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CLINICAL ARTICLE

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Cytologic DNA methylation for managing minimally abnormal cervical cancer screening results

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Abstract

Objectives: To explore the role of a DNA methylation assay for managing minimally abnormal cervical cancer screening results in a prospective cohort undergoing opportunistic cervical cancer screening.

Methods: In the cohort of the METHY2 and METHY3 screening studies of women undergoing opportunistic cervical cancer screening, cervical cytology samples were sent for high-risk human papillomavirus (hrHPV) DNA assays, cytologic pathology and methylation assays of *PAX1/JAM3* (CISCER). This study evaluated the discriminative power of CISCER in managing women with minimally abnormal cervical cancer screening results for CIN3+. Absolute CIN3+ risks and colposcopy referrals within one screening round were calculated.

Results: A total of 1857 women with minimally abnormal cervical cancer findings had cervical histologic outcomes and were included in the analysis. In women with a minimally abnormal cervical cancer result, the sensitivity and specificity of CISCER was 74.9% (95% confidence interval [CI], 68.3%–81.4%) and 89.1% (95% CI 87.6%–90.6%) for detecting CIN3+. CISCER analysis discriminated well for minimally abnormal cervical cancer results, yielding a CIN3+ risk of 40.5% (95% CI 34.9%–46.2%) after a positive result and a CIN3+ risk of 2.7% (95% CI 2.0%–3.6%) after a negative result.

Conclusions: In women with a minimally abnormal cervical cancer screening result, the CISCER provides excellent detection of CIN3+. The use of CISCER in women with a minimally abnormal cervical cancer screening result can lead to a substantial reduction in the number of direct colposcopy referrals.

KEYWORDS
DNA methylation, high-risk human papillomavirus, immediate risk, minimally abnormal cervical cancer test result

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1 | INTRODUCTION

Uterine cervical cancer is the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in women, with an estimated 604000 new cases and 342000 deaths worldwide in 2020.[1] The age-standardized incidence and mortality of cervical cancer in China in 2020 were 10.7 and 5.4 per 100000, respectively, causing a great disease burden.[2] Cervical cancer screening strategies have changed substantially over the past decade. Current screening strategies for individuals older than 30 years include cytology (cervical screening tests), high-risk (oncogenic) human papillomavirus (hrHPV), or both.[3] However, the combination of cytology and hrHPV results leads to complex management algorithms and excessive referral to colposcopy evaluation, especially for minimally abnormal test results. Minimally abnormal screening results were usually defined as an hrHPV-positive test result with a concurrent normal cytologic interpretation (negative for intraepithelial lesion or malignancy), atypical squamous cells of undetermined significance (ASCUS), and low-grade squamous intraepithelial lesion (LSIL).[4] In the 2019 ASCCP Risk-Based Management Consensus Guidelines, more than 1.5 million individuals aged 25–65 years were enrolled for cervical cancer screening. It was discovered that 90% of the results were normal, 0.75% were severely abnormal, and the remainder were minimally abnormal.[5] As a result of the low prevalence of cervical precancerous lesions or cancer in women with minimally abnormal screening results, their management is of great challenge.

Hypermethylation of promoter regions of certain tumor suppressor genes is a crucial step in cervical carcinogenesis.[6] Methylation levels have been shown to increase with the duration of HPV infection and with increasing grades of cervical lesions.[7] Moreover, there is a synergistic effect between HPV infection and methylation, which together contribute to the development of cervical cancer.[8] The DNA methylation assay has been approved as a potential screening method for cervical cancer in numerous studies. In July 2021, WHO endorsed DNA methylation detection as a promising strategy in the future.[9] Several studies have applied methylation assays for triage of minimally abnormal findings. *LINE-1* and *Alu*, *CGB3* and *NOP56*, and *PAX1* methylation status can be used for triage of ASCUS.[10] *ZNF582* methylation was the better triage tool than hrHPV for LSIL for referral to colposcopy.[11] The *PAX1* methylation assay was also superior to HPV DNA for ASCUS triage.[12] However, the sample sizes of these studies limited their application in more clinical situations.

The aim of our study was to extend validation of the diagnostic performance of the *PAX1/JAM3* methylation assay (CISCER) and to find new applications in triage of women on a large cervical cancer screening program. In this study, we explored the utility of CISCER triage for minimally abnormal screening results.

2 | MATERIALS AND METHODS

2.1 | Participants and study design

This is a cross-sectional study based on the METHY2 and METHY3 screening study of women, all of whom underwent colposcopy (Figure 1). The participants were from two cohorts (METHY2 and METHY3) at Peking Union Medical College Hospital from May 2019 to December 2022. The Institutional Review Board of Peking Union Medical College Hospital approved the present study (Nos JS-1954 and JS-2380). The registration numbers are NCT03960879 and NCT04646954 (clinicaltrials.gov, registered on May 23, 2019, and November 26, 2020, respectively). All participants provided written informed consent before participating in the study and gave consent for publication.

The inclusion criteria were as follows: aged 18 years or more; an intact uterine cervix; with both definite HPV and cytologic results, and cytologic results defined as NILM (negative for intraepithelial lesion or malignancy), ASCUS (atypical squamous cells of undetermined significance), or LSIL (low-grade squamous intraepithelial lesion); and the patient had fully consented. The exclusion criteria consisted of pregnancy, positive HIV status, and organ transplant patients who were on antirejection medications. For the follow up and treatment, the results of CISCER would not be indicated for colposcopy referrals in this study. The follow up and treatment were conducted with reference to and in compliance with the Guidelines for Cervical Cancer Screening in China.[13] The STARD 2015 guidelines were used in this study.[14]

2.2 | Sample collection, cytologic pathology assay, and hrHPV genotyping

All eligible participants underwent gynecologic examinations and collection of cervical cytology. Both hrHPV and cytologic pathology assays were performed on the cytologic samples. The residual samples were sent for CISCER.

A liquid-based cytology (LBC) test and imaging system manual was used for cytology tests (Hologic Inc., MA, USA). The cytopathologic results were classified based on the 2014 Bethesda system.[15] Two cytologists independently reviewed the cytology and determined the final results: NILM, ASCUS, or LSIL.

The fully automated real-time polymerase chain reaction (PCR)-based Cobas® 4800 HPV assay (Roche, Switzerland) was used for HPV detection and genotyping according to the manufacturer's manuals. The HPV DNA test results were reported as negative for hrHPV (hrHPV–), or HPV16 and/or 18 (HPV16/18+), and/or others (HPV31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68 as a whole).

2.3 | DNA methylation testing

Methylation was detected in a certified DNA laboratory while the operators and staff members were blinded to the patients' clinical information, LBC test, HPV genotyping, and cervical histopathology results. Genomic DNA (gDNA) was extracted from the exfoliated cervical sample using the JH-DNA Isolation and Purifying Kit (OriginPoly Bio-Tec Co., Ltd., Beijing, China) according to the manufacturer's instructions. The DNA concentration was quantified using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, DE, USA). The $PAX1^m$ and $JAM3^m$ levels were determined using the Human *PAX1* and *JAM3* gene methylation detection kit (Real-time PCR) (CISCER®) for Cervical Cancer (Class III medical

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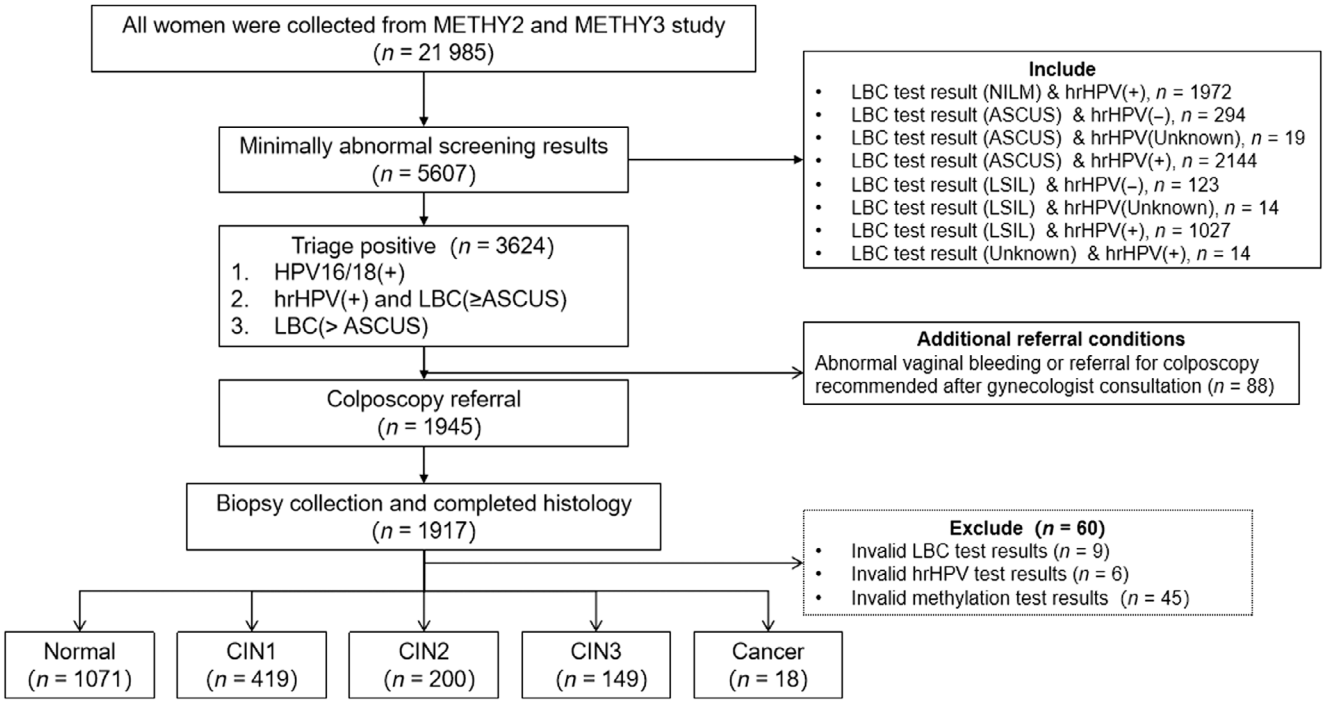

FIGURE 1 Flowchart of the enrollment process for this study, showing the number of women at each step. Triage-positive women included HPV16/18-positive ($n = 506$), hrHPV-positive and LBC (ASCUS+) ($n = 952$), LBC (>ASCUS) ($n = 523$), and abnormal vaginal bleeding or referral for colposcopy recommended after gynecologist consultation ($n = 88$). Then, 1919 women out of 3712 who underwent colposcopy had histology results recorded. Ultimately, 1857 patients with microscopic abnormalities of cervical cancer who underwent colposcopy and histologic assessment were included in the subsequent analysis of this study. ASCUS, atypical squamous cells of undetermined significance; Cancer, including squamous cell carcinoma and adenocarcinoma; CIN1, cervical intraepithelial neoplasia grade 1; CIN2, cervical intraepithelial neoplasia grade 2; CIN3, cervical intraepithelial neoplasia grade 3; HPV16/18 (+), HPV16 and/or HPV18 positive; hrHPV, high-risk human papillomavirus, including types HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68; LBC, liquid-based cytology LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy; Normal, no intraepithelial tissue lesions.

devices approved by the National Medical Products Administration [No. 20233400253]) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (OriginPoly Bio-Tec Co., Ltd., Beijing, China) by SLAN-96S automatic medical PCR analysis system (Shanghai Hongshi Med Tech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The hypermethylation level of the *PAX1* and *JAM3* genes was determined by the difference between the two Ct values (ΔCt *PAX1* = Ct *PAX1* − Ct *GAPDH* and ΔCt *JAM3* = Ct *JAM3* − Ct *GAPDH*). In accordance with the manufacturer's instructions, the positive result of the CISCER test is defined as ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0.

2.4 | Histology evaluations

Cervical histology samples were collected by colposcopy performed according to current guidelines[16,17] or by other surgical procedures, that is, conization, loop electronic cervical excision, or hysterectomy for cervical lesions. Two pathologists independently reviewed the histologic results. The results of CISCER would not be indicated for colposcopy referrals in this study.

2.5 | Statistical analysis

SPSS 26.0 (IBM Corp., Armonk, NY, USA) and R (version 4.1.2, Vienna, Austria) were used for all statistical analyses. The ΔCt values of methylated *PAX1* and *JAM3* comparison among each group were analyzed by one-way ANOVA. The measurement data are shown as percentages, and the comparison among each group was analyzed by the χ^2 test. Pearson's correlation coefficient was used to assess the correlation between *PAX1* and *JAM3* methylation levels. Receiver operating characteristic (ROC) curves were used to evaluate the area under the ROC curve (AUC) of $PAX1^m$, $JAM3^m$, and CISCER detected for CIN3+. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for detecting CIN2+ or CIN3+ were calculated with a 95% confidence interval (CI). The sensitivity and specificity for detecting CIN3+ between the different triage methods were compared using McNemar test. The immediate risk of CIN3+ was determined by various screening methods and their combinations. Direct referral percentage was calculated as the percentage of positives from each screening strategy and the number of referrals needed to detect one CIN3+ was calculated by dividing the number of screen positives by the number of true positives. All tests were two-sided,

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and differences were considered statistically significant at p values less than 0.05 with 95% CI.

3 | RESULTS

3.1 | Demographic and clinical characteristics of the participants

The flowchart of the study design is shown in Figure 1. A total of 1857 women with minimally abnormal cervical cancer results who underwent colposcopy and histologic evaluations were included in the analysis. The median age was 42 years (the interquartile range [IQR] 34.0–54.0 years). The screening results consisted of ASCUS (980, 52.8%), LSIL (523, 28.2%), NILM with hrHPV infection (354, 19.1%). The histologic results consisted of normal (1071, 57.7%), CIN1 (419, 22.6%), CIN2 (200, 10.8%), CIN3 (149, 8.0%), and squamous cell carcinoma or adenocarcinoma (18, 1.0%). The clinical characteristics of the participants are presented in Table 1. According to the various histologic results, cytology results, HPV genotype, age, and DNA methylation test results all had significant differences ($p < 0.05$).

3.2 | | The relationship of $PAX1^m/JAM3^m$ test with cervical pathology

As the results in Table 1 show, the medians of ΔCt *PAX1* and ΔCt *JAM3* methylation were 13.72 (IQR 9.70–17.69) and 115.08 (IQR 13.25–17.34) in the normal group, 12.67 (IQR 9.36–17.64) and 15.10 (IQR 13.16–17.01) in the CIN1 group, 8.31 (IQR 4.23–14.17) and 12.56 (IQR 6.98–14.89) in the CIN2 group, 5.24 (IQR 3.56–9.27) and 8.24 (IQR 5.12–13.55) in the CIN3 group, and 3.74 (IQR 2.43–6.94) and 5.85 (IQR 3.95–7.50) in the cervical cancer group. The median of ΔCt *PAX1* and ΔCt *JAM3* methylation were significantly different between the CIN1, CIN2, and CIN3 groups (Figure 2a,b, $p < 0.01$), and the median of ΔCt *JAM3* methylation significantly differed between the CIN3 and cervical cancer groups (Figure 2b, $p = 0.0097$). The correlation analysis revealed a significantly and highly correlated relationship between the methylation of *PAX1* and *JAM3* ($R = 0.61$, $p < 0.001$, Figure S1).

3.3 | | The diagnostic effects of CISCER for CIN3+ at different scenarios

ROC analysis of the CISCER for detecting CIN2+ and CIN3+ gave AUC of 0.775 (95% CI 0.749–0.801) and 0.820 (95% CI 0.786–0.854), respectively. The results show that the performance of CISCER for CIN3+ detection is superior to single *PAX1* or *JAM3* methylation assays (0.778, 95% CI 0.745–0.808 and 0.777, 95% CI 0.745–0.808, Figure 3). The detection effects of CISCER for CIN3+ at different scenarios are shown in Table 2. The sensitivity and specificity of CISCER for the diagnosis of CIN3+ in women with minimally abnormal cervical cancer findings were 74.9% (95% CI 68.3%–81.4%) and 89.1% (95% CI 87.6%–90.6%), respectively. Comparing the different cytologic results, the performance of CISCER in diagnosing CIN3+ in NILM (AUC 0.867, 95% CI 0.788–0.945) is superior to ASCUS (AUC 0.812, 95% CI 0.776–0.849), and LSIL (AUC 0.787, 95% CI 0.732–0.842). In different age subgroups, the AUC and sensitivity of CISCER for detecting CIN3+ in women older than 50 years was higher than in women younger than 50 years.

TABLE 1 Clinical characteristics of different histopathology subgroups.[a]

Pathologic result	Overall ($n = 1857$)	Normal ($n = 1071$)	CIN1 ($n = 419$)	CIN2 ($n = 200$)	CIN3 ($n = 149$)	Cancer ($n = 18$)	p Value
Age, year	42 (34–54)	44 (36–56)	40 (33–51)	40 (33–48)	41 (34–51)	40 (38–45)	<0.001
Cytology result							
NILM	354 (19.1)	245 (22.9)	58 (13.8)	31 (15.5)	15 (10.1)	5 (27.8)	<0.001
ASCUS	980 (52.8)	594 (55.5)	222 (53.0)	88 (44.0)	66 (44.3)	10 (55.6)	
LSIL	523 (28.2)	232 (21.7)	139 (33.2)	81 (40.5)	68 (45.6)	3 (16.7)	
HPV genotype							
HPV16/18 (+)	506 (27.2)	244 (22.8)	99 (23.6)	74 (37.0)	75 (50.3)	14 (77.8)	<0.001
Non-16/18 hrHPV (+)	1290 (69.5)	783 (73.1)	308 (73.5)	124 (62.0)	72 (48.3)	3 (16.7)	
HrHPV (–)	61 (3.3)	44 (4.1)	12 (2.9)	2 (1.0)	2 (1.3)	1 (5.6)	
ΔCt $PAX1^m$	11.34 (8.71–17.47)	13.72 (9.70–17.69)	12.67 (9.36–17.64)	8.31 (4.23–14.17)	5.24 (3.56–9.27)	3.74 (2.43–6.94)	<0.001
ΔCt $JAM3^m$	14.48 (12.50–16.95)	15.08 (13.25–17.34)	15.10 (13.16–17.01)	12.56 (6.98–14.89)	8.24 (5.12–13.55)	5.85 (3.95–7.50)	<0.001
CISCER(+) (%)	309 (16.6)	63 (5.9)	23 (5.5)	98 (49.0)	107 (71.8)	18 (100.0)	<0.001

Abbreviations: ASCUS, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; CISCER, ΔCt PAX1 ≤ 6.6 or ΔCt JAM3 ≤ 10.0; HPV16/18 (+), HPV16 and/or HPV18-positive; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy; Non-16/18 hrHPV (+), HPV31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68 (any one or more type positive).

[a]Data are presented as median (interquartile range) or as number (percentage).

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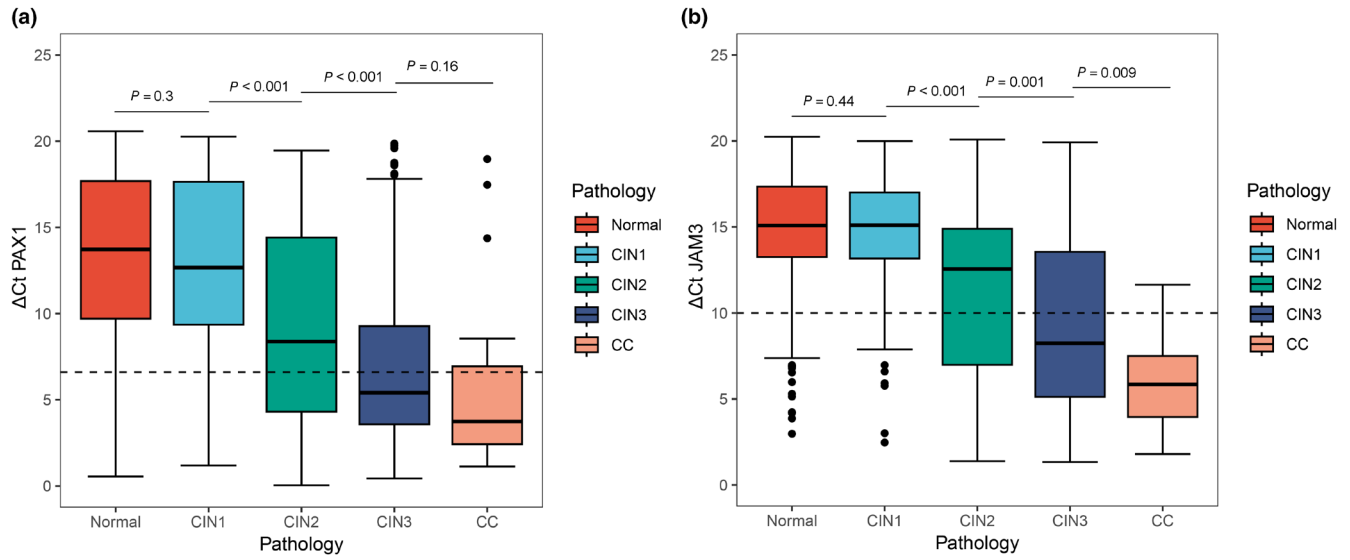

FIGURE 2 The relationship of the value ΔCt *PAX1* and *JAM3* test with cervical pathology. CC, including squamous cell carcinoma and adenocarcinoma; CIN1, cervical intraepithelial neoplasia grade 1; CIN2, cervical intraepithelial neoplasia grade 2; CIN3, cervical intraepithelial neoplasia grade 3; Normal, no intraepithelial tissue lesions. The comparisons between two groups were performed with the nonparametric Mann–Whitney *U* test, *p* values less than 0.05 differences were considered statistically significant.

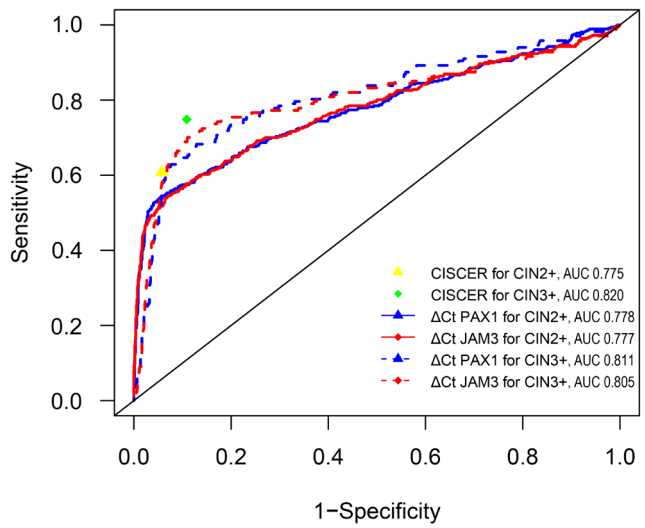

FIGURE 3 Receiver operator characteristic (ROC) and area under the curve (AUC) of CISCER for detecting CIN2+ or CIN3+. The yellow triangle denotes the sensitivity and specificity of CISCER for detecting CIN2+, the AUC of CISCER detecting CIN2+ was 0.775 (95% confidence interval [CI] 0.749–0.801). The green rhomboid denotes the sensitivity and specificity of CISCER for detecting CIN3+, the AUC of CISCER detecting CIN3+ was 0.820 (95% CI 0.786–0.854).

3.4 | Effect of CISCER on the immediate risk of CIN3+ in women with microscopic anomalies in different scenarios

Table 3 lists the underlying risks of CIN3+ estimated by the various screening methods and their combinations. Figure 4 visualizes the pre- and post-test CIN3+ risks after application of CISCER triage strategies. A positive CISCER resulted in the highest absolute CIN3+ risk (40.5%, 95% CI 34.9%–46.2%), and significantly increased absolute CIN3+ risk in women with minimally abnormal cervical cancer results (9.0%, 95% CI 7.7%–10.4%). Before bypass with CISCER triage, the highest absolute CIN3+ risk among women with a finding of minimally abnormal cervical cancer was in women with LBC (LSIL) with hrHPV positivity, with an absolute CIN3+ risk of 14.1% (11.1%–17.5%). The lowest was in women with LBC (ASCUS) and hrHPV-negative with an absolute CIN3+ risk of 3.6% (95% CI 0.1%–18.3%). Triage of women with a minimally abnormal cervical cancer result using CISCER was effective in reducing the number of referrals needed to detect one CIN3+ (11.1 versus 2.5).

4 | DISCUSSION

Several studies have explored the role of a panel that includes *PAX1*[18–20] or *JAM3*[21,22] for the screening or triage of CIN2, CIN3, and/or cervical cancer. However, there have been no reports on the combined use of *PAX1* and *JAM3* genes for cytologic minimally abnormal cervical cancer. The preliminary results of this study indicated that CISCER has good prospects in cervical cancer detection, with good efficacy in detecting CIN3+ for minimally abnormal cervical cancer screening results. Additional risk stratification of women with minimally abnormal cervical cancer screening results could use CISCER to reduce direct colonoscopy referral rates while maintaining high sensitivity for CIN3+ detection.

The establishment of routine cytology screening has resulted in a significant decrease in cervical cancer deaths. Similar to the 2019 ASCCP Risk-Based Management Consensus Guidelines, minimally

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TABLE 2 Evaluating the efficacy of CISCER in detecting CIN3+ in different scenarios.

Screening strategies	*N*	*n* for CIN3+	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	AUC (95% CI)	OR (95% CI)
All minimally abnormal samples	1857	167	74.9 (68.3–81.4)	89.1 (87.6–90.6)	40.5 (35–45.9)	97.3 (96.5–98.1)	0.820 (0.786–0.854)	24.359 (16.632–35.678)
LBC (NILM)	363	26	80.8 (65.6–95.9)	92.6 (89.8–95.4)	45.7 (31.3–60.0)	98.4 (97.1–99.8)	0.867 (0.788–0.945)	52.416 (18.215–150.835)
LBC (ASCUS)	1503	147	74.1 (67.1–81.2)	88.3 (86.6–90.0)	40.7 (34.8–46.6)	96.9 (96.0–97.9)	0.812 (0.776–0.849)	21.594 (14.409–32.364)
LBC (LSIL)	523	71	71.8 (61.4–82.3)	85.6 (82.4–88.9)	44.0 (34.9–53.0)	95.1 (93.0–97.2)	0.787 (0.732–0.842)	15.182 (8.501–27.116)
hrHPV positive	1796	164	74.4 (67.7–81.1)	88.8 (87.3–90.4)	40.1 (34.6–45.6)	97.2 (96.3–98.0)	0.816 (0.782–0.851)	56.142 (15.778–33.943)
Age <50 years	1242	123	73.2 (65.3–81.0)	89.0 (87.2–90.8)	42.3 (35.6–48.9)	96.8 (95.7–97.9)	0.811 (0.771–0.851)	32.514 (14.918–70.862)
Age ≥50 years	615	44	79.5 (67.6–91.5)	89.3 (86.8–91.9)	36.5 (26.8–46.1)	98.3 (97.1–99.4)	0.844 (0.783–0.906)	22.084 (14.214–34.313)

Abbreviations: AUC, the area under the ROC curve; CI, confidence intervals; CIN3+, cervical intraepithelial neoplasia of grade 3 or worse; CISCER-positive, ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; hrHPV-positive, high-risk human papillomavirus positive; *N*, total numbers; *N* for CIN3+, the numbers of CIN3+; NPV, negative predictive value; OR, odds ratio; PPV, positive predictive value.

TABLE 3 CIN3+ immediate risk assessment based on various test results.

LBC result	hrHPV genotyping result	DNA methylation result	*N*	*n* of CIN3+	Absolute risk for CIN3+ (%)	Referrals needed to detect one CIN3+[a]
Minimally abnormal		/	1857	167	9.0 (7.7–10.4)	11.1
Minimally abnormal		CISCER+	309	125	40.5 (34.9–46.2)	2.5
Minimally abnormal		CISCER−	1550	42	2.7 (2.0–3.6)	36.9
NILM	hrHPV+	/	354	20	5.6 (3.5–8.6)	17.7
NILM	hrHPV+	CISCER+	41	16	39.0 (24.2–55.5)	2.6
NILM	hrHPV+	CISCER−	313	4	1.3 (0.3–3.2)	78.2
ASCUS	hrHPV−	/	28	1	3.6 (0.1–18.3)	28
ASCUS	hrHPV−	CISCER+	1	1	100.0 (2.5–100.0)	1
ASCUS	hrHPV−	CISCER−	27	0	0.0 (0.0–12.8)	/
ASCUS	hrHPV+	/	952	75	7.9 (6.2–9.8)	12.7
ASCUS	hrHPV+	CISCER+	151	57	37.7 (30.0–46.0)	2.6
ASCUS	hrHPV+	CISCER−	801	18	2.2 (1.3–3.5)	44.5
LSIL	hrHPV−	/	33	2	6.1 (0.7–20.2)	16.5
LSIL	hrHPV−	CISCER+	4	2	50.0 (6.8–93.2)	2
LSIL	hrHPV−	CISCER−	29	0	0.0 (0.0–11.9)	/
LSIL	hrHPV+	/	490	69	14.1 (11.1–17.5)	7.1
LSIL	hrHPV+	CISCER+	112	49	43.8 (34.4–53.4)	2.3
LSIL	hrHPV+	CISCER−	378	20	5.3 (3.3–8.1)	18.9

Abbreviations: ASCUS, atypical squamous cells of undetermined significance. CI, confidence interval; CIN3+, cervical intraepithelial neoplasia of grade 3 or worse; CISCER, ΔCt *PAX1* ≤ 6.6 or ΔCt *JAM3* ≤ 10.0; hrHPV-positive, high; risk human papillomavirus positive; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy.

[a]Referrals needed to detect one CIN3+, refers to the number of patients who need to be referred for further diagnostic procedures (such as colposcopy and biopsy) in order to identify one case of CIN3+.

abnormal cervical cancer screening test results were reported in approximately 9.25%.[5] In China, ASCUS was reported in approximately 9.5%–10.7%,[23] and ASCUS was reported in approximately 1.7%–5.8%. Referring all these women for colposcopy is cumbersome, concerning, and expensive; the past alternative of repeated cytologic testing for women diagnosed with ASCUS has been

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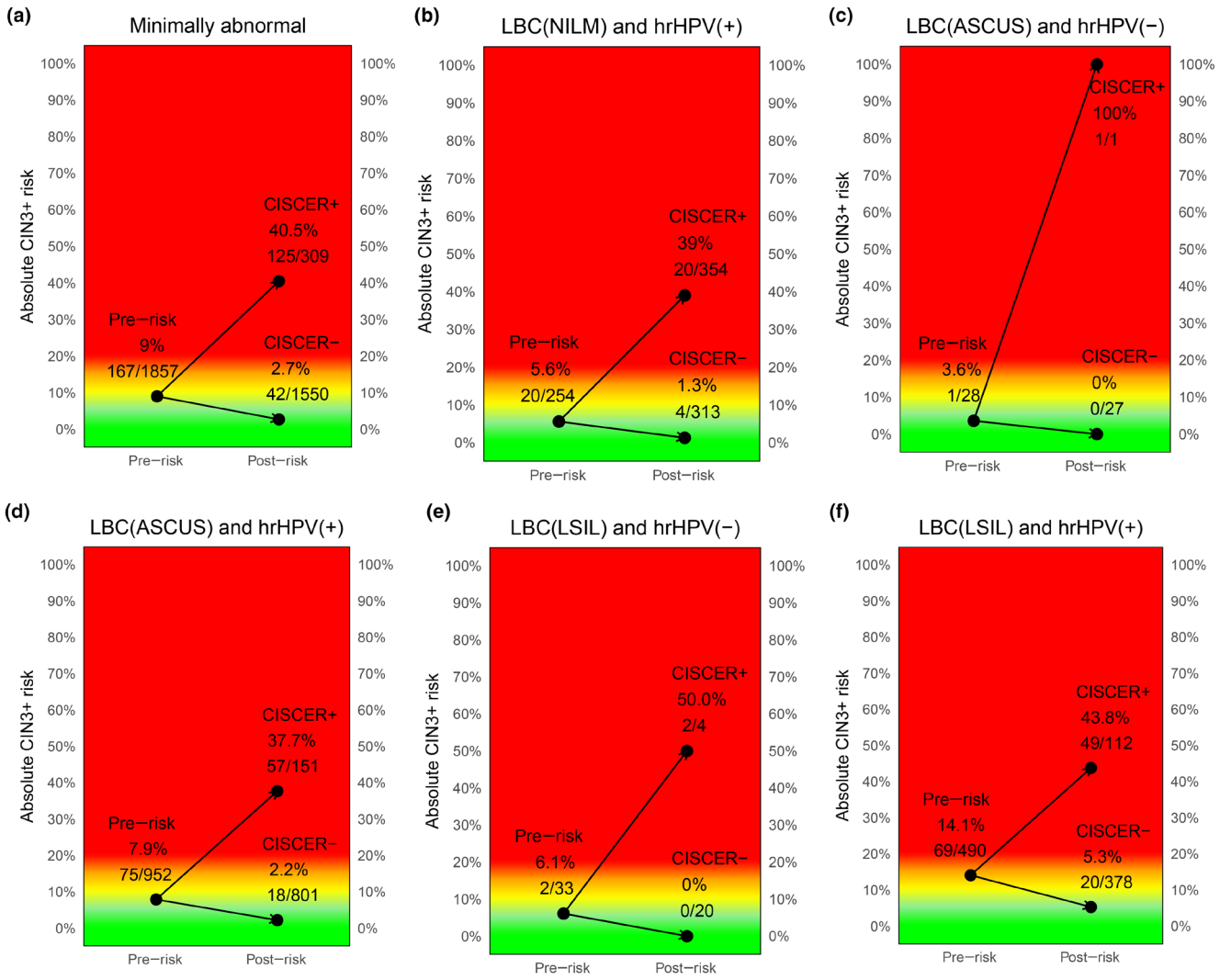

FIGURE 4 Pre- and post-test CIN3+ risk plots for CISCER triage strategies in different scenarios. Color legend: Green, low CIN3+ risk; orange, intermediate CIN3+ risk.

supplanted by hrHPV testing for secondary screening. However, the alternative of repeated cytologic testing is insufficient for preventing invasive cancers.[24] The adoption of hrHPV testing in combination with cytology provides sensitivity but results in complicated algorithms for clinical decision making.[4] More efficient triage solutions are still needed to avoid over-referral to colposcopy while ensuring proper follow up of higher-risk ASCUS cases. Based on this, methylation detection in exfoliated cervical cells may be a feasible approach. Detection of methylation of host genes can predict cervical carcinogenesis and progression and is a potential alternative to existing screening methods such as cytology and HPV testing.[25] In this study, methylation detection had higher specificity (89.1%) and NPV (97.3%) for CIN3+ in women with minimally abnormal cervical cancer screening results.

Existing studies have shown that for HPV(+) women with minor cytologic abnormalities, S5 detection and HPV16/18 have good triage performance for colposcopy diagnosis of CIN3+.[26] HPV infection and p16 methylation are high-risk factors for the progression of cytologic ASCUS/LSIL to HSIL.[27] *CGB3* and *NOP56* methylation can serve as triage tools for ASCUS. *LINE-1* and *Alu* methylation status can be used to triage ASCUS.[28] For cytologic LSIL, *ZNF582* methylation is the best triage tool and is superior to hrHPV.[10] For HPV(+) minor cytologic abnormalities (ASCUS or LSIL), *FAM19A4/miR124-2* methylation and/or HPV genotyping can significantly reduce colposcopy utilization.[11] For cytologic ASCUS, *WT1* and *PCDH10* methylation triage for detecting CIN3+ outperforms HPV testing.[29] *PAX1* methylation detection is superior to HC2 for triaging ASCUS or ASC-H,[30] and another study also found *PAX1* methylation detection to be better than HPV DNA for triaging ASCUS.[12] The results of this study suggest that methylation testing has a high diagnostic performance as a triage method for CIN3+ in women with minimally abnormal cervical cancer screening results, the AUC of CIN3+ was 0.820 (95% CI 0.786–0.854).

CIN3 is the most advanced precancerous lesion of the cervix and a direct precursor of cervical cancer. Detecting CIN3 in time provides an opportunity to treat these lesions and intervene before

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potential progression to invasive cancer.[31] In this study, the absolute CIN3+ risk was 9.0% in all women with minimally abnormal cervical cancer screening results. CISCER analysis discriminated well for minimally abnormal cervical cancer results, yielding a CIN3+ risk of 40.5% (95% CI 34.9%–46.2%) after a positive result and a CIN3+ risk of 2.7% (95% CI 2.0%–3.6%) after a negative result. Triage of women with minimally abnormal cervical cancer results using CISCER was effective in reducing the number of referrals needed to detect one CIN3+. Detecting one CIN3+ patient without triage using methylation required 11.1 referrals of women with minimally abnormal cervical cancer screening results. However, with CISCER triage, the detection of a CIN3+ patient required only 2.5 referrals of CISCER-positive women with minimally abnormal cervical cancer screening results. As an adjunct test, it can improve the specificity of screening and avoid excessive diagnostic procedures in low-risk women, which would be a large saving for each individual, hospital, and region, not only because of lower financial costs but also because of the overall lower clinical burden.

Older women (age 50+) tend to have a higher incidence of advanced cervical cancer, and their precancerous lesions may be more difficult to detect with standard screening approaches.[32,33] This is because postmenopausal cervical atrophy and associated anatomical changes present detection challenges for CIN3 lesions in women over 50 years, including poor cytology sampling,[34] reduced colposcopy visibility,[35] and lower accuracy of HPV testing.[36] This necessitates more effective screening approaches, such as methylation biomarker testing, to compensate for deficiencies in traditional methods and avoid missed detection of advanced lesions.[37] The results of this study indicate that the CISCER test has high sensitivity (79.5%) and specificity (89.3%) for CIN3+ in older women.

LSIL (also known as CIN1) are now recognized as a histologic diagnosis of benign viral replication that should be managed conservatively. The clinical course and biologic behavior of CIN2 are less well understood, whereas CIN3 is recognized as a true pre-invasive precursor with the potential to progress to cancer.[38] Treatment of CIN lesions is associated with cervical morbidity and preterm birth.[39] Therefore, the level of overtreatment should be kept as low as possible. In this study, we found that methylation was able to distinguish well between all levels of cervical lesions. The median of ΔCt *PAX1* and *JAM3* methylation were significantly different between the CIN1, CIN2, and CIN3 groups ($p < 0.01$), and the median of ΔCt *JAM3* methylation significantly differed between CIN3 and cervical cancer group ($p < 0.01$). In summary, the ability of *PAX1* and *JAM3* methylation assays to distinguish between different levels of cervical lesions supports their use in clinical practice to improve the accuracy of cervical cancer screening and management, minimizing overtreatment and ensuring timely intervention for high-risk lesions.

However, this study also has some points to be improved. First, not all women with minimally abnormal cervical cancer underwent colposcopy and histopathologic examination. This study included only those with histopathologic information, which may introduce a selection bias. Second, there was a lack of long-term follow up, so the subsequent development of methylation-positive cases was not described. Finally, the study sample was drawn from the gynecology outpatient clinic of a metropolitan general hospital. Hence, the results of this study need to be validated in a wider population. In conclusion, although there are still some limitations, this study provides some clinical experiences for the application of methylation markers in cervical cancer screening.

In conclusion, in women with a minimally abnormal cervical cancer screening result, the CISCER provides excellent detection of CIN3+. The use of CISCER in women with a report of minimally abnormal cervical cancer can lead to a substantial reduction in the number of direct colposcopy referrals.

AUTHOR CONTRIBUTIONS

L.L. and J.L. conceived the original idea for the study, interpreted results, carried out the statistical analysis, edited the paper, and were overall guarantors. X.S. obtained ethical approval, contributed to the preparation of the dataset, interpreted results, and contributed to drafts of the paper. L.K., Y.L., X.J., and P.L. contributed to the study design and interpretation of results, and commented on drafts of the paper. Y.Y. and H.W. conducted the pathologic evaluation and reviewed the original materials. All authors have approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are fully available within the article and its Supporting Information.

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