervical lesions

$PAX1^m$ distributions among cytological results and pathological results are presented in Fig. 2A and B. $PAX1^m$ was separated into three levels based on the degree of methylation: low [ΔCp>15.0] ($n=188$), moderate [7.2<ΔCp≤15.0] ($n=49$), and high [ΔCp≤7.2] ($n=44$). Cytology results were similarly separated into three groups, including NILM ($n=147$), ASC-US ($n=89$), and LSIL+ ($n=45$). Linear trends were observed between

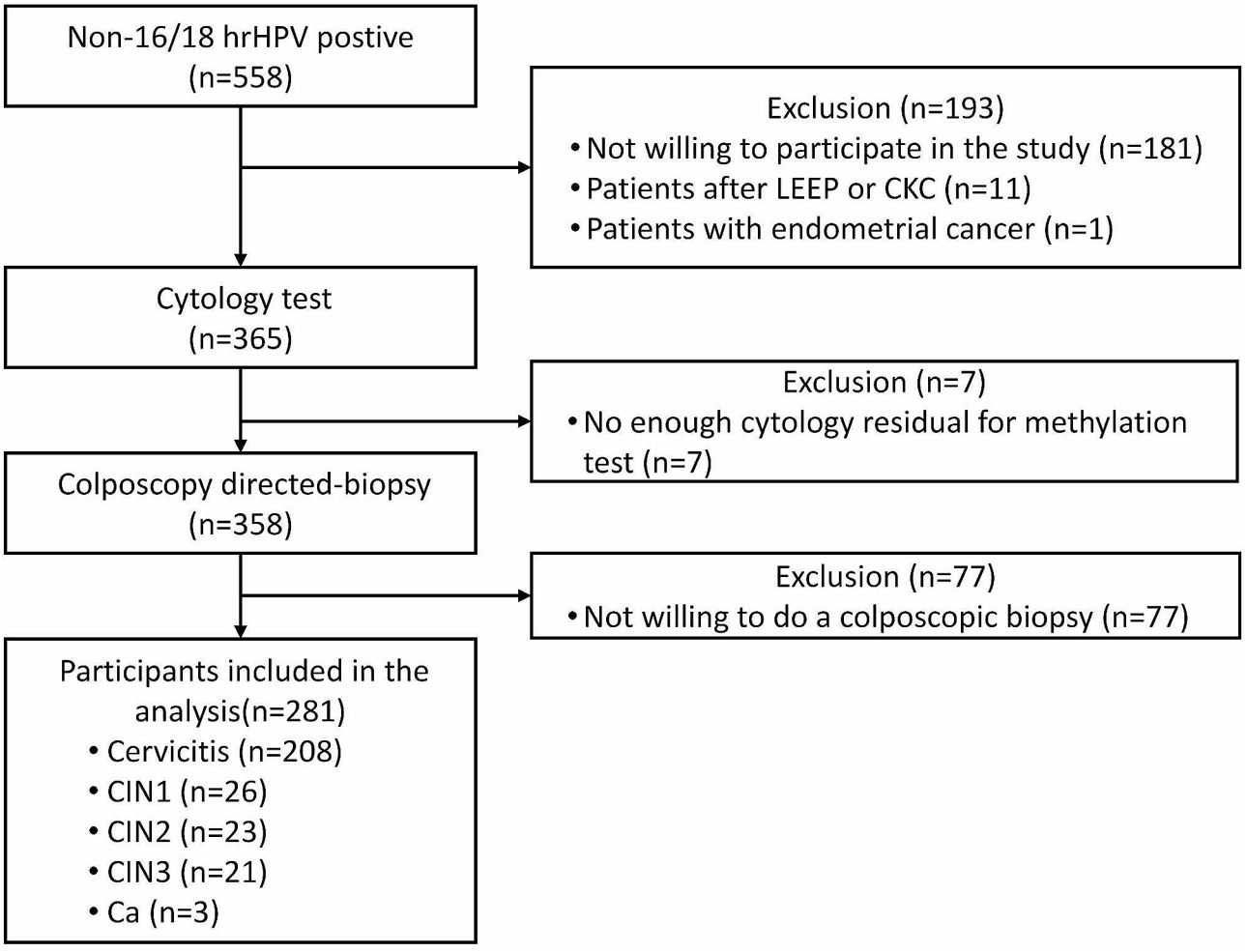

Fig. 1 Study flow chart. Abbreviations LEEP, loop electrosurgical excision procedure; CKC, cold knife conization; CIN1, cervical intraepithelial neoplasm grade 1; CIN2, cervical intraepithelial neoplasm grade 2; CIN3, cervical intraepithelial neoplasm grade 3; Ca, cervical cancer

both $PAX1^m$ and cytology results and biopsy pathology findings (<CIN2 vs., CIN2+($P<0.001$, $P<0.001$), < CIN3 vs. CIN3+ ($P<0.001$ $P=0.002$, respectively)). In cases of moderate and high levels of $PAX1^m$, respective ORs for CIN2+/CIN3+were 8.86(95%CI=2.24–42.17, $P<0.001$)/1.87(95%CI=0.03–36.68, $P=0.512$) and 166.32 (95%CI=47.09-784.97, $P<0.001$)/ 196.31(95%CI=29.11-7990.65, $P<0.001$) (Table 2). When cytology findings were ASC-US and LSIL+, the respective ORs for CIN2+/CIN3+were 6.27(95%CI=2.66–16.14, $P<0.001$)/10.42(95%CI=2.85–57.56, $P<0.001$) and 5.51(95%CI=1.95–16.18, $P<0.001$) / 5.93(95%CI=1.10–39.80, $P=0.018$).

Clinical performance of *PAX1* methylation and cytology results for women positive for non-16/18 hrHPV

The AUC values for $PAX1^m$ status when used to diagnose CIN2+and CIN3+were 0.948 (95%CI=0.895–0.999) and 0.927 (95%CI=0.876–0.977) respectively (Fig. 2C and D). The performance of $PAX1^m$ and cytology results as tools for the triage of non-16/18 hrHPV-positive women is detailed in Table 3. No significant differences in the sensitivity of these two triage tools were evident (89.4% vs. 80.9% for CIN2+, $P=0.206$; 95.8% vs. 87.5%, $P=0.317$). With respect to specificity, $PAX1^m$ (CIN2+: 87.2% and CIN3+: 80.9%) was significantly superior to cytology findings (CIN2+: 59.0% and CIN3+: 56.0%) (all $P<0.001$). The negative predictive performance of $PAX1^m$ and cytology was comparable (97.6% vs. 93.9% for CIN2+, $P=0.056$; 99.5% vs. 98.0%, $P=0.216$), but the positive predictive value of $PAX1^m$ was significantly higher than that of cytology results (58.3% vs. 28.4% for CIN2+, $P<0.001$; 31.9% vs. 15.7%, $P<0.001$).

Table 4 shows that 47.7% (134/281) of women testing positive for non-16/18 hrHPV had abnormal cytological results, necessitating colposcopy referrals, with a 95% confidence interval of 41.7-53.7%. Based on triage according to $PAX1^m$ status, 25.6% required colposcopy referral (95%CI=20.6-31.1%). Neither $PAX1^m$ nor cytology analyses missed cervical cancer diagnoses, with

Table 1 Basic patient clinical characteristics

Characteristics	*n*	Proportion
Total	281	100.0%
Age Median (min-max)	42(19–75)	
Cytology		
-NILM	147	52.3%
-ASC-US	89	31.7%
-LSIL	32	11.4%
-ASC-H	9	3.2%
-HSIL	4	1.4%
***PAX1*^{m}** (ΔCp) Median (IQR)	19.2(9.7–21.0)	
Pathology		
-Cervicitis	208	74.0%
-CIN1	26	9.3%
-CIN2	23	8.2%
-CIN3	21	7.4%
-Ca	3	1.1%

Abbreviations: NILM, negative for intraepithelial lesions or malignancy; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells cannot exclude HSIL; HSIL, high-grade squamous intraepithelial lesion; CIN1, cervical intraepithelial neoplasm grade 1; CIN2, cervical intraepithelial neoplasm grade 2; CIN3, cervical intraepithelial neoplasm grade 3; Ca, cervical cancer

respective requirements for 3.13 and 6.18 referrals to detect one instance of CIN3+.

Predictive performance of different triage tools in non-16/18 hrHPV positive women of different ages

An additional analysis of the clinical performance of *PAX1* and cytology results was also conducted according to age group (Table 5). For CIN2+, no significant differences in sensitivity or NPV were detected between the two for individuals under 45 years of age ($P=0.414$, $P=0.197$, respectively), whereas significant differences in specificity and PPV were detected (all $P<0.001$). The same was also true for individuals 45+years of age. The respective sensitivity and NPV values of *PAX1*^{m} for the 45+age group were 90.9% (20/22) and 97.7% (86/88).

Discussion

The HPV test, recommended for cervical cancer screening, lacks specificity without subsequent triage, potentially leading to an excessive number of colposcopy examinations [20]. Thus, triage tools play a crucial role in minimizing unnecessary colposcopy referrals [21, 22]. For women testing positive for non-16/18 hrHPV, cytology is the primary triage method used in most countries. However, alternatives like extended genotyping [23], dual staining (p16/Ki67) [24], and HPV E6/E7 mRNA testing [25] are currently being explored. Here, *PAX1*^{m} analyses of cytology specimens and compared to the corresponding cytology results in order to gauge the triage performance of *PAX1*^{m}. This study included 13 HPV genotypes among non-16/18 high-risk HPV-positive patients, with the three most common being HPV52 (34.52%), HPV58 (25.27%), and HPV53 (11.39%) (Supplementary Table 1). This distribution is generally in line with the rates of HPV infection observed across various regions of China [26, 27].

In this study cohort, we found that elevated *PAX1* methylation levels were associated with an increased risk of both CIN2+and CIN3+lesions, as detailed in Table 2. Among the 188 and 49 enrolled patients with low-degree *PAX1*^{m} [$\Delta Cp>15$] and moderate-degree *PAX1*^{m} [$7.2<\Delta Cp\leq 15$], there were a total of 1 and 0 cases of CIN3+, respectively. Among 147 cytologically normal and 89 cytologically ASC-US women, there were 3 and 16 cases of CIN3+, respectively. This suggests that triaging patients according to low- and moderate-degree *PAX1*^{m} is potentially safer than doing so based on NILM and ASC-US cytology findings. Relative to patients exhibiting low-degree *PAX1*^{m}, the OR for patients with high-degree *PAX1*^{m} [$\Delta Cp\leq 7.2$] was 196.31 (29.11-7990.65), which was markedly higher than the corresponding OR when comparing the cytology LSIL+and NILM designations. This suggests a potential correlation between high-degree *PAX1*^{m} in CIN3+incidence. High methylation of the *PAX1* gene is associated with an increased risk of cervical intraepithelial neoplasia grade 3 or more severe (CIN3+). Clinically available methylation tests can be utilized in the following ways [28]: (1). For the preliminary classification of high-risk HPV (HRHPV)-positive women to detect cervical cancer and advanced cervical intraepithelial neoplasia (CIN). (2). As a secondary triage for women with mild cytological abnormalities to determine their risk of CIN3 or more severe disease. (3). As an exit test for women who opt out of the screening program to identify those with cervical cancer or advanced CIN. (4). To support the management of CIN. There have been several reports demonstrating that TSGs are associated with the incidence of cervical lesions [29–32]. Specifically, the expression of high levels of the hrHPV E6/E7 oncogenes can regulate methyltransferase activity, resulting in the aberrant methylation of many TSGs. This, in turn, can result in the silencing or inactivation of these TSGs such that their antitumor functions are absent [11, 33, 34]. This mechanism can lead to the incidence of transformed cervical intraepithelial neoplasia (tCIN), including certain cases of CIN2 and CIN3 [8–10, 35]. This may explain the increased risk of CIN3+that was found to be associated with high-degree *PAX1*^{m}. High expression of *PAX1*^{m} positivity correlated with increased severity of lesions, as shown in Supplementary Tables 2–3.

With respect to the clinical performance of these two approaches to triaging non-16/18 high-risk HPV-positive women, *PAX1*^{m} and cytology results yielded

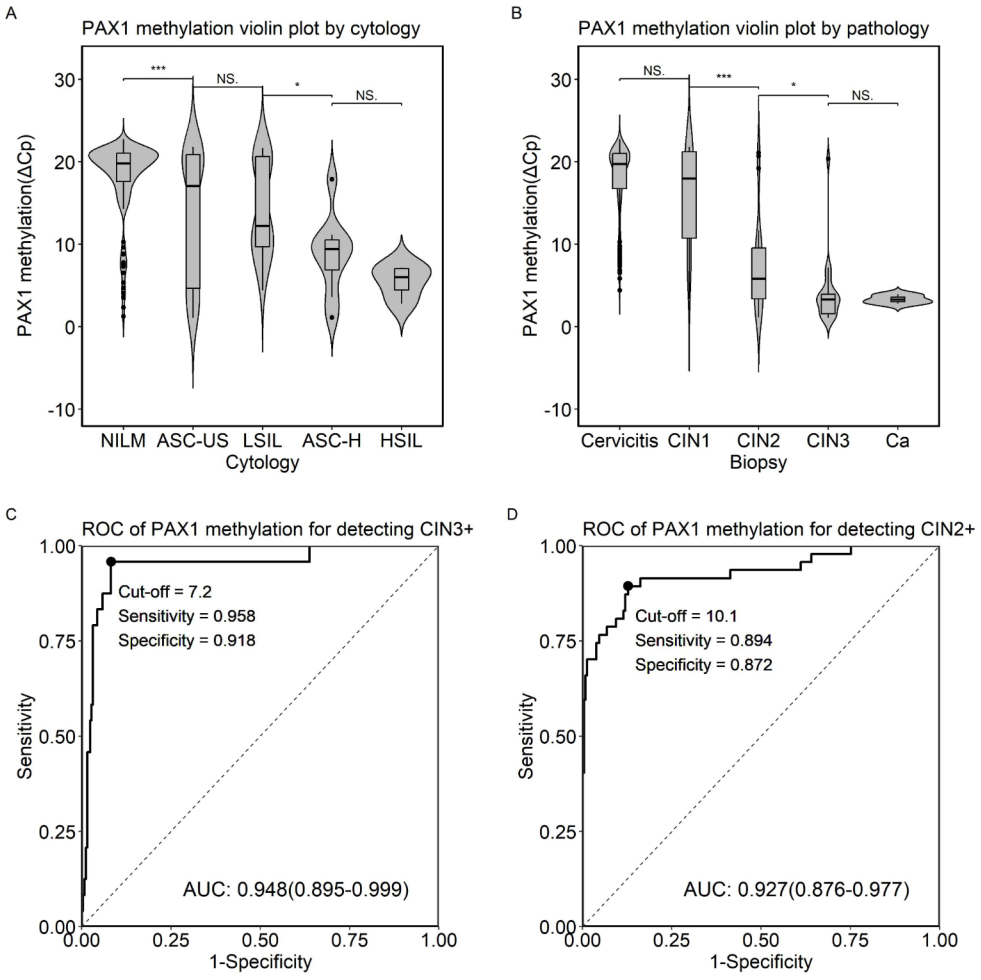

Fig. 2 *PAX1* methylation distribution plots and ROC curves for CIN2+/CIN3+detection. **A**, Violin plots of *PAX1*^{m} (ΔCp) levels grouped according to cytology results. **B**, Violin plots of *PAX1*^{m} (ΔCp) levels grouped according to biopsy results. **C**, ROC curve for the *PAX1*^{m}-based detection of CIN2+lesions. **D**, ROC curve for the *PAX1*^{m}-based detection of CIN3+lesions. Abbreviations: NILM, negative for intraepithelial lesions or malignancy; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells cannot exclude HSIL; HSIL, high-grade squamous intraepithelial lesion; CIN1, cervical intraepithelial neoplasm grade 1; CIN2, cervical intraepithelial neoplasm grade 2; CIN2+, cervical intraepithelial neoplasm grade 2 or worse; CIN3, cervical intraepithelial neoplasm grade 3; CIN3+, cervical intraepithelial neoplasm grade 3 or worse; Ca, cervical cancer; ROC, receiver operator characteristic curve; AUC, area of under the ROC. Statistical identification: NS., no significance; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$

comparable sensitivity for the detection of CIN2+and CIN3+ ($P=0.206$, $P=0.317$, respectively), but *PAX1*^{m} offered greater specificity than cytology (Table 3). In cervicitis and CIN1 cases, cytological abnormality rates were also higher than *PAX1*^{m} positivity rates (37.5% vs. 11.5%, 69.2% vs. 23.1%) (Supplementary 2). All participants in this study were women who were positive for hrHPV, and cellular abnormalities caused by hrHPV infection can contribute to cytological abnormalities being more frequently present in cervicitis and CIN1 cases (cytology≥ASC-US) [36]. The NPV of *PAX1*^{m} in this study was comparable to that of cytology, underscoring the reliability of this alternative triage strategy. In addition, the PPV of *PAX1*^{m} was superior to that of cytology, suggesting that those women with positive *PAX1*^{m} results may face a higher risk of CIN2+/CIN3+incidence as compared to those women with positive cytology findings. These methylation results thus offer a higher degree of clinical accuracy that can be leveraged to guide colposcopy. When stratifying participants according to age, for both individuals≥45 and <45 years, *PAX1*^{m} and cytology exhibited comparable sensitivity and NPV for the detection of CIN2+, while the specificity and PPV of *PAX1*^{m} were superior to those for cytology. These results support previously published results suggesting that methylation testing can serve as a more effective criterion for the discontinuation of further screening in older women [28].

The management of cervical cancer remains a significant challenge. Tertiary prevention strategies for cervical cancer include HPV vaccination, screening methods

Table 2 *PAX1* methylation odds ratios and cytology results for CIN2+/CIN3 + patients

Tests	OR (95%CI)†			$P_{for\ trend}$‡
$PAX1^m$	Low ($n=188$) [ΔCp > 15.0]	Moderate ($n=49$) [7.2 < ΔCp ≤ 15.0]	High ($n=44$) [ΔCp ≤ 7.2]	
$n_{<CIN2}$/ n_{CIN2+}	184/4	41/8	9/35	< 0.001
OR (95%CI)	1.0	8.86(2.24–42.17)	166.32(47.09-784.97)	
P		< 0.001	< 0.001	
$n_{<CIN3}$/ n_{CIN3+}	187/1	49/0	21/23	< 0.001
OR (95%CI)	1.0	1.87(0.03–36.68)	196.31(29.11-7990.65)	
P		0.512	< 0.001	
Cytology	NILM($n=147$)	ASC-US($n=89$)	LSIL+($n=45$)	
$n_{<CIN2}$/ n_{CIN2+}	138/9	63/26	33/12	< 0.001
OR (95%CI)	1.0	6.27(2.66–16.14)	5.51(1.95–16.18)	
P		< 0.001	< 0.001	
$n_{<CIN3}$/ n_{CIN3+}	144/3	73/16	40/5	0.002
OR (95%CI)	1.0	10.42(2.85–57.56)	5.93(1.10–39.80)	
P		< 0.001	0.018	

Abbreviations: NILM, negative for intraepithelial lesions or malignancy; ASC-US, atypical squamous cells of undetermined significance; ASC-US+, atypical squamous cells of undetermined significance or worse; CIN2+, cervical intraepithelial neoplasm grade 2 or worse; CIN3+, cervical intraepithelial neoplasm grade 3 or worse; OR, odds ratio. Statistical annotation: †, Fisher's exact test, using low $PAX1^m$ or NILM cytology results as the reference; ‡, Cochran-Armitage test for trend

Table 3 Comparisons of the performance of $PAX1^m$ status and cytology results for the triage of non-16/18 hrHPV-positive women ($n=281$)

Tests	Cut-off	Sensitivity % (95%CI)	Specificity % (95%CI)	PPV % (95%CI)	NPV % (95%CI)
CIN2 + versus < CIN2					
$PAX1^m$	ΔCp ≤ 10.1	89.4(77.4–95.4)	87.2(82.3–90.9)	58.3(46.8–69.0)	97.6(94.5–99.0)
Cytology	ASC-US+	80.9(67.4–89.6)	59.0(52.6–65.1)	28.4(21.4–36.5)	93.9(88.8–96.8)
P		0.206	< 0.001	< 0.001	0.056
CIN3 + versus < CIN3					
$PAX1^m$	ΔCp ≤ 10.1	95.8(79.7–99.3)	80.9(75.7–85.3)	31.9(22.3–43.4)	99.5(97.3–99.9)
Cytology	ASC-US+	87.5(69.0-95.7)	56.0(49.9–62.0)	15.7(10.5–22.8)	98.0(94.2–99.3)
P		0.317	< 0.001	< 0.001	0.216

Abbreviations: ASC-US+, atypical squamous cells of undetermined significance or worse; $PAX1^m$, PAX1 methylation; PPV, Positive Predictive Value; NPV, Negative Predictive Value

for early detection, and treatments for CIN and cancer, such as surgery, minimally invasive surgery (MIS), and diathermy therapy. The highly immunogenic nature of the HPV vaccine has led to its promotion by the WHO, resulting in a worldwide decrease in the incidence of cervical cancer. The efficacy of the bivalent, quadrivalent, and non-valent vaccines against HPV 16/18 has been found to be similar. Real-world data shows a significant reduction of HPV 6/11/16/18 in vaccinated women compared to unvaccinated women, indicating that the vaccine is highly effective. Moreover, the direct effect of the non-valent vaccine with the cross-protection of bivalent and quadrivalent vaccines results in the reduction of HPV 6/11/16/18/31/33/45/52/58. HPV vaccination has also been shown to provide herd protection as well [37]. Surgical treatment of CIN and cervical cancer is important according to global guidelines recommendations; however, it may pose a risk of adverse outcomes for pregnant women. Furthermore, women who undergo hysterectomy are more likely to develop high levels of lesions in the vulva, vagina, and anus - including a 6% increased risk of HPV-related lesions, a 9% increased risk of vaginal HPV-related lesions, and a 20% risk of vaginal cancer [38]. According to LACC study MIS had four times higher recurrence rates and six times higher all-cause mortality rates than laparotomy [39]. A growing body of evidence supports introducing vaccination in an "adjuvant" setting after initial treatment for CIN. Preliminary data suggest that vaccination after conization reduces the 50–80% risk of CIN recurrent. HPV vaccination can also prevent lower reproductive tract dysplasia and high levels of lesions in the vulva, vagina, and anus [38]. Given the dominance of the bivalent HPV vaccine worldwide, it underscores the importance of methylation testing in women with high-risk HPV genotypes other than HPV 16 and 18 for detecting high-grade cervical lesions and cancers. The integration of vaccination and methylation testing as part of post-treatment management for cervical lesions and cancers is increasingly crucial, and warrants further investigation through larger clinical studies.

There are some limitations to these analyses. For one, many of the women eligible for enrollment did not complete the entirety of the trial, with 181 non-16/18 hrHPV-positive women declining to participate, potentially owing to the novelty of the $PAX1^m$ analysis strategy. In addition, 77 participants refused to undergo colposcopy-guided biopsy. These factors may have introduced bias into the study results. In addition, this study was not a randomized controlled trial and instead relied on residual cytological specimens, potentially contributing to the underestimation or overestimation of the clinical performance of $PAX1^m$-based triage strategies. Lastly, no follow-up was conducted for patients who did not reach the clinical study endpoint (CIN2+), and as such, the

Table 4 Colposcopy referral percentages and referrals required for the detection of one CIN2+/CIN3 + case when using two triage strategies for non-16/18 hrHPV-positive women ($n = 281$)

Tests	Colposcopy referral indicators	Colposcopy referral (%) (95%CI)	Referrals needed to detect one CIN2+	Referrals needed to detect one CIN3+	Missed CIN2/ CIN3/ Ca
PAX1^m	ΔCp ≤ 10.1	25.6(20.6–31.1)	1.71	3.13	4/1/0
Cytology	ASC-US+	47.7(41.7–53.7)	3.53	6.38	6/3/0

Abbreviations: ASC-US+, atypical squamous cells of undetermined significance or worse; *PAX1*^{m}, PAX1 methylation; CIN2, cervical intraepithelial neoplasm grade 2; CIN3, cervical intraepithelial neoplasm grade 3; Ca, cervical cancer; CIN2+, cervical intraepithelial neoplasm grade 2 or worse; CIN3+, cervical intraepithelial neoplasm grade 3 or worse

Table 5 Sensitivity, specificity, PPV, and NPV of cytology- and *PAX1*^{m}-based triage strategies for the detection of CIN2 + among non-16/18 hrHPV-positive women in different age groups

Tests	Cut-off	Sensitivity % (*n*/*N*)	Specificity % (*n*/*N*)	PPV % (*n*/*N*)	NPV % (*n*/*N*)
Age < 45 years (n = 150)					
PAX1^m	ΔCp ≤ 10.1	88.0 (22/25)	94.4 (118/125)	75.9 (22/29)	97.5 (118/121)
Cytology	ASC-US+	80.0 (20/25)	65.6 (43/125)	31.7 (20/63)	94.3 (82/87)
P		0.414	< 0.001	< 0.001	0.197
Age ≥ 45 years (n = 131)					
PAX1^m	ΔCp ≤ 10.1	90.9 (20/22)	78.9 (86/109)	46.5 (20/43)	97.7 (86/88)
Cytology	ASC-US+	81.8 (18/22)	51.4 (56/109)	25.4 (18/71)	93.3 (56/60)
P		0.317	< 0.001	< 0.001	0.155

Abbreviation ASC-US+, atypical squamous cells of undetermined significance or worse; *PAX1*^{m}, PAX1 methylation; CIN2+, cervical intraepithelial neoplasm grade 2 or worse; PPV, Positive Predictive Value; NPV, Negative Predictive Value

long-term reliability of negative *PAX1*^{m} results remains to be assessed.

Even with these limitations, the results of this study have profound implications for cervical cancer screening efforts in China. At present, the standard cervical cancer screening strategy at the national level in China consists of initial hrHPV testing with subsequent cytology. However, cytology is dependent on prolonged and comprehensive training such that cytologist shortages in different healthcare systems represent an important barrier to the widespread implementation of cervical cancer screening. Integrating artificial intelligence systems, such as the Hologic Genius AI, into cervical cytology could alleviate the impact of cytologist shortages and facilitate broader implementation of cervical cancer screening. *PAX1*^{m} analyses, in contrast, are based on a fluorescent qPCR technique that has matured significantly such that it can be performed in a largely automated manner, thereby abrogating this dependence on specialized cytologists to gauge patient risk. Among non-16/18-positive patients, the cytological indication for colposcopy referral is ASC-US or higher, contributing to high rates of colposcopy referral. In this study, cytological abnormalities were detected in 47.7% of non-16/18-positive patients, indicating that almost half of this cohort would require colposcopy referral. In contrast, the *PAX1*^{m} testing positivity rate was just 25.6%, representing a 46.3% reduction in the colposcopy referral rate (Table 4). The implementation of *PAX1*^{m} testing would thus enable to implementation of fully automated molecular testing-based cervical cancer screening while also reducing the number of unnecessary colposcopy referrals, thereby preventing the inefficient use of medical resources.

Conclusions

In summary, the present results revealed that *PAX1*^{m} is significantly associated with cervical lesion severity. *PAX1*^{m} exhibited good diagnostic performance for the detection of CIN2 + and CIN3 + lesions in women positive for non-16/18 hrHPV. As such, the use of *PAX1*^{m} analyses of cervical specimens represents a viable approach to triaging non-16/18 hrHPV-positive women, especially in areas where shortages of cytologists or large-scale screening concerns exist.

Abbreviations

ASC-US	Atypical squamous cells of undetermined significance
ASC-US+	Atypical squamous cells of undetermined significance or worse
ASC-H	Atypical squamous cells cannot exclude HSIL
AUC	Area of under the ROC
Ca	Cervical cancer
CIN1	Cervical intraepithelial neoplasm grade 1
CIN2	Cervical intraepithelial neoplasm grade 2
CIN3	Cervical intraepithelial neoplasm grade 3
CIN2+	Cervical intraepithelial neoplasm grade 2 or worse
CIN3+	Cervical intraepithelial neoplasm grade 3 or worse
CKC	Cold knife conization
HSIL	High-grade squamous intraepithelial lesion
LEEP	Loop electrosurgical excision procedure
LSIL	Low-grade squamous intraepithelial lesion
NILM	Negative for intraepithelial lesions or malignancy
NPV	Negative Predictive Value
OR	Odds ratio
PAX1^m	*PAX1* methylation
PPV	Positive Predictive Value
ROC	Receiver operator characteristic curve

Supplementary Information

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Supplementary Material 1

Author contributions
CD designed the study and finalized the paper. HM performed data analysis, reviewed the literature, and drafted the article. WT, LM, QM and DS collected clinical data. All authors contributed to the article and approved the submitted version.

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Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the Zhuzhou Hospital Affiliated to Xiangya School of Medicine, Central South University (No: ZD2022001-01) and informed consent was signed by the patients.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, Jemal A. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2024;74(3):229–63.
2. Zhang J, Zhao Y, Dai Y, Dang L, Ma L, Yang C, Li Y, Kong L, Wei L, Zhang S, et al. Effectiveness of high-risk human papillomavirus testing for Cervical Cancer Screening in China: a Multicenter, Open-label, Randomized Clinical Trial. JAMA Oncol. 2021;7(2):263–70.
3. Chinese Cervical Cancer Screening Guidelines (1). Chinese Clinical Journal of Obstetrics and Gynecology 2023, Volume 24, Issue 4. July 2023:437–442.
4. Güzel C, van Sten-Van J, de Kok I, Govorukhina NI, Boychenko A, Luider TM, Bischoff R. Molecular markers for cervical cancer screening. Expert Rev Proteomics. 2021;18(8):675–91.
5. Zhao Y, Bao H, Ma L, Song B, Di J, Wang L, Gao Y, Ren W, Wang S, Wang HJ, et al. Real-world effectiveness of primary screening with high-risk human papillomavirus testing in the cervical cancer screening programme in China: a nationwide, population-based study. BMC Med. 2021;19(1):164.
6. Sørbye SW, Suhrke P, Revå BW, Berland J, Maurseth RJ, Al-Shibli K. Accuracy of cervical cytology: comparison of diagnoses of 100 pap smears read by four pathologists at three hospitals in Norway. BMC Clin Pathol. 2017;17:18.
7. Wei LH, Zhao Y, Shen DH, Zh FH, Geng L, Bi H, Xu HM, Li JR. Expert consensus on cervical cancer screening and abnormal management in China (1). Chin J Obstet Gynecol. 2017;18(02):190–2.
8. Pietropaolo V, Prezioso C, Moens U. Role of Virus-Induced host cell epigenetic changes in Cancer. Int J Mol Sci 2021, 22(15).
9. Wilting SM, Steenbergen RDM. Molecular events leading to HPV-induced high grade neoplasia. Papillomavirus Res. 2016;2:85–8.
10. Steenbergen RD, Snijders PJ, Heideman DA, Meijer CJ. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. Nat Rev Cancer. 2014;14(6):395–405.
11. Su PH, Lai HC, Huang RL, Chen LY, Wang YC, Wu TI, Chan MWY, Liao CC, Chen CW, Lin WY, et al. Paired Box-1 (PAX1) activates multiple phosphatases and inhibits kinase cascades in Cervical Cancer. Sci Rep. 2019;9(1):9195.
12. Li B, Guo R, Lai T, Qiao L, Fu H. The application of PAX1 methylation detection and HPV E6/E7 mRNA detection in cervical cancer screening. J Obstet Gynaecol Res 2021.
13. Fu K, Lei M, Wu LS, Shi JC, Yang SY, Yang WQ, Xu JY, Kang YN, Yang ZY, Zhang X, et al. Triage by PAX1 and ZNF582 methylation in Women with Cervical Intraepithelial Neoplasia Grade 3: a Multicenter Case-Control Study. Open Forum Infect Dis. 2022;9(5):ofac013.
14. Li M, Zhao C, Zhao Y, Li J, Wang J, Luo H, Tang Z, Guo Y, Wei L. The role of PAX1 methylation in predicting the pathological upgrade of cervical intraepithelial neoplasia before cold knife conization. Front Oncol. 2022;12:1064722.
15. Bukowski A, Hoyo C, Vielot NA, Graff M, Kosorok MR, Brewster WR, Maguire RL, Murphy SK, Nedjai B, Ladoukakis E, et al. Epigenome-wide methylation and progression to high-grade cervical intraepithelial neoplasia (CIN2+): a prospective cohort study in the United States. BMC Cancer. 2023;23(1):1072.
16. Chang CL, Ho SC, Su YF, Juan YC, Huang CY, Chao AS, Hsu ZS, Chang CF, Fwu CW, Chang TC. DNA methylation marker for the triage of hrHPV positive women in cervical cancer screening: real-world evidence in Taiwan. Gynecol Oncol 2021.
17. Yang L, Tao H, Lin B, He X, Chen Y, Fan X. Utilization of PAX1 methylation test for cervical cancer screening of non-HPV16/18 high-risk HPV infection in women. Future Oncol 2023.
18. Wang Y, Chuan J, Zhu B, Zu Y, Zhang R, Tang L, Wen Y, Yang T, Chen K, Chen X, et al. Evaluation of PAX1/ST6GALNAC5 methylation as a triage test for cervical intraepithelial neoplasia and cervical cancer. Epigenomics. 2023;15(3):131–45.
19. Su Y, Tang Y, Zhang T, Xu Y, Zhang Y, Shen Y, Qin L, Zhang L, Cao L, Zhou Y et al. Methylated ZNF582 as a triage marker for occult cervical cancer and advanced cervical intraepithelial neoplasia. Future Oncol 2022.
20. Koliopoulos G, Nyaga VN, Santesso N, Bryant A, Martin-Hirsch PP, Mustafa RA, Schünemann H, Paraskevaidis E, Arbyn M. Cytology versus HPV testing for cervical cancer screening in the general population. Cochrane Database Syst Rev. 2017;8(8):Cd008587.
21. Ebisch RM, Siebers AG, Bosgraaf RP, Massuger LF, Bekkers RL, Melchers WJ. Triage of high-risk HPV positive women in cervical cancer screening. Expert Rev Anticancer Ther. 2016;16(10):1073–85.
22. Luttmer R, De Strooper LM, Steenbergen RD, Berkhof J, Snijders PJ, Heideman DA, Meijer CJ. Management of high-risk HPV-positive women for detection of cervical (pre)cancer. Expert Rev Mol Diagn. 2016;16(9):961–74.
23. Stoler MH, Parvu V, Yanson K, Andrews J, Vaughan L. Risk stratification of HPV-positive results using extended genotyping and cytology: data from the baseline phase of the Onclarity trial. Gynecol Oncol. 2023;174:68–75.
24. Wright TC Jr., Stoler MH, Ranger-Moore J, Fang Q, Volkir P, Safaeian M, Ridder R. Clinical validation of p16/Ki-67 dual-stained cytology triage of HPV-positive women: results from the IMPACT trial. Int J Cancer. 2022;150(3):461–71.
25. Sørbye SW, Falang BM, Antonsen M. Performance of a 7-Type HPV mRNA test in triage of HPV DNA primary screen positive women compared to liquid-based cytology. J Mol Pathol. 2023;4(2):69–80.
26. Li K, Li Q, Song L, Wang D, Yin R. The distribution and prevalence of human papillomavirus in women in mainland China. Cancer. 2019;125(7):1030–7.
27. Zhao S, Zhao X, Hu S, Lu J, Duan X, Zhang X, Chen F, Zhao F. Distribution of high-risk human papillomavirus genotype prevalence and attribution to cervical precancerous lesions in rural North China. Chin J cancer Res = Chung-kuo Yen Cheng Yen Chiu. 2019;31(4):663–72.
28. Kremer WW, Steenbergen R, Heideman D, Kenter GG, Meijer C. The use of host cell DNA methylation analysis in the detection and management of women with advanced cervical intraepithelial neoplasia: a review. BJOG: Int J Obstet Gynecol. 2021;128(3):504–14.
29. Shi L, Yang X, He L, Zheng C, Ren Z, Warsame JA, Suye S, Yan L, Cai H, Xiao X, et al. Promoter hypermethylation analysis of host genes in cervical intraepithelial neoplasia and cervical cancers on histological cervical specimens. BMC Cancer. 2023;23(1):168.
30. Verhoef L, Bleeker MCG, Polman N, Steenbergen RDM, Meijer C, Melchers WJG, Bekkers RL, Molijn AC, Quint WG, van Kemenade FJ, et al. Performance of DNA methylation analysis of ASCL1, LHX8, ST6GALNAC5, GHSR, ZIC1 and SST for the triage of HPV-positive women: results from a Dutch primary HPV-based screening cohort. Int J Cancer. 2022;150(3):440–9.
31. van den Helder R, Steenbergen RDM, van Splunter AP, Mom CH, Tjiong MY, Martin I, Rosier-van Dunné FMF, van der Avoort IAM, Bleeker MCG, van Trommel NE. HPV and DNA methylation testing in urine for cervical intraepithelial neoplasia and cervical Cancer detection. Clin cancer Research: Official J Am Association Cancer Res. 2022;28(10):2061–8.
32. Loaeza-Loaeza J, Illades-Aguiar B, Del Moral-Hernández O, Castro-Coronel Y, Leyva-Vázquez MA, Dircio-Maldonado R, Ortiz-Ortiz J, Hernández-Sotelo

D. The CpG island methylator phenotype increases the risk of high-grade squamous intraepithelial lesions and cervical cancer. Clin Epigenetics. 2022;14(1):4.
33. Lai HC, Lin YW, Huang TH, Yan P, Huang RL, Wang HC, Liu J, Chan MW, Chu TY, Sun CA, et al. Identification of novel DNA methylation markers in cervical cancer. Int J Cancer. 2008;123(1):161–7.
34. Tirado-Magallanes R, Rebbani K, Lim R, Pradhan S, Benoukraf T. Whole genome DNA methylation: beyond genes silencing. Oncotarget. 2017;8(3):5629–37.
35. Lorincz AT. Virtues and weaknesses of DNA methylation as a test for Cervical Cancer Prevention. Acta Cytol. 2016;60(6):501–12.
36. Stanley M. Pathology and epidemiology of HPV infection in females. Gynecol Oncol. 2010;117(2 Suppl):S5–10.
37. Kamolratanakul S, Pitisuttithum P. Human papillomavirus vaccine efficacy and effectiveness against Cancer. Vaccines 2021, 9(12).
38. Bogani G, Sopracordevole F, Ciavattini A, Ghelardi A, Vizza E, Vercellini P, Casarin J, Pinelli C, Ghezzi F, De Vincenzo R, et al. HPV-related lesions after hysterectomy for high-grade cervical intraepithelial neoplasia and early-stage cervical cancer: a focus on the potential role of vaccination. Tumori. 2024;110(2):139–45.
39. Golia D'Augè, Cuccu T, Etrusco I, D'Amato A, Laganà A, D'Oria AS, Bogani O, Di Donato G, Muzii V, Giannini L. A: State of the art on HPV-related cervical lesions. Ital J Gynaecol Obstet https://doi.org/10.36129/jog.2024.161

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