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The current state of DNA methylation biomarkers in self-collected liquid biopsies for the early detection of cervical cancer: a literature review

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Abstract

Cervical cancer (CC) is a preventable disease and treatable cancer. Most of the new cases and deaths from CC occur in Low- and Middle-Income Countries (LMICs) due to cultural and systematic barriers leading to low CC screening uptake. In recent years, self-sampling has been proposed as a method to increase CC screening uptake and is slowly being implemented into screening programmes worldwide. Simultaneously, DNA methylation has been proposed as a novel biomarker that could be used for the triage of self-collected samples that test positive for high-risk types of Human Papillomavirus (HPV). In this paper, we conducted a literature review of studies assessing the efficacy of DNA methylation markers to detect Cervical Intraepithelial Neoplasia (CIN) in self-collected cervicovaginal swabs or urine (2019–2024). Our review showed that, of the available data, DNA methylation together with self-sampling could perform as well as cytology in the detection of CIN as well as improve uptake of CC screening and reduce loss to follow up, especially in LMICs. However, more data is still needed to understand which methylation tests are most efficacious. Future studies should assess the full potential of DNA methylation and self-sampling in large, diverse screening cohorts.

Keywords Cervical Cancer screening, Self-sampling, Methylation, Triage, Urine, Cervicovaginal swab

Background

Cervical Cancer (CC) is a preventable and treatable cancer, yet it remains the fourth most diagnosed cancer and the fourth most prevalent among women aged 35–44 [1]. In 2018, the World Health Organization (WHO) reported that there were around 569,000 new cases diagnosed with 311,000 new deaths due to CC [2]. In 2022, the number of new cases and the number of deaths increased to 660,000 and 350,000, respectively [2]. Not only is the burden of cervical cancer increasing, but it is also increasingly inequitable. Low- and Middle-Income Countries (LMICs) accounted for 90% of new cases and deaths in 2022 [2]. The success of High-Income Countries (HICs) at controlling cervical cancer originates from

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their coordinated screening programmes and high capacity to recruit, screen, and follow up with screening-eligible women. In LMICs, resources limitations, insufficient funding, and cultural preferences underpin low uptake of screening for CC.

While cytology is primarily used in HICs to screen for Cervical Intraepithelial Neoplasia (CIN), most LMICs utilize a test and treat method using Visual Inspection with Acetic acid (VIA) [3]. Recently, however, HPV testing has been recognized as the new preferred method of cervical screening by the WHO, especially for young women [4]. Compared to VIA and cytology, HPV testing is a simpler test that is more objective and more cost-effective [4]. HPV testing has been implemented with high efficacy as a primary screening test for CC in multiple HICs [5] and has the potential to be as, if not more, effective in low and middle-income contexts. Another benefit of HPV testing is the opportunity to move toward self-collected samples. The COVID-19 pandemic has ushered in a new era of home testing [6]; there is increased trust and understanding around collection and shipment of self-collected specimens, and many countries have new infrastructure aimed at population-wide sample collection with centralized processing. In the context of cervical cancer screening, self-sampling provides many advantages such as flexibility of time and place of collection, increased bodily autonomy, and reduced demand on skilled clinicians [7]. Liquid biopsies for cervical cancer include both urine and cervicovaginal swabs, and both have high been validated across diverse populations to have high acceptance among women [8, 9]. With respect to the sample quality, it has been well documented that HPV testing in urine and swab samples yields a comparable sensitivity and specificity to that in clinician-collected material for the detection of CIN2 and higher (CIN2+) [7, 10, 11].

In addition to different testing modalities, understanding of the natural history of CC has also improved tremendously, especially the role of epigenetic alteration in driving disease progression. Among these epigenetic alterations, DNA methylation has been consistently shown to play an important role in the development of CC [12]. DNA methylation at specific sites of cytosine-guanine repeats (CpG islands) across the genome can silence tumor suppressor genes or activate oncogenes, depending on the affected locus, leading to the initiation or progression of CC. Multiple different methylation assays that were developed in cervical cells have been shown to correlate with disease severity, predict disease progression, and detect cervical pre-cancer with high accuracy when combined with HPV testing or cytology [13–15]. Two host gene panels have since been commercialized: GynTect (*ASTN1/DLX1/ITGA4/RXFP3/SOX17/ZNF671*; oncgnostics GmbH, Jena, Germany) and QIAsure (*FAM19A4*/miR124-2; Qiagen, Hilden, Germany). Another panel, the S5 classifier, uniquely assesses both host (EPB41L3) and viral (late regions of HPV genotypes 16, 18, 31, and 33) gene methylation (London, United Kingdom). The advantage of both the QIAsure and S5 classifier is their validation in worldwide cohorts of both high- and LMICs [16, 17]. In particular, the S5 classifier has also been optimized to address regional screening demands reflected by the variable distribution of HPV types globally and higher disease prevalence [18]. Next, a study on a long term cohort using the S5 classifier has also shown good potential in predicting hr-HPV positive women that are at the highest risk of developing into CIN3 [19]. Finally, S5 classifier has shown good potential as a triage strategy especially in women that are negative for the hr-HPV genotype of 16 and 18 which could play an important role in improving the colposcopy referral [20] .

In addition to other trained dual and triple markers, methylation panels such as these could aid in reducing the overtreatment of lesions suspected to regress and add objective clinical value to the assessment of the heterogenous CIN2 subset of lesions. More importantly, if combined with HPV-testing in self-collected material, methylation triage could enable high-grade CIN to be detected in one high-throughput, non-invasive test collected at home. With the encroaching 2030 WHO deadline, many studies have begun to examine not only which methylation markers perform best at diagnosing CIN, but also in what self-sample types can methylation analysis be performed; however, no manuscript to date has synthesized these results. Thus, this review seeks to interrogate the validity of methylation tests that have thus far been assessed in self-collected cervicovaginal swabs or urine samples to provide insight into the status of methylation testing in self-sampled material for CC screening as well as to highlight future steps needed for the use of both tools synergistically for global CC elimination.

Methods

A semi-systematic literature review was conducted between June 6th and June 24th, 2024. The search was conducted in PubMed, SCOPUS, and Google Scholar using the search terms self-sampling, cervical cancer screening, and methylation and their related MESH terms. Articles were included if they principally performed host or viral gene methylation analysis on cervicovaginal swab or urine samples collected by the patient. Included articles were also published in the last 5 years and conducted on a referral population, a screening population, or nonattending/under-screened women. Articles were only included if they reported a quantitative measure, including but not limited to sensitivity, specificity, or Area Under the Curve (AUC). Articles were

excluded if they focused on economic outcomes, were performed in a population niche (e.g. pregnant or immunosuppressed women) or used methylation for detection of uterine cancers other than the cervix. Grey literature, editorials, and review papers were also excluded from the search. Although vaginal lavage has been explored as a potential device for self-sampling, articles which assessed methylation in lavage samples were excluded due to the inferiority of this sampling device compared to urine and swab. All three searches were duplicated by Z. Yim.

Results

The literature search yielded a total of 1278 results, of which only 20 articles were included after title and abstract scan. A full text review was then conducted of each article leading to the exclusion of nine additional papers. This revealed a total of 11 articles that were consistent with the search criteria. Only three studies were conducted in LMICs (Papua New Guinea [21] and Thailand [22, 23]) and eight took place in Europe (Netherlands [24–29], Germany [30], Belgium [31]).

Of 11 studies, four examined urine samples and seven examined cervicovaginal swab sampling. One paper [26] assessed both swabs and urine. Less than half of the papers reported all three measures of AUC, sensitivity and specificity. Eight studies examined a CIN2/3+endpoint, three studies used an HSIL+endpoint (when final diagnosis was determined by cytology), and three studies used a cervical cancer endpoint. All studies except Van den. Helder et al. assessed their methylation markers on high-risk HPV-positive (hr-HPV+) self-samples [26].

A total of 31 individual gene markers were investigated in self-samples along with nine combination markers. Individual markers included one endocrine gene (Thyrotropin-Releasing Hormone, TRH), one RNA gene (miR124-2) and 29 tumor suppressor genes. Of these, 15 markers of which were confirmed by de Waard et al. to have had a sensitivity of ≥70% and specificity of ≥60% in clinician-collected samples in at least one study [24]. To date, the efficacy of the S5 classifier, which includes viral genes, has not yet been published in self-samples, thus all included markers are human host genes. Combination markers included two panels (*ANKRD18CP/LHX8/EPB41L3* and *ITGA4/ASCL1/FAM19A4*) that were developed through recursive partitioning to behave as good or better than cytology [24, 25]. One study utilized multivariate logistic regression with stepwise selection to obtain the optimal marker panel, *ASCL1/LHX8* [26]. Two studies also tested one or both of two commercially available methylation panels, GynTect and QIAsure, on cervicovaginal swabs [24, 30]. The remaining marker panels were selected for their superiority among earlier studies performed in clinician-collected cervical samples [21].

Does DNA methylation level correlate with disease severity in DNA collected from self-samples?

Fifteen gene markers (*ANKRD18PC*, *ASCL1*, *C13OFR18*, *EPB41L3*, *GHSR*, *JAM3*, *LHX8*, *PAX1*, *POU4F3*, *SOX1*, *SST*, *STGALNAC5*, *ZIC1*, *ZNF582*, and *ZSCAN1*) demonstrated that the methylation levels of self-samples increased significantly with increasing disease grade in at least one study (Supplementary Table 1). *ASCL1*, *GHSR*, *LHX8* and *SST* showed methylation correlated with lesion severity in four or more studies, including at least one using urine and one using cervicovaginal swab samples. Other genes were not reported on or showed inconsistency between methylation level and disease severity in one or more studies.

Can self-collected cervicovaginal swabs be tested for DNA methylation to stratify and detect cervical disease?

Seven studies from Netherlands, Germany, Papua New Guinea and Thailand reported on the efficacy of methylation triage in cervicovaginal swabs [21–25, 28, 30]. Five studies were conducted in screening populations of 43–593 persons and two studies were conducted in referral populations of 275–280 persons. All studies agreed on the use of dry brush sampling; three studies specified using the Evalyn Brush [24, 25, 31] and one study used the Cytobrush [21]. All methylation tests were performed on hr-HPV+self-samples.

CIN3+endpoint

Two studies reported on the efficacy of methylation triage of hr-HPV+self-collected cervicovaginal swabs from a screening population via AUC [24, 25]. There were ten individual markers and two combination panels that achieved an AUC of 0.7 or greater for CIN3+ (Table 1). Four studies quantified the performance of their methylation marker on cervicovaginal swab samples by sensitivity and specificity [24, 25, 28, 30]. Verhoef et al. [28] assessed the usefulness of *ASCL1* and *LHX8*, both separately and as a combined panel and reported a sensitivity and specificity of 51.2% and 80.5% for *ASCL1*, 65.1% and 69.2% for *LHX8*, and 73.3% and 61.2% for *ASCL1*/*LHX8* combination marker (Table 1). Similarly, de Waard et al. [24, 25] reported the sensitivity and specificity of the two currently available commercial assays, QIAsure and GynTect, to compare to their two trained classifiers, *ITGA4*/*ASCL1*/*FAM19A4* and *ANKRD18CP*/*LHX8*/*EPB41L3* [24, 25]. A comparison of this subset of markers revealed that *ITGA4*/*ASCL1*/*FAM19A4* had the highest sensitivity (84.0%) with a pre-set specificity of 70.0% while the GynTect assay had the highest specificity (91-95.5%) with a sensitivity of 31.6–59.0% [24, 30] (Table 2). Moreover, *ITGA4*/*ASCL1*/*FAM19A4* did not differ significantly in sensitivity or specificity from

Table 1 Performance of individual and combination host gene methylation markers in discriminating CIN3 + for self-collected cervicovaginal swabs by Area under the curve, sensitivity and specificity

Marker	*n*=	AUC	95% CI	Study
SOX17	304	0.615	0.543–0.687	de Waard, 2024
C13ORF18	304	0.587	0.514–0.659	de Waard, 2023
has-miR124-2	304	0.618	0.551–0.686	de Waard, 2024
ITGA4	304	0.622	0.550–0.695	de Waard, 2024
DLX1	304	0.625	0.553–0.697	de Waard, 2024
JAM3	304	0.635	0.565–0.705	de Waard, 2023
GHSR	304	0.645	0.576–0.714	de Waard, 2023
ANKRD18PC	304	0.646	0.575–0.718	de Waard, 2023
ASTN1	304	0.654	0.583–0.726	de Waard, 2024
RXPF3	304	0.67	0.599–0.741	de Waard, 2024
ST6GALNAC5	304	0.676	0.609–0.743	de Waard, 2023
PAX1	304	0.676	0.605–0.746	de Waard, 2023
SST	304	0.7	0.637–0.764	de Waard, 2023
EPB41L3	304	0.72	0.655–0.790	de Waard, 2023
ZNF671	304	0.761	0.695–0.826	de Waard, 2024
ZNF582	304	0.731	0.665–0.798	de Waard, 2023
POU4F3	304	0.745	0.682–0.808	de Waard, 2023
SOX1	304	0.746	0.680–0.812	de Waard, 2023
ZSCAN1	304	0.762	0.701–0.823	de Waard, 2023
LHX8	304	0.781	0.721–0.841	de Waard, 2023
ZIC1	304	0.787	0.729–0.844	de Waard, 2023
ASCL1	304	0.806	0.749–0.863	de Waard, 2023
ITGA4/ASCL1/FAM19A4	304	0.83		de Waard, 2024
ANKRD18CP/LHX8/EPB41L3	304	0.84		de Waard, 2023

Marker	Pop	n=	Sens.	95% CI	Spec.	95% CI	Study
GynTect	Referral	68	31.6%	15–54%	95.9%	85–99%	Klischke, 2021
ASCL1	Screening	593	51.2%	40.6–61.7%	80.5%	77.0-83.9%	Verhoef, 2023
GynTect	Screening	304	59.0%		91.0%		de Waard, 2024
QIAsure	Screening	304	65.0%		72.0%		de Waard, 2024
LHX8	Screening	593	65.1%	55.0-75.2%	69.2%	65.2–73.2%	Verhoef, 2023
ASCL1/LHX8	Screening	593	73.3%	63.9–82.6%	61.1%	56.9–65.4%	Verhoef, 2023
ANKRD18CP/LHX8/EPB41L3	Screening	304	82.0%		74.0%		de Waard, 2024
ITGA4/ASCL1/FAM19A4	Screening	304	84.0%		70.0%		de Waard, 2024

N: population size; AUC: area under the curve; CI: confidence interval

ANKRD18CP/*LHX8*/*EPB41L3*, but its sensitivity was significantly higher than that of both commercial assays [24].

Other endpoints

Only one gene (miR124-2) was assessed for all endpoints. miR124-2 performed statistically similarly but with increasing accuracy in the detection of HSIL+, CIN3+, and cervical cancer [24, 31]. *ASCL1* performed the best for both CIN2+and CIN3+endpoints with AUC of 0.758 and 0.806, respectively [25] (Supplementary Table 2). Molano et al. and Oranratanaphan et al. also published the sensitivity and specificity of single, dual and triple marker panels for diagnosing HSIL+in cervicovaginal swabs. Of these markers, the panel of miR124-2/*MAL*/*CADM1* had the highest sensitivity (90.9%), but a poor specificity (23.8%) (Supplementary Table 3). For all combination markers except miR124-2/*MAL*, high sensitivity (81.8-100%) was achieved by sacrificing specificity (4.8-23.8%) (Molano et al., 2024). This has important implications for the utility of each test in LMICs. Although a high sensitivity ensures that cases of HSIL+, especially cervical cancer, are captured, a low specificity could amplify resource strain. Given the small sample size and low precision of the data, however, further investigation into the accuracy of these markers is still needed.

Can urine samples be tested for DNA methylation to stratify and detect cervical disease?

Four studies in Netherlands and Belgium reported on the usefulness of methylation markers to triage urine samples [26, 27, 29, 31]. Three studies examined hr-HPV+urine

Table 2 Methylation detection of CIN3 + in hrHPV + urine samples from referral populations

Marker	*n*=	AUC	95% CI	Study
ST6GALNAC5	33	0.628	0.453–0.804	Van Keer, 2021
ZIC1	33	0.558	0.400-0.742	Van Keer, 2021
ZIC1	74	0.62	0.47–0.77	van den Helder, 2020
ZIC1	245	0.72	0.66–0.78	van den Helder, 2022
LHX8	74	0.68	0.54–0.82	van den Helder, 2020
LHX8	33	0.682	0.465–0.842	Van Keer, 2021
LHX8	245	0.78	0.72–0.83	van den Helder, 2022
SST	33	0.634	0.473–0.809	Van Keer, 2021
SST	245	0.72	0.65–0.79	van den Helder, 2022
SST	74	0.73	0.59–0.86	van den Helder, 2020
GHSR	74	0.7	0.56–0.84	van den Helder, 2020
GHSR	33	0.769	0.561–0.897	Van Keer, 2021
GHSR	245	0.79	0.72–0.84	van den Helder, 2022
ASCL1	33	0.566	0.473–0.849	Van Keer, 2021
ASCL1	74	0.79	0.67–0.91	van den Helder, 2020
ASCL1	245	0.83	0.77–0.88	van den Helder, 2022
ASCL1/LHX8	245	0.84	0.78–0.89	van den Helder, 2022

N: population size; AUC: area under the curve; CI: confidence interval

samples [27, 29, 31]. All four studies used AUC as their final quantifier and utilized a population of 33–245 persons. Only one study used first void urine [31]. The remaining three studies extracted DNA from the sediment of full void urine [26, 27, 29]. A total of 8 individual host genes (*ASCL1*, *FAM19A4*, *GHSR*, *LHX8*, *PHACTR3*, *PRDM14*, *SST*, *ST6GALNAC5*, and *ZIC1)* and one combination panel (*ASCL1*/*LHX8*) were assessed in urine.

CIN3 + endpoint

All markers achieved an AUC of 0.7 or more in at least one study except *STGALNAC6* (Table 2). Among these genes, the combination of *ASCL1*/*LHX8* performed the best, followed by *ASCL1* [26]. Variability in AUC across studies is most likely due to discrepancies in sample size.

Other endpoints

For a CC endpoint, the best performing marker was *LHX8*, with an AUC of 0.97 [27] (Supplementary Table 4). Three host genes, *GHSR*, *ZIC1*, and *SST*, were evaluated in both Snoek et al. and Van den Helder et al. and showed highly similar results [26, 29]. All markers (*FAM19A4*, *GHSR*, *PRDM14*, *ZIC1*, *ASCL1*, *SST* and *LHX8*) except *PHACTR3* achieved an AUC of greater than 0.8 [26, 29]. Van Keer et al. reported on the ability of six individual host genes to discern HSIL + from ≤HSIL [31]. All genes, *ZIC1*, *LHX8*, *ST6GALNAC5*, *ASCL1*, *GHSR*, and *SST*, reported an AUC of less than 0.7 (Supplementary Table 4). This may reflect variation in sample size or inconsistencies in cytological grading for HSIL+, as other markers (*GHSR* and *LHX8*) distinguished CIN2 + from ≤CIN2 with an AUC of 0.801 and 0.763, respectively [29]. Thus, retesting these markers in a study with higher power is necessary.

Does DNA methylation in self-sampled material reflect that of clinician-collected cervical scrapes?

Only one study directly compared the validity of their methylation tests in cervicovaginal swabs with clinician-collected samples [21]. Molano et al. demonstrated that the sensitivity and specificity of methylation markers (*MAL*, miR124-2, *CADM1*, and their combinations) in clinician-collected samples was slightly higher than in cervicovaginal swabs but statistically insignificant [19] (Supplementary Table 6). Another study published data on the performance of *CCNA1* methylation in an earlier study [32]. Comparison of clinician-samples and self-collected samples revealed well-coordinated sensitivity (19.0% and 19.7%) and specificity (99.3% and 99.54%) [22, 33].

Alternatively, multiple studies quantified how well the methylation level of 8 genes (*ASCL1*, *SST*, *GHSR*, *ZIC1*, *PRDM14*, *LHX8*, *FAM19A4*, and *PHACTR3*) correlated between self-samples and clinician-collected cervical scrapes by Spearman coefficient (Table 3). Correlation between self-samples and clinician-collected samples ranged from weak to strong (0.42–0.717), with most having a moderate agreement. *LHX8* has the highest correlation with clinician-collected samples in cervicovaginal swabs [26] while *PHACTR3* had the highest correlation in urine [29]. Host genes that were assessed in multiple studies generally had similar correlation level, with slightly lower coordination seen in studies with a larger population size.

Importantly, some discrepancy in the methylation level of self-samples compared to clinician samples is expected and can be attributed by the heterogeneity of cells collected in the self-sample. However, variability in sample correlation may also highlight the quality of sample preparation or the sensitivity of different assays to DNA concentration. Considerable debate has occurred around how to optimize self-sample processing to preserve DNA quality and is reflected in the diverse methods reported of the included studies. Of seven studies that used dry brush collection, four quoted suspending the brush in 20mL of ThinPrep PreservCyt media during sample preparation [24, 25] and two studies used only 1.5mL [26, 28]. Of the four studies investigating urine, two used an EDTA preservative [26, 27] and one used Universal Collection Medium as a preservative [31]. Only Van Keer et al. processed the samples immediately upon collection whereas other self-samples were transported at room temperature and processed within, on average, 72 h [31]. Eventually it will be important to confirm the validity of methylation tests under the conditions that can be achieved in screening programmes.

Table 3 Reported Spearman correlation coefficient for DNA methylation levels in urine or cervicovaginal swab self-samples as compared to clinician-collected cervical scrapes and in urine as compared to cervicovaginal swabs

Marker	Sample	Comparator	Spearman Co.	Significance	Study
ASCL1	Urine	LBC	0.42	$p < 0.001$	Van den Helder, 2022
SST	Urine	LBC	0.46	$p < 0.001$	Van den Helder, 2022
GHSR	Urine	LBC	0.48	$p < 0.001$	Van den Helder, 2022
ZIC1	Urine	LBC	0.5	$p < 0.001$	Van den Helder, 2022
PRDM14	Urine	LBC	0.508		Snoek, 2019
LHX8	Urine	LBC	0.52	$p < 0.001$	Van den Helder, 2022
GHSR	Urine	LBC	0.543		Snoek, 2019
LHX8	Swab	LBC	0.55		Verhoef, 2023
ASCL1	Swab	LBC	0.56	$p < 0.001$	Van den Helder, 2022
ASCL1	Swab	LBC	0.563		Verhoef, 2023
ZIC1	Swab	LBC	0.59	$p < 0.001$	Van den Helder, 2022
SST	Swab	LBC	0.6	$p < 0.001$	Van den Helder, 2022
GHSR	Swab	LBC	0.61	$p < 0.001$	Van den Helder, 2022
ZIC1	Urine	LBC	0.613		Snoek, 2019
SST	Urine	LBC	0.621		Snoek, 2019
LHX8	Swab	LBC	0.63	$p < 0.001$	Van den Helder, 2022
FAM19A4	Urine	LBC	0.674		Snoek, 2019
PHACTR3	Urine	LBC	0.717		Snoek, 2019
SST	Urine	Swab	0.59	$p < 0.001$	Van den Helder, 2022
ASCL1	Urine	Swab	0.61	$p < 0.001$	Van den Helder, 2022
ZIC1	Urine	Swab	0.61	$p < 0.001$	Van den Helder, 2022
LHX8	Urine	Swab	0.62	$p < 0.001$	Van den Helder, 2022
GHSR	Urine	Swab	0.65	$p < 0.001$	Van den Helder, 2022

LBC: liquid based cytology sample

How do the methylation results compare between urine and cervicovaginal swab self-samples?

CIN3 + endpoint

Four studies, one using cervicovaginal swab and three using urine, assessed the same five different host genes (*GHSR*, *SST*, *ZIC1*, *LHX8*, and *ASCL1*) and two studies, one swab and one urine, assessed *ST6GALNAC5* (Table 4). Both urine and cervicovaginal swab yielded similar AUCs for the same marker. Where variability in AUC was observed, there were notable differences in population size.

Other endpoints

Concerning the HSIL+and CC endpoint, no studies of urine and swab self-samples assessed the same methylation markers. Two studies, one swab and one urine, assessed the performance of the same five markers (*SST*, *ZIC1*, *ASCL1*, *LHX8*, and *ST6GALNAC5*) in diagnosing CIN2+ [25, 31] (Supplementary Table 2). The agreement of AUC for swab and urine samples was best for *LHX8* (0.733 and 0.76, respectively) and worst for *ZIC1* (0.728 and 0.62, respectively) [25, 31].

Methylation level

One study assessed how the methylation level of 5 genes correlated between cervicovaginal swab and urine self-samples by Spearman coefficient [26] (Table 3). The result suggests that *GHSR* has the highest correlation (0.65) between these samples with moderate correlation (0.59–0.62) for the remaining genes [26].

Future studies contrasting the performance of swabs and urine will be useful to inform longitudinal guidance about self-sample quality for methylation analysis. Country capacity for laboratory analysis and international agreement around sample processing will also be critical to consider. To this effect, there may be some preference towards swab-based self-sampling as multiple countries have already begun to implement dry swab sampling for HPV testing into their cervical cancer screening programmes [34]. Nonetheless, future studies contrasting the two types should optimize their sample processing methods to both HPV genotyping and DNA methylation testing.

Could methylation testing be paired with another triage strategy to improve the validity of cervical screening from self-collected samples?

Multiple alternative methods of triage have been examined as candidates for a more accessible and more objective cervical screening test, including HPV genotyping, miRNA analysis, and viral load, and most have been validated in studies using self-collected samples. Two papers in this review assessed if the sensitivity and specificity of their given methylation test was increased by

Table 4 Comparative performance of host gene methylation markers to discern CIN3 + in urine and cervicovaginal swab self-samples

Marker	Sample type	*n*=	Population	AUC	Confidence interval (95%)	Study
GHSR	Urine	74	Referral	0.7	0.56–0.84	van den Helder, 2020
GHSR	Cervicovaginal swab	304	Screening	0.645	0.576–0.714	de Waard, 2023
GHSR	Urine	33	Referral	0.769	0.561–0.897	Van Keer, 2021
GHSR	Urine	245	Referral	0.79	0.72–0.84	van den Helder, 2022
ST6GALNAC5	Urine	33	Referral	0.628	0.465–0.725	Van Keer, 2021
ST6GALNAC5	Cervicovaginal swab	304	Screening	0.676	0.609–0.627	de Waard, 2023
SST	Urine	74	Referral	0.73	0.59–0.86	van den Helder, 2020
SST	Urine	33	Referral	0.634	0.473–0.809	Van Keer, 2021
SST	Cervicovaginal swab	304	Screening	0.7	0.637–0.764	de Waard, 2023
SST	Urine	245	Referral	0.72	0.65–0.79	van den Helder, 2022
ZIC1	Urine	74	Referral	0.62	0.47–0.77	van den Helder, 2020
ZIC1	Urine	33	Referral	0.558	0.400–0.742	Van Keer, 2021
ZIC1	Urine	245	Referral	0.72	0.66–0.78	van den Helder, 2022
ZIC1	Cervicovaginal swab	304	Screening	0.787	0.729–0.863	de Waard, 2023
LHX8	Urine	74	Referral	0.68	0.54–0.82	van den Helder, 2020
LHX8	Urine	33	Referral	0.682	0.465–0.842	Van Keer, 2021
LHX8	Urine	245	Referral	0.78	0.72–0.83	van den Helder, 2022
LHX8	Cervicovaginal swab	304	Screening	0.781	0.721–0.841	de Waard, 2023
ASCL1	Urine	74	Referral	0.79	0.67–0.91	van den Helder, 2020
ASCL1	Urine	33	Referral	0.566	0.473–0.849	Van Keer, 2021
ASCL1	Cervicovaginal swab	304	Screening	0.806	0.749–0.863	de Waard, 2023
ASCL1	Urine	245	Referral	0.83	0.77–0.88	van den Helder, 2022

adding HPV genotyping [21, 28]. Molano et al. additionally tested its dual triage test against clinician-collected cervical scrapes and found good correlation with self-samples regardless of triage strategy [21]. Uniformly, the addition of HPV16/18 genotyping and extended genotyping to methylation of single and dual combinations of miR124-2, *MAL*, and *CADM1* lead to increased sensitivity with drastically reduced specificity in the detection of HSIL+ [21] (Table 4 and Supplementary Table 5). For *MAL*, miR124-2 and miR124-2/*MAL*, specificity dropped more than 50% points with the addition of HPV16, 18, 31, 33, 45, 52, and 58 genotyping for both self-samples and clinician-collected samples. Where sensitivity was modest (32-57.1%, *MAL*), the decline in specificity lead to a rise in sensitivity of more than 40% (95.5–100%) [21]. Where sensitivity was already high (77.3–81.0%, miR124-2 and 81.8–90.5%, miR124-2/*MAL*), sensitivity rose less than 20% [21]. This effect was also seen to a lesser extent with the addition of HPV16/18 genotyping to methylation and may reflect the low study power. In the detection of CIN3+, Verhoef et al. documented an increase in sensitivity of *ASCL1*/*LHX8* (73.3–88.9%) with minimal loss in specificity (61.1–57.0%) with the addition of HPV 16/18 genotyping in a population of 593 persons [28].

Discussion

Globally, there has been a move towards self-testing for cervical cancer screening. A recent study revealed that at least 27 countries have implemented or plan to implement national guidance for the use of self-sampling for hr-HPV testing to replace cytology or VIA. Nonetheless, most countries still employ cytology as a triage test. While hr-HPV testing will have a significant impact on increasing surveillance of among non-attending women, the prevalence of cervical HPV among women remains high at 14–24%. As such, many women will still need to be recalled for a smear test in the clinic. However, since the initiation of hrHPV self-sampling as a first line test in the Netherlands in 2017, 10–20% of those women who are recalled for cytology are lost to follow up [28]. Taken together, there is still room to optimize the global monitoring of this preventable and inequitable disease.

This review argues that there is an opportunity to do so with methylation triage. To date, 40 host gene methylation markers or marker panels have been investigated in self-collected samples as candidates for the triage of women who test hr-HPV+on during screening. This report demonstrates that not only do a wide range of candidate markers perform comparably to clinician-collected samples in both urine and swab self-samples, but also methylation triage of hr-HPV+self-samples could perform as well as the current mainstay tests at detecting disease. Despite significant variation among methylation markers, the best performing marker in self-swabbed

material (*ITGA4/ASCL1/FAM194A*) [24] outcompeted the pooled sensitivity of cytology (72.9%) [7] and the pooled sensitivity of VIA in LMICs (72.3%) [3] for the detection of CIN3+. This marker demonstrated superior sensitivity to both the GynTect and QIAsure commercial assays. Unpublished data suggests that the performance of the S5 classifier may outperform the panel by de Waard et al. [24], yielding a CIN3+sensitivity of 85.27% in dry cervicovaginal swab (AUC=0.84). Across all three methylation markers, GynTect had the superior specificity. This review provides further evidence that exploration into the optimal methylation marker, sample type, and processing methods is warrented. Implementation of methylation triage of hr-HPV+self-samples has the potential to increase screening attendance, reduce loss to follow up, and progress LMICs toward the goal of CC elimination by the next century.

There were multiple limitations in this review. In general, there was poor sample variability and global representation. HICs, specifically the Netherlands, were far overrepresented. Due to the recent incorporation of self-sampling into the Netherlands' population screening guidelines, there has been an increase in studies using their screening cohort; data from six of nine articles from European nations were produced in the Netherlands. There were also two sets of two papers which were published by the same research group [24–27]. Moreover, AUC, specificity and sensitivity were not used consistently across all studies; this, in addition to the differences in population type, self-sample type, population size, and endpoint, made cross-marker comparison often not possible. It is also important to note that cytological grading of CIN2 lesions is often inconsistent and final diagnoses made using cytology are inferior to histology; thus, conclusions drawn from the HSIL+endpoint may have limited utility. Another disadvantage was the divergence in population cohorts used by studies assessing urine versus those assessing swabs. All urine studies were conducted in referral populations and, as a result, we could expect slightly inferior AUCs to be seen in urine when tested in a population with a lower prevalence of high-grade CIN. Future studies should seek to repeat the analysis of the most successful markers with more power, concentrating on LMICs. Ultimately, more unified guidelines around self-sampling collection and processing strategies will aid countries in their decision to (or not to) implement this testing modality and continued exploration for the most robust markers with efficacy in both HICs and LMICs pre- and post-vaccination cohorts could rapidly progress us toward global cervical cancer elimination [35, 36].

Supplementary Information

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Supplementary Material 1

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Author contributions
E.S. conducted the literature review, data analysis, prepared main text and supplementary tables, and wrote the main manuscript. Z.Y. duplicated the literature search and contributed to the writing of the background and abstract. All authors reviewed the manuscript. HM and BN reviewed the paper and contributed to the design of the study.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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