Received: 20 September 2023 | Revised: 9 January 2024 | Accepted: 8 February 2024

DOI: 10.1002/ijc.34913

RESEARCH ARTICLE

Cancer Epidemiology

IJC INTERNATIONAL JOURNAL of CANCER | UICC

Long-term prediction by DNA methylation of high-grade cervical intraepithelial neoplasia: Results of the ARTISTIC cohort

Clare Gilham[1] | Belinda Nedjai[2] | Dorota Scibior-Bentkowska[2] | Caroline Reuter[3] | Rawinder Banwait[4] | Adam R. Brentnall[2] | Jack Cuzick[2] | Julian Peto[1] | Attila T. Lorincz[2]

[1]Faculty of Epidemiology and Population Health, London School of Hygiene & Tropical Medicine, London, UK

[2]Wolfson Institute of Population Health, Queen Mary University of London, London, UK

[3]Wolfson Institute for Biomedical Research, University College London, London, UK

[4]Blizzard Institute, Centre for Genomics and Child Health, Queen Mary University of London, London, UK

Correspondence
Belinda Nedjai and Attila T. Lorincz, Queen Mary University of London, London, UK, Email: b.nedjai@qmul.ac.uk and a.lorincz@qmul.ac.uk

Funding information
Health Technology Assessment Programme, Grant/Award Number: 15.59.47; Cancer Research UK, Grant/Award Numbers: C8162/A4609, C8162/A10406, C569/A10404, C236/A

Abstract

Methylation markers have shown potential for triaging high-risk HPV-positive (hrHPV+) women to identify those at increased risk of invasive cervical cancer (ICC). Our aim was to assess the performance of the S5 DNA methylation classifier for predicting incident high-grade cervical intraepithelial neoplasia (CIN) and ICC among hrHPV+ women in the ARTISTIC screening trial cohort. The S5 classifier, comprising target regions of tumour suppressor gene *EPB41L3* and L1 and L2 regions of HPV16, HPV18, HPV31, and HPV33, was assayed by pyrosequencing in archived hrHPV+ liquid-based samples from 343 women with high-grade disease (139 CIN2, 186 CIN3, and 18 ICC) compared to 800 hrHPV+ controls. S5 DNA methylation correlated directly with increasing severity of disease and inversely with lead time to diagnosis. S5 could discriminate between hrHPV+ women who developed CIN3 or ICC and hrHPV+ controls (p <.0001) using samples taken on average 5 years before diagnosis. This relationship was independent of cytology at baseline. The S5 test showed much higher sensitivity than HPV16/18 genotyping for identifying prevalent CIN3 (93% vs. 61%, $p = .01$) but lower specificity (50% vs. 66%, p <.0001). The S5 classifier identified most women at high risk of developing precancer and missed very few prevalent advanced lesions thus appearing to be an objective test for triage of hrHPV+ women. The combination of methylation of host and HPV genes enables S5 to combine the predictive power of methylation with HPV genotyping to identify hrHPV-positive women who are at highest risk of developing CIN3 and ICC in the future.

KEYWORDS

cervical screening, genotyping, HPV, methylation

Clare Gilham and Belinda Nedjai contributed equally to this work.

What's new?

Most human papillomavirus infections do not lead to pre-cancer, so a test is essential to triage high-risk HPV-positive women for treatment or further testing. While methylation markers have shown some success in triaging high-risk HPV samples for prevalent disease, their potential for long-term prediction of incident CIN3 and invasive cancer has yet to be revealed. In this case-control study, S5 DNA methylation correlated with increasing disease severity, and inversely with lead time to diagnosis. The S5 classifier could discriminate between high-risk HPV-positive women who developed CIN3 or invasive cancer and high-risk HPV-positive controls on average 5 years before diagnosis.

1 | INTRODUCTION

Primary HPV testing is increasingly regarded as the preferred approach to cervical screening.[1,2] HPV testing is highly sensitive, moderately specific, and objective. There are many different testing platforms available, including fully automated versions in the USA and Europe, as well as low-tech options to address needs in the better-funded of the low-resource settings.[3] Most HPV infections do not lead to precancer, so a test is essential in any HPV screening setting to triage high-risk HPV (hrHPV) positive women for treatment or further testing.[4] Cytology is the default triage test in many countries, including the United Kingdom[5] and the Netherlands, mainly because of a long history and proven adequate performance. However, referring all hrHPV-infected women with borderline/ASCUS or low-grade cytology entails excessive colposcopy referrals.[6,7] In some countries, self-sampling for HPV testing has been introduced into screening programmes[8] but triage with cytology requires hrHPV-positive women to attend a clinic for a cervical cytology sample to be taken. This limitation of cytology triage creates additional costs and can lead to a substantial loss-to-follow-up.[9] Referral to colposcopy for HPV 16 and/or HPV 18 or to cytology for other hrHPVs is used by the Australian cervical screening programme.[10]

A sensitive molecular triage for hrHPV-infected women with better specificity than cytology that can be done on clinician and self-taken samples is urgently needed to improve cervical screening worldwide. Although several potential prognostic tests have been evaluated, no optimum triage strategy has been identified.[4] DNA methylation of host and viral genes has emerged as a promising biomarker that can distinguish between advanced transforming CIN3 and lesser lesions, including CIN2 or CIN3 with low methylation levels, which have a low risk of becoming cervical cancer.[11] Many studies have shown that prevalent CIN3 can be detected with high accuracy using host methylation.[12–14] DNA methylation has also been reported to increase with disease progression over time,[15–17] allowing this epigenetic event to be used as a temporal biomarker, with a potential not only to accurately detect prevalent CIN3 and cancer but also to predict whether hrHPV infection will progress to high-grade disease and cancer.[18–20] DNA methylation testing is feasible on other types of samples including self-collected vaginal swabs and urine,[14] and could become the preferred option for triage of hrHPV-positive women.[21] An ability to identify women at an earlier stage of their disease progression, if combined with acceptable specificity, might be used cost-effectively to prevent invasive cancer and hence minimise the need for radical treatments.

As well as testing for methylation on the host tumour suppressor gene *EPB41L3*,[22] the S5 DNA-methylation classifier also tests for methylation of the viral late genes (L1 and L2) of HPV16, HPV18, HPV31, and HPV33.[23] The main aim of our study was to evaluate the ability of S5 to predict progression of hrHPV-positive women to CIN3 or cancer. We used archived liquid-based cytology (LBC) material from the 20-year longitudinal ARTISTIC screening trial cohort and compared methylation results to follow-up data on clinical outcomes.

2 | MATERIALS AND METHODS

2.1 | ARTISTIC cohort

Women attending routine cervical screening were recruited to the ARTISTIC trial in Greater Manchester, UK in 2001–2003. LBC samples were collected for cytology and HPV testing, and women were randomly allocated in a ratio of 3:1 to have the HPV result revealed and acted upon or concealed until the end of the trial in 2009. Histological diagnoses of CIN2+ were obtained from local laboratories until 2009, and CIN3 and cancer diagnoses have been notified through national cancer registration until 2020. The majority of disease identified within the ARTISTIC cohort was diagnosed following abnormal cytology, while a small number of cases were identified following referral on the basis of HPV screening alone in the revealed arm of the trial. Women were screened routinely every 3 years, those testing HPV positive (with hybrid capture 2 (HC2)) in the revealed arm were recalled after 12 and 24 months, and all those with low-grade abnormal cytology were recalled every 6 months. Thus, women provided multiple samples over the 8-year trial period. ARTISTIC women are unlikely to have been vaccinated against HPV since the cohort were all aged over 25 when the school-based vaccination programme was started in the United Kingdom in 2008. A detailed description of the trial intervention and management protocol has been published elsewhere.[24]

2.2 | HPV detection

Following the trial protocol, samples were classified as HPV positive based on the result of the HC2 test (Qiagen, Hilden, Germany), and then HC2-positive samples were genotyped for hrHPV. Three genotyping

assays were used at various stages of the trial: Line Blot Assay (Roche Molecular Diagnostics, Pleasanton, CA, USA), PapilloCheck (Greiner Bio-One GmbH, Frickenhausen, Germany), and Linear Array (Roche, Molecular Diagnostics, Pleasanton, CA, USA). Samples positive for hrHPV detected by any of the assays were included in the analyses.

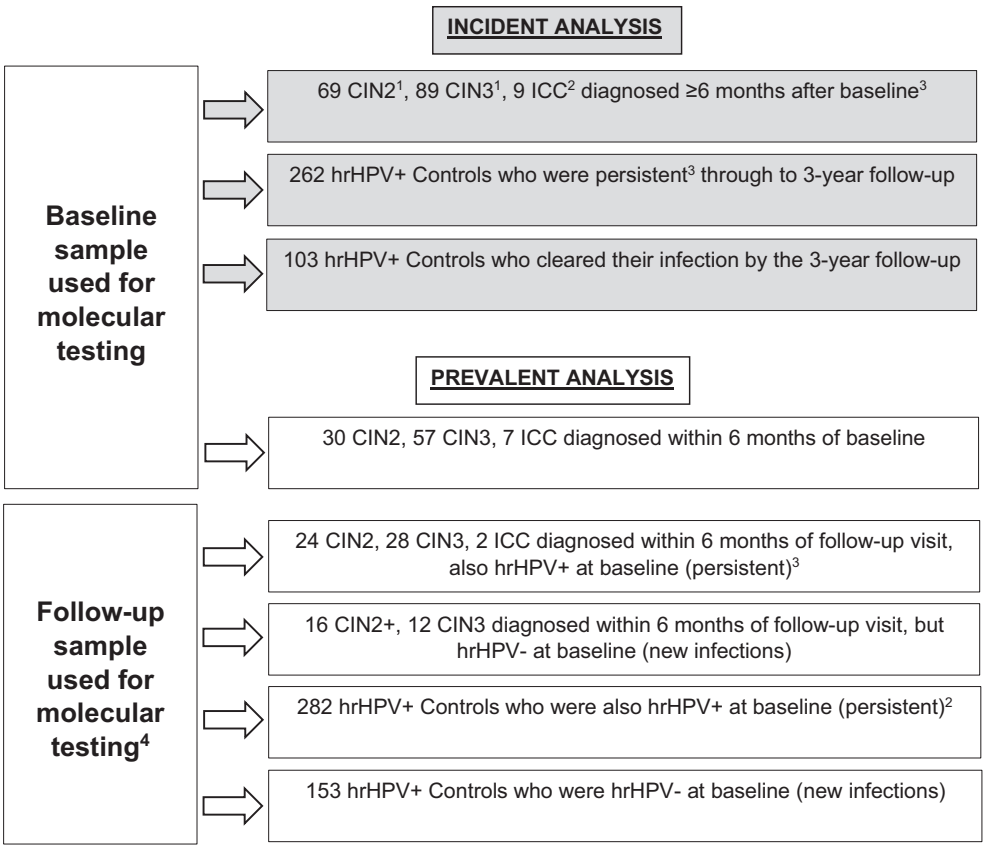
2.3 | Sample selection

The primary aim of this case-control study was to evaluate the performance of S5 to predict incident cervical intraepithelial grade 2, grade 3, and invasive cervical cancer (CIN2+) among hrHPV-positive cervical samples. The disease endpoint was defined as incident if it was diagnosed at least 6 months after the baseline sample. Baseline samples were analysed from 167 CIN2+ cases (69 CIN2, 89 CIN3, and 9 ICC) and 365 controls including 262 women whose hrHPV infections persisted for at least 3 years and 103 who had cleared their infections by the follow-up round about 3 years later (Figure 1, Table S1). A secondary aim was to evaluate the performance of S5 to detect prevalent CIN2 and CIN3+ using a sample taken within 6 months of the histological diagnosis. A sample of 94 women with CIN2+ diagnosed at baseline (30 CIN2, 57 CIN3, and 7 ICC) and 82 diagnosed later in the trial (40 CIN2, 40 CIN3, and 2 ICC) were compared to follow-up control samples from women with both persistent ($n = 282$) and new ($n = 153$) hrHPV infections (Figure 1, Table S1). Thus, only hr-HPV-positive samples were analysed.

Controls did not have any identified pre-cancer during the study and were randomly selected, stratified by hrHPV results at entry and follow-up screening round, and by year of birth (born before or after 1960) to frequency-match the cases; additional samples from hrHPV persistent controls were also assayed (Table S1). Women were aged between 20 and 66 years at the time of their sample collection, with a median age of 30 years. Some women contributed different samples to both prevalent and incident analyses (19 CIN2, 22 CIN3, 2 ICC, and 246 controls).

2.4 | Measurement of S5 DNA methylation classifier

The S5 methylation classifier and methods have been described in detail elsewhere[23]: DNA was extracted from aliquots of the LBC samples with the QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany)



[1] Samples from 26/69 CIN2 and 18/89 CIN3 were diagnosed 6-30 months after baseline, the remaining 43 CIN2 and 71 CIN3 were persistently hrHPV positive and diagnosed >30 months after the baseline sample.
[2] hrHPV+ baseline samples from 9 ICC ranged from 6.4 – 15.7 years before cancer diagnosis, median 12.1 years.
[3] S5 results were obtained from both baseline and follow-up samples from 19 women with CIN2, 22 women with CIN3, 2 with ICC and 246 controls. These women thus contributed to both analyses.
[4] Median time from baseline to follow-up was 3.1 years for controls

FIGURE 1 Numbers of samples taken from CIN2+ cases and control women included in the incident and prevalent analyses.

and bisulfite conversion of genomic DNA was done using 200ng DNA with the EZ DNA Methylation Kit (Zymo Research, CA, USA) following the manufacturer's instructions. We used previously optimised PCR conditions for the markers included in the S5 classifier. The S5 classifier targets CpGs in the promoter region of *EPB41L3* (CpG sites 425, 427, and 438 relative to transcription start site) and viral regions of HPV16 (L1: CpG sites 6367, 6389 and L2: CpG sites 4238, 4259, 4275), HPV18 (L2: CpG sites 4257, 4262, 4266, 4269, 4275, 4282), HPV31 (L1: CpG sites 6352 and 6354), and HPV33 (L2: CpG sites 5557, 5560, 5566). Amplifications were done using the PyroMark PCR kit (QIAGEN, Germany) with 20 ng input of converted DNA in a 25 µL volume of PCR reaction. The PCR products were pyrosequenced using a PyroMark™Q96 ID (Qiagen) instrument which included controls to allow standardised direct comparisons between different primer sets.

2.5 | Statistical analyses

The S5 score is a weighted average of six components: the proportion of the three CpGs in the HPV16 L2 region in which any methylation was detected, and the mean methylation of the CpGs within the other 5 targeted regions.[25] Methylation levels of S5 are thus expressed on a linear scale from 0 (no methylation) to 100 (hypermethylation). The S5 DNA methylation classifier was calculated as

$$S5 = (30.9 * \text{EPB41L3}) + (13.7 * \text{HPV16}_{L1}) + (4.3 * \text{HPV16}_{L2}) + (8.4 * \text{HPV18}_{L2}) + (22.4 * \text{HPV31}_{L1}) + (20.3 * \text{HPV33}_{L2}).$$

Median (and inter-quartile range) of S5 by disease outcome were tabulated. The diagnostic accuracy (sensitivity and specificity) of the S5 classifier for CIN2+ outcomes was calculated for hrHPV-positive women using a previously validated cut-point of ≥0.8 for methylation test positivity.[23,26] Additional cut-points of 0.6, 1.5, and 3.0 were explored in secondary analyses, as a higher value conferring better specificity may be preferred for low and middle-income countries (LMICs).[18–20]

The control set were chosen to allow multiple research questions to be answered and were stratified such that a higher proportion were selected with persisting hrHPV infections. Methylation scores were slightly higher among persisters. We were thus able to determine whether methylation could distinguish between women with persistent hrHPV who did and did not develop CIN3+. To evaluate the S5 methylation classifier as a triage test for predicting incident disease required the reconstruction of a control set representative of all hrHPV+ women who would not go on to develop CIN3+. In ARTISTIC 73% of women who were hrHPV+ at entry tested negative at round 2.[27] The estimates of test specificity were therefore weighted using the control sampling proportions in order to correct for this imbalance. Receiver operator characteristic curves for S5 were plotted for CIN3+ and the area under the curve calculated. Scenarios with and without prior knowledge of genotyping were considered, and methylation of *EPB41L3* alone was also considered.

Comparisons were made using two-sample Wilcoxon rank sum tests and were stratified for new or persisting infection among prevalent CIN2+ and controls at follow-up. *p*-values were presented to one significant figure and trends were assessed by the Cuzick test.[28] Box plots were drawn using the method defined by Tukey[29] where the box represents the interquartile range (IQR) with lines (or 'whiskers') drawn to span all data points within 1.5 IQR of the nearer quartile. Points outside this range are shown as additional points on the plot. Box plots were drawn on a log (base 10) scale, with S5 and *EPB41L3* scores of zero replaced with 0.05 before taking logs. All analyses were done in Stata V17.0 (StataCorp 2021).

3 | RESULTS

3.1 | S5 methylation classifier to predict future CIN2+

The primary aim was to evaluate the performance of S5 to predict incident CIN2+ among hrHPV-positive cervical samples. The time from the baseline sample which was tested using S5 to the histological diagnosis of 167 women with CIN2+ is shown in Figure S1. The median time to diagnosis was 3.1 years for CIN2, 4.9 years for CIN3, and 12.1 years for ICC (footnote of Figure S1). S5 methylation was higher in HPV-positive samples taken in advance of both CIN3 and invasive cancer diagnoses (median S5 values of 3.78 and 10.85, respectively, p <.0001 and $p = .0003$, respectively, compared to women in the control series, Table 1). In contrast, S5 was not elevated among baseline samples of women who were later diagnosed with CIN2 (Table 1, $p = .9$). Figure 2A shows median S5 score and S5 test sensitivity at a 0.8 cut-off in relation to interval from cervical sample to diagnosis. Median S5 declined with increasing interval but remained elevated up to 10 years before diagnosis of CIN3. In contrast, the sensitivity declined steeply with increasing interval from sample to diagnosis of CIN2 (p for trend = .0007).

Similar numbers of incident CIN3 presented with negative ($n = 43$) or borderline/low-grade cytology ($n = 37$) at baseline. Baseline S5 methylation of women with incident CIN3 was similar in women with baseline borderline / low-grade cytology (median S5 = 4.38, 75.7% with S5 >0.8) to women with normal cytology (median S5 = 2.93, 79.8% with S5 >0.8) (Table 1).

As expected, methylation scores were higher for samples positive for the four HPV genotypes included in the classifier (HPVs 16, 18, 31, and 33). Among the controls, the median S5 was highest among those with HPV16 (median S5 = 4.96) followed by HPV33 (median S5 = 2.49), HPV31 (median S5 = 1.65), and HPV 18 (median S5 = 1.11), and lowest in controls with other hrHPV infections (median S5 = 0.39) (Table 2). Stratifying by genotype, median S5 methylation levels were consistently higher among those with incident CIN3 than among the controls (Table 2).

3.2 | S5 methylation classifier to detect prevalent CIN2+

The secondary aim was to compare S5 at the time of prevalent CIN2+ diagnosis (<6 months in 176 women) to 435 HPV-positive

TABLE 1 Prediction of incident CIN2+ by S5 DNA methylation overall (and stratified by cytology[a]) in baseline HPV+ samples taken at least 6 months before diagnosis.

	Total	S5 ≥0.8	S5 score		
	n	*n* (%)	median (IQR)	*p*-value against all controls[b]	*p*-value against persisting controls[c]
All women					
Controls clearing by follow-up	103	39 (37.9)	0.66 (0.37–1.45)		
Controls persisting to follow-up	262	145 (55.3)	1.16 (0.42–4.68)		
CIN2	69	38 (55.1)	0.91 (0.35–5.18)	0.15	0.9
CIN3	89	71 (79.8)	3.78 (0.99–7.00)	<0.0001	<0.0001
ICC[d]	9	8 (88.9)	10.85 (6.60–12.87)	<0.0001	0.0003
Negative cytology					
Controls clearing by follow-up	64	21 (32.8)	0.63 (0.36–1.28)		
Controls persisting to follow-up	180	97 (53.9)	1.07 (0.44–4.51)		
CIN2	31	19 (61.3)	0.91 (0.34–6.42)	0.1	0.7
CIN3	43	34 (79.1)	2.93 (0.94–6.60)	<0.0001	0.002
ICC[e]	6	5 (83.3)	9.70 (4.39–12.87)	0.005	0.01
Borderline/low-grade cytology					
Controls clearing by follow-up	34	15 (44.1)	0.69 (0.33–2.56)		
Controls persisting to follow-up	75	45 (56.0)	1.18 (0.35–4.69)		
CIN2	37	18 (48.6)	0.72 (0.35–4.34)	0.8	0.7
CIN3	37	28 (75.7)	4.38 (0.81–7.77)	0.0005	0.004
ICC	2	2 (100)	9.16 (7.47–10.85)		

Abbreviations: CIN, cervical intraepithelial neoplasia; IQR, interquartile range (25th–75th percentile); ICC, invasive cervical cancer.

[a]5 clearing, 7 persisting controls, 1 CIN2, 9 CIN3s and 1 HPV positive ICC presented with high-grade cytology at baseline with median S5 scores of 1.10, 2.80, 2.93, 4.48, and 14.18, respectively. The histological diagnosis of these 11 CIN2+ was on average 3 years (range 0.6–7.5 years) after the sample was taken.

[b]Two-sample Wilcoxon rank sum test versus control group (adjusted for persistence among controls).

[c]Two-sample Wilcoxon rank sum test versus controls who persisted to follow-up round.

[d]Two-sample Wilcoxon rank sum test versus CIN3 group gives $p = .006$.

[e]Two sample Wilcoxon rank sum test versus CIN3 group gives $p = .1$.

control samples (Table S1). S5 was higher in women with CIN2 (median S5 = 3.37) and CIN3 (median S5 = 4.86) than among control women ($p = .0002$ and $p < .0001$ respectively, Table S2). The median S5 score was 13.11 within 6 months of ICC diagnosis, which was much higher than among the prevalent CIN3 cases (median S5 = 4.86; $p = .003$, Table S2). In the stratified analysis, S5 methylation remained elevated among women with CIN3 diagnosis compared to controls regardless of HPV genotype. In addition, 10 of the 16 CIN3 cases with none of the four HPV genotypes included in S5 (HPV16/18/31/33) had elevated methylation above the 0.8 cut-off (compared to only 31 of 206 among the controls, $p = .0001$, Table S2). The median S5 was lower among prevalent CIN3 cases with a new HPV infection at follow-up (HPV negative at baseline, median S5 was 3.43 in 12 CIN3) compared to those with an infection persisting from baseline (median S5 was 4.87 in 28 CIN3, $p = .03$, footnote of Table S2). S5 levels were highest among the 57 CIN3 who were diagnosed at baseline (footnote of Table S2).

Figure 2B compares the distribution of S5 methylation among the incident and prevalent cases compared to the combined control series. The distribution of S5, and in particular the median methylation, was similar in the controls and the incident CIN2 (diagnosed with CIN2 approximately 3 years after the sample was taken). In contrast, methylation increased markedly in the prevalent CIN2 compared to the controls, increased further in both the incident and prevalent CIN3s, and was highest in the invasive cancers. This trend ($p < .0001$) reflects the commonly accepted natural history model in which many CIN2s and some CIN3s are transient and cancer arises in persistent CIN3. The distribution of S5 methylation among the women with prevalent CIN2 disease was similar to women with incident CIN3 (diagnosed after approximately 5 years), and the S5 methylation distributions were similar for prevalent CIN3 and incident ICC.

3.3 | Threshold of S5 methylation for referral

Table 3 compares four thresholds of S5 for referral (0.6, 1.5, and 3.0 in addition to the proposed threshold of 0.8) for prevalent and incident CIN3, respectively. The S5 data can be compared directly to the

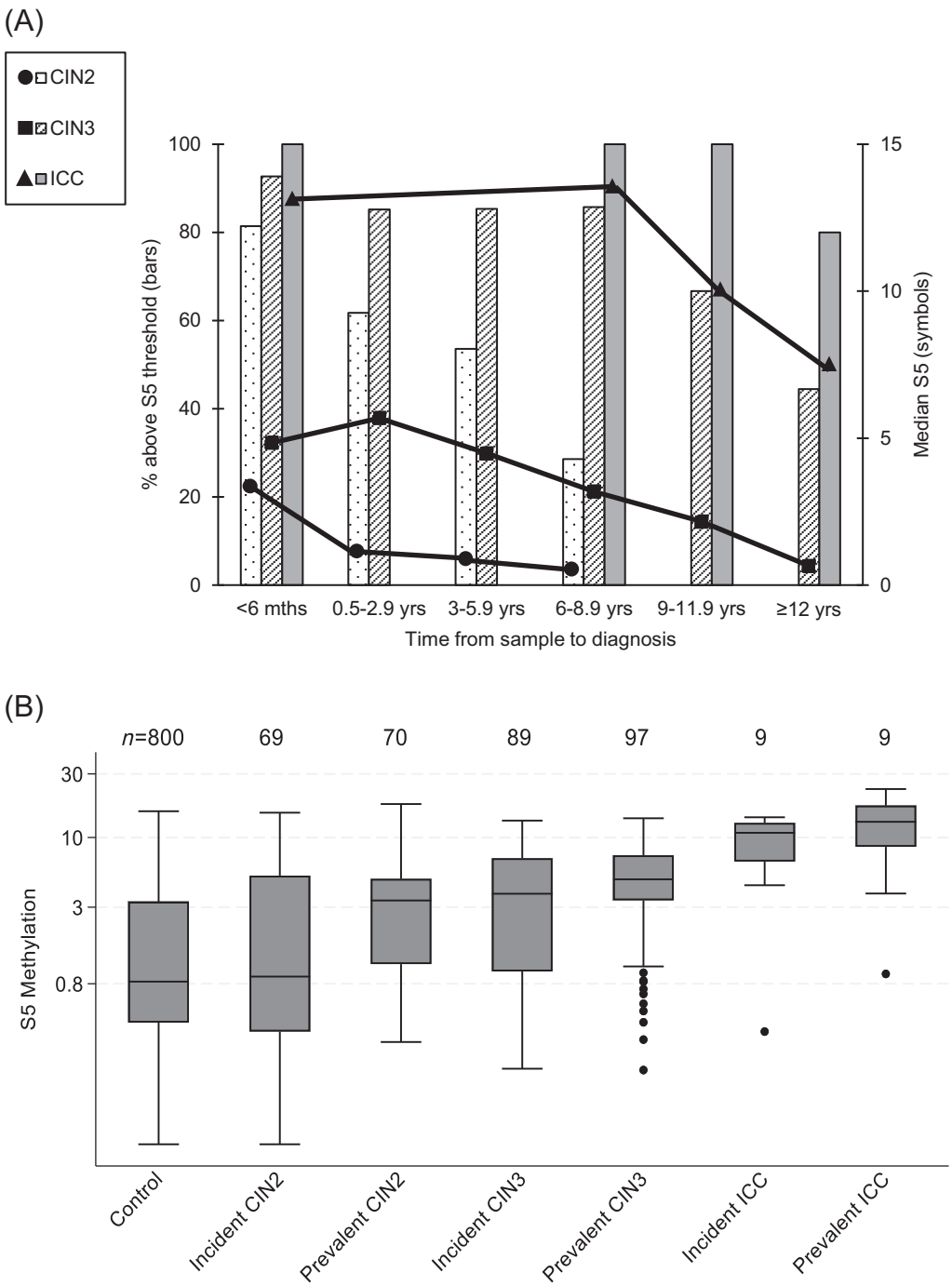


FIGURE 2 Distribution of S5 DNA methylation levels by timing of diagnosis of CIN2, CIN3 and ICC. (A) Median S5 score (symbols) and percentage of those above the 0.8 cut-off (bars) by time interval to diagnosis. (B) Box plots of S5 methylation levels including 800 controls (trend across all groups: p <.0001). Numbers contributing to each group are shown on the graph. A $\log_{10}$ scale has been used, the 6 samples (5 control samples, 1 incident CIN2) with zero readings for S5 are shown by the lowest whisker on the graph for the control and incident CIN2 series.

performance of partial HPV genotyping (referral if positive for various combinations of HPV16, HPV18, HPV31, and HPV33). At the 0.8 cut-off, the S5 classifier was much more sensitive for both detecting prevalent CIN3 and predicting incident CIN3 than HPV16/18 genotyping (92.8% vs. 60.8%, relative sensitivity 0.66 (95%CI:0.56–0.77), p <.0001 for prevalent CIN3 and 79.8% vs. 64.0%, relative sensitivity 0.80 (95%CI:0.70–0.92), $p = .002$ for incident CIN3, Table 3). However, at this threshold, S5 was less specific than HPV16/18 genotyping (50.0% vs. 65.8%, relative specificity 1.31 (95%CI:1.20–1.44), p <.0001 for prevalent CIN3 and 57.9% vs. 66.3%, relative specificity 1.15 (95%CI:1.04–1.26), $p = .004$ for incident CIN3, Table 3, Figure 3). The sensitivity of the S5 classifier at the 0.8 threshold was similar to that of genotyping for the four types included in the classifier (HPV 16, 18, 31, and 33) for the detection of incident CIN3 (relative sensitivity 1.00, 95%CI:0.93–1.07, p >.9 and relative specificity 0.91, 95%CI:0.84–0.99, $p = .02$), but more sensitive for detecting prevalent CIN3 (relative sensitivity 0.90, 95%CI:0.83–0.97, $p = .007$).

TABLE 2 S5 DNA methylation in HPV positive controls versus incident CIN3 stratified by HPV genotype (samples with multiple types are categorised by the hierarchical order as given in the table).

	Total	S5 ≥ 0.8	S5 score	
HPV genotypes	***n***	***n* (%)**	**median (IQR)**	***p*-value[a]**
Controls[b]				
16	94	84 (89.0)	4.96 (3.45–5.90)	
18	48	30 (62.5)	1.11 (0.69–2.02)	
31	43	40 (93.0)	1.65 (1.37–4.49)	
33	13	13 (100)	2.49 (1.61–3.30)	
16/18/31/33	198	167 (84.3)	3.05 (1.30–5.2)	
45/52/58	45	5 (11.1)	0.32 (0.26–0.50)	
35/39/51/56/59/68	122	12 (9.8)	0.43 (0.30–0.61)	
non-S5 HPV types[c]	167	17 (10.2)	0.39 (0.28–0.61)	
CIN3				
16	46	44 (95.7)	6.48 (4.48–8.52)	0.006
18	11	10 (90.9)	1.54 (0.94–3.13)	0.3
31	9	9 (100)	2.78 (1.63–6.38)	0.2
33	5	5 (100)	4.24 (2.68–4.38)	0.3
16/18/31/33	71	68 (95.8)	5.04 (2.78–8.04)	0.0001
45/52/58	8	2 (25.0)	0.55 (0.36–0.77)	0.1
35/39/51/56/59/68	10	1 (10.0)	0.56 (0.31–0.65)	0.5
non-S5 HPV types[c]	18	3 (16.7)	0.55 (0.31–0.71)	0.2

Note: IQR, interquartile range (25th–75th percentile).
[a]Two-sample Wilcoxon rank sum test versus control group.
[b]Controls were women HPV positive at baseline who did not develop detectable CIN during follow-up.
[c]HPV Types 35, 39, 45, 51, 52, 56, 58, 59, 68.

Table 3 shows that S5 with a cut-off of 3.0 was both more sensitive and more specific than HPV16/18 genotyping for prevalent CIN3 (sensitivity of 77.3% vs. 60.8%, respectively, $p = .0006$; specificity of 76.3% vs. 65.8%, respectively, $p < .0001$). In fact, the sensitivity of S5 for identifying prevalent CIN3 did not fall below 60.8% (the sensitivity of HPV16/18 genotyping) until the S5 threshold was raised to 4.65, and at this threshold, the specificity of the S5 was much higher at 87.6% compared to 65.8% for HPV16/18 genotyping ($p < .0001$). For incident CIN3 the sensitivity of S5 at cut-off 3.0 was slightly lower than for HPV16/18 genotyping (sensitivity of 57.3% vs. 64.0%, $p = 0.2$) but the specificity of S5 was still much higher than for HPV16/18 genotyping (81.1% vs. 66.3%, $p < .0001$).

In women testing positive for HPV16, the sensitivity of S5 is extremely high (95.7% for incident and 98.1% for prevalent CIN3), but the specificity was very low even at a threshold of 3.0 (10.5% and 27.7% for incident and prevalent CIN3, respectively). Among women with a non-16/18 hrHPV type, S5 achieved high sensitivity (84.2%) and moderate specificity (64.6%) for detecting prevalent CIN3 (using a referral threshold of 0.8), but relatively lower sensitivity for identifying future CIN3 (53.1%).

3.4 | Host methylation

Methylation of the host gene, *EPB41L3*, was not elevated in samples taken on average 3 years before CIN2 diagnosis, but marginally increased among incident CIN3 cases (median 1.61, $p = .06$) and HPV-positive invasive cancers (median 3.37, $p = 0.06$) compared to the control series (Table S3, Figure S2). However, *EPB41L3* methylation was elevated among those with prevalent disease, particularly among the prevalent ICC (medians in CIN2, CIN3, and ICC were 2.05, 2.68, and 19.07 respectively, $p = .07$, $p < .0001$, and $p = .0001$ compared to control series) (Table S3, Figure S2).

3.5 | HPV persistence among control series

We evaluated the ability of the S5 classifier to predict HPV persistence by comparing the control women in each of the analyses (Tables S1 and 1). Median S5 methylation appeared higher among the 282 control samples with persisting HPV infections (median 1.2) compared to infections among the 103 women which went on to clear (median 0.7), but this difference disappeared after stratification by HPV genotype since a higher proportion of the persisters were HPV16 positive. At follow-up, the median S5 was slightly higher among 282 controls with persisting HPV infections (median 0.9) compared to the 153 controls with new HPV infections (median 0.7, $p = .02$, Table S2) and this relationship remained among the small proportion of HPV16 positives after stratification.

TABLE 3 Sensitivity and specificity for prevalent and incident CIN3 by the S5 methylation test using various cut-off thresholds. A cut-off threshold of 0.8 has been suggested to be appropriate for referral in developed countries while a cut-off of 3.0 has been suggested for LMICs. Sensitivity and specificity[a] using genotyping alone are also given.

	Prevalent CIN3 (<6 months elapsed before diagnosis) 97 CIN3s, 435 controls		Incident CIN3 (≥6 months elapsed before diagnosis) 89 CIN3s, 365 controls	
S5 threshold	Sensitivity	Specificity	Sensitivity	Specificity
All samples				
0.6	94.8% (88.3–98.3)	42.0% (37.4–46.9)	84.3% (75.0–91.1)	42.8% (37.6–48.0)
0.8	92.8% (85.7–97.0)[b]	50.0% (45.0–54.7)	79.8% (69.9–87.6)[c]	57.9% (52.5–62.9)
1.5	87.6% (79.4–93.4)	66.4% (61.8–70.9)	69.7% (59.0–79.0)	71.5% (66.6–76.1)
3.0	77.3% (67.7–85.2)	76.3% (72.0–80.2)	57.3% (46.4–67.7)	81.1% (76.7–85.0)
HPV 16				
0.6	98.1% (90.1–100)	18.8% (11.7–27.1)	95.7% (85.2–99.5)	7.2% (3.0–14.7)
0.8	98.1% (90.1–100)	18.8% (11.7–27.1)	95.7% (85.2–99.5)	8.1% (3.7–16.1)
1.5	98.1% (90.1–100)	23.2% (15.6–32.2)	95.7% (85.2–99.5)	8.7% (3.7–16.1)
3.0	98.1% (90.1–100)	27.7% (19.6–37.2)	89.1% (76.4–96.4)	10.5% (5.2–18.7)
HPV 18				
0.6	100.0% (47.8–100)	21.0% (10.7–35.7)	90.9% (58.7–99.8)	9.2% (2.3–20.0)
0.8	100.0% (47.8–100)	30.5% (17.3–44.9)	90.9% (58.7–99.8)	45.2% (31.4–60.8)
1.5	80.0% (28.4–99.5)	72.4% (57.4–84.4)	54.6% (23.4–83.3)	81.1% (67.4–91.1)
3.0	60.0% (14.7–94.7)	89.9% (76.9–96.4)	27.3% (6.0–61.0)	95.2% (85.7–99.5)
Non 16/18 hrHPV				
0.6	89.5% (75.2–97.1)	54.0% (47.9–59.9)	65.6% (46.8–81.4)	60.0% (53.3–66.6)
0.8	84.2% (68.7–94.0)	64.6% (58.7–70.2)	53.1% (34.7–70.9)	73.4% (67.2–79.2)
1.5	73.7% (56.9–86.6)	80.8% (75.6–85.2)	37.5% (21.1–56.3)	84.7% (79.4–89.2)
3.0	50.0% (33.4–66.6)	91.3% (87.5–94.4)	21.9% (9.3–40.0)	95.3% (91.9–97.8)
Genotyping alone				
HPV 16	55.7% (45.2–65.8)	77.0% (72.8–80.9)	51.7% (40.8–62.4)	82.3% (77.9–86.0)
HPV 16/18	60.8% (50.4–70.6)	65.8% (61.1–70.2)	64.0% (53.2–73.9)	66.3% (61.2–71.1)
HPV 16/18/31/33	83.5% (74.6–90.3)	48.0% (43.3–52.9)	79.8% (69.9–87.6)	52.8% (47.6–58.1)
Relative to S5[d]				
HPV 16/18	0.66 (0.56–0.77)	1.31 (1.20–1.44)	0.80 (0.70–0.92)	1.15 (1.04–1.26)
HPV 16/18/31/33	0.90 (0.83–0.97)	0.96 (0.89–1.04)	1.00 (0.93–1.07)	0.91 (0.84–0.99)

[a]Specificity adjusted for sampling proportion of controls.
[b]The sensitivity was 100% (95%CI: 76.8–100) in women aged <30 and 91.6% (95CI: 83.4–96.5) in women aged ≥30 years.
[c]The sensitivity was 81.1% (95%CI: 64.8–92.0) in women aged <30 and 78.8% (95CI: 65.3–88.9) in women aged ≥30 years.
[d]Relative sensitivity and specificity to S5 with a threshold of 0.8.

4 | DISCUSSION

ARTISTIC is one of the largest cervical screening trials with up to 16 years of follow-up for CIN3 and invasive cancer through UK cancer registries.[27] Here, we report a case-control study of DNA methylation for predicting disease from 139 CIN2, 186 CIN3, 18 invasive cancer cases, and 800 controls.

Elevation of S5 DNA methylation correlates directly with increasing severity of disease and inversely with lead time to diagnosis (Figure 2). S5 methylation testing has 92.8% (95CI: 85.7–97.0) sensitivity for detecting prevalent CIN3 and 100% sensitivity (95%CI: 66.4–100) for detecting prevalent ICC (based on 97 women with CIN3 and 9 with ICC), which is consistent with earlier reports of S5 methylation performance.[15,18–20,30,31] The accumulating results thus indicate that as a triage test, S5 would miss a few prevalent CIN3s and almost no invasive cancers.

S5 outperformed other assays as reported in a review article (sensitivity of 84.5% (Table S4) vs. 71.1% as estimated by Kelly et al.[11] with fixed specificity at 70%), but showed slightly lower sensitivity compared to the WID-CIN test, a DNA methylation signature comprising 5000 CpG sites (sensitivity of 78.4% (Table S4) vs. 89.7% as estimated by Barrett et al.[12] with fixed specificity at 75%) for detecting prevalent CIN3.

The major strength of our analysis is our evaluation of methylation on samples taken on average 5 years before CIN3 and 10 years

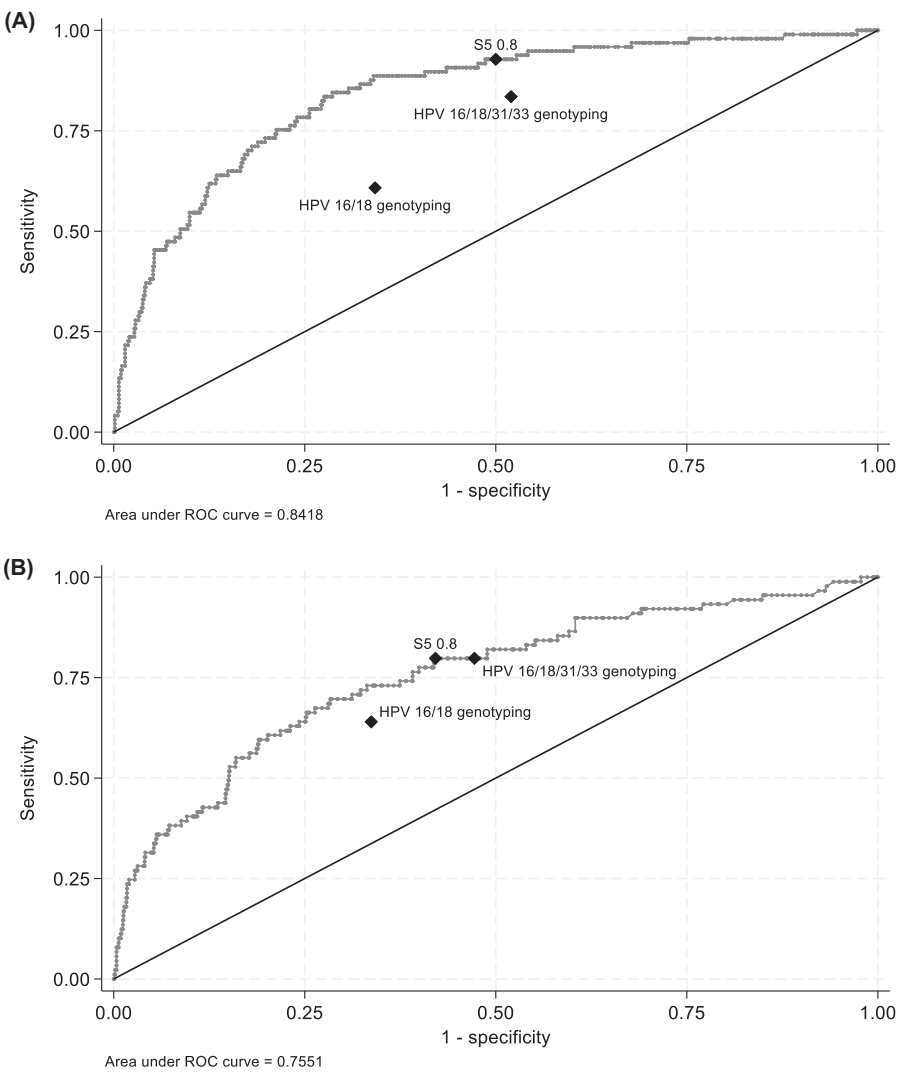


FIGURE 3 ROC curve for the S5 methylation classifier for prevalent CIN3 (A) and incident CIN3 (B). Point estimates are shown for S5 with 0.8 cut-off and for HPV genotyping.

before ICC diagnoses, almost all of which showed negative, borderline, or low-grade cytology. Few other studies have assessed methylation for such long-term prediction of incident disease. FAM19A4/miR124-2 methylation at baseline showed good sensitivity for identifying CIN3 in the first round of the POBASCAM trial (94/116 = 81.0%) but not at subsequent rounds of screening at 5 and 10 years, where HPV 16/18 genotyping was much more predictive of CIN3 (sensitivity of methylation 22/76 = 28.9% vs. genotyping 51/76 = 67.1% over rounds 2 and 3, calculated from Dick et al.[32]). On Swedish samples taken 1–4 years before CIN3+ diagnosis the WID-CIN methylation test achieved slightly lower sensitivity than S5 (74.3% sensitivity vs. 82.0% sensitivity of S5 after fixing specificity to 50%, Table S4), with lower sensitivity among women aged under 30 years.[12] In contrast, the S5 classifier performs better in younger women because a higher proportion present with the four HPV types (16/18/31/33) included in the test (footnote of Table 3). In a Chinese study, a methylation panel of six human genes showed good sensitivity for identifying CIN3+ up to 3 years after testing (32/34 = 94.1% at baseline and 18/29 = 62.1% over the 3-year follow-up).[33]

The S5 classifier achieved a 100% sensitivity for identifying the 9 prevalent cancers and 88.9% sensitivity (8/9) in HPV-positive samples taken 6–16 years before cancer diagnosis. These eight identified by S5 were positive for HPV 16, 18, 31, or 33. The remaining cancer with S5 <0.8 was positive for HPV45. Among 18 cancers diagnosed among women who were HPV positive at baseline in the POBASCAM trial, FAM19A4/mir124-2 methylation identified 100% (9/9) of the cancers diagnosed within 4 years of baseline but may have lower sensitivity (56%, 5/9) in HPV positive samples taken 5–14 years before cancer diagnosis.[34]

4.1 | Host and viral methylation

As far as we are aware, ours is the first study to show elevated risk in samples taken as long as an average of 5 years before incident CIN3 diagnosis and 12 years before cancer diagnosis. Our data show that the lead time for predicting incident CIN3 and cancer may be quite short for *EPB41L3* methylation, similar to other studies based only on methylation of human genes.[12,32,33] The S5 test combines methylation of a host gene, *EPB41L3*, with methylation of HPV16, HPV18, HPV31, and

HPV33 genes. Thus, S5 combines the predictive probability of HPV genotyping, enhanced with methylation of HPV genomes, with methylation of a tumour suppressor gene (*EPB41L3*). About half of our controls had HPV 16, 18, 31, or 33 infections compared to 80% of the CIN3 cases. HPV genotypes HPV16, 18, 31, 33, and 45 have been estimated to account for 85% of worldwide cervical cancer cases,[35] and it has been argued that these types should be triaged ahead of other genotypes.[36] Many partial genotyping assays used by screening programmes around the world identify HPV16 and HPV18 separately, but few also identify types 31 and 33. As vaccinated cohorts are entering the screening programme in the United Kingdom and elsewhere, there is a need for cost-effective extended genotyping assays to further stratify the women at highest risk. The sensitivity of the S5 classifier was similar to genotyping with expanded partial genotyping to include HPV 16/18/31/33 for identifying incident CIN3 ($p > .9$, Table 3) and was slightly better for detecting prevalent CIN3 ($p = .007$, Table 3).

4.2 | Non HPV16/18 infections

The majority of hrHPV-positive women have non-HPV16/18 infections, ranging from approximately 60% in unvaccinated women such as the ARTISTIC cohort[37] to virtually 100% in vaccinated cohorts. Approximately half of these women may have abnormal cytology, yet their risk of CIN3+ is 2–3 times lower than following HPV16/18 infections.[37] We have shown that the S5 classifier is sensitive for identifying prevalent CIN3+ in women without HPV16/18 infection (84.2%, 95%CI:68.7–94.0), but the confidence intervals were wide for estimating the performance for identifying future CIN3+ (53.1%, 95% CI:34.7–70.9, based on detecting 17/32 cases, Tables 2 and 3). These results are driven by the inclusion of types 31 and 33 in the S5 classifier. Expanding the classifier to include other HPV types may improve the predictive power among vaccinated cohorts, though it may be cost-effective to simply rely on extended genotyping. S5 was higher in women with prevalent CIN3 and non-S5 genotypes (Table S2) presumably due to the contribution of the human gene methylation to the S5 score. Further data on non-S5 genotypes are required to determine whether a lower threshold for referral would increase the sensitivity for early CIN3 while retaining acceptable specificity.

In less developed countries, a threshold for S5 of 3.0 has been suggested for triaging hrHPV-positive women.[18–20] At this cut-off, S5 has the ability to identify prevalent CIN3+ with reasonable sensitivity and specificity (77.3% and 76.3% respectively) and is clearly more effective than HPV16/18 genotyping alone. At a threshold of 3.0, S5 was less sensitive but more specific than HPV 16/18 genotyping for predicting incident CIN3 (Table 3), so a slightly lower threshold might be preferable depending on the resources available.

4.3 | Strengths and weaknesses

The main strengths of our analysis are the long follow-up and the provision of multiple samples within the ARTISTIC trial cohort, enabling methylation to be performed on stored samples taken up to 16 years before histological diagnosis. ARTISTIC was a pragmatic trial, which has the advantage that the cohort is representative of screened women in the United Kingdom, but the disadvantage that women were largely diagnosed following abnormal cytology. Diagnosis of histological endpoints may therefore have been delayed and incomplete, leading to a degree of misclassification where a proportion of CIN2+ classified as incident, may be undiagnosed prevalent disease. Figure 2A shows that similar results would have been obtained if the 6-month cut-off was increased to 12 months. All CIN3 and cancers recorded by UK cancer registries up to 16 years after baseline were identified. Figure 2 shows a methylation pattern that correlates with the natural history model, with the S5 score increasing with increasing disease severity and highest in prevalent disease.

DNA methylation testing is promising as a triage test, and perhaps eventually as a screening test in an automated system in which methylation testing of HPV and human genes replaces methylation-agnostic HPV DNA testing. There are ongoing improvements in DNA methylation testing making such tests more suitable for routine use. The S5 test is based on pyrosequencing, which is excellent as a research tool but not as a routine diagnostic test. There are near-term routine technology solutions already developed that have converted the S5 test to a qMSP-based format, and advances in next-generation sequencing promise direct detection of methylated and non-methylated CpG sites in native DNA without bisulfite conversion or PCR amplification. These advances may provide automated quantitation of methylation in all hrHPV types as well as in a larger panel of human genes.

4.4 | Clinical utility

Assuming an automated test can be produced at a reasonable cost, the S5 classifier would be best placed as a triage test for HPV-positive women, particularly in circumstances where cytology cannot be done (for example on self-taken vaginal or urine tests, or in low resource settings). S5 uses the strong predictive power of genotyping to identify those at highest risk of disease (i.e., those with types 16, 18, 31, and 33). If the classifier were extended to include HPV 45, 52, and 58 an estimated 91% of all cancers could be identified.[35] In the long-term, the possibility that non-vaccine types may become more common in vaccinated cohorts should be kept under review.[38] An extended genotyping assay may be more cost-effective for predicting future disease, however the inclusion of human gene methylation is able to identify those with prevalent cancer and pre-cancer, who should be immediately referred for colposcopy and treatment. The remaining women can be re-screened after a longer interval thus reducing the number of immediate referrals to colposcopy. The NHS CSP lacks evidence for managing women with persistent HPV infection and negative cytology.[5] Methylation may be a better triage strategy than cytology by reducing the number of women attending repeated colposcopy appointments.

5 | CONCLUSION

The S5 classifier is promising as a triage test for hrHPV-positive women in cervical cancer screening programmes and modified versions may be suitable for some low-resource settings. We have shown that S5 DNA methylation can identify women in the ARTISTIC cohort at highest risk of developing disease 5 or more years in the future, with acceptable specificity while missing very few prevalent advanced lesions. The combination of methylation of host and HPV genes enables the S5 classifier to combine the predictive power of methylation with HPV genotyping to identify hrHPV-positive women who are at highest risk of developing CIN3 and ICC in the future.

AUTHOR CONTRIBUTIONS

ATL and JC conceived and designed the study. CG performed the final data analysis following an initial analysis by ARB. CG and BN drafted the manuscript, and ATL and JP revised it. DSB, CR and RB performed the laboratory analysis under the supervision of BN and ATL. All authors reviewed and approved the final version of the manuscript. The work reported in the paper has been performed by the authors unless clearly specified in the text.

ACKNOWLEDGEMENTS

We thank the women who participated in the ARTISTIC trial and the many people who worked on the data collection and laboratory analysis during the active phases of the trial, particularly Henry Kitchener, Alexandra Sargent, Mina Desai, Andrew Bailey, and Maribel Almonte.

FUNDING INFORMATION

The Molecular Epidemiology Laboratory at Queen Mary University of London was funded by CRUK grants C8162/A4609, C8162/A10406, C569/A10404, and C236/A11795. The long-term follow-up of the ARTISTIC cohort was funded by NIHR-HTA (15.59.47).

CONFLICT OF INTEREST STATEMENT

Jack Cuzick received funds from grants, cooperative agreements, or subcontracts related to cervical screening and triage through his institution. He received grants and personal fees from Qiagen, grants from Hologic, grants and personal fees from Becton Dickinson (BD), grants and personal fees from Genera Biosystems (GB), grants from Gene First, and Trovagene, outside the submitted work. The other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical approval was granted by the London—Brighton & Sussex Research Ethics Committee (14/LO/0627 & 17/LO/1483). Women gave consent to participate in the original trial. Section 251 approval for continued follow-up of the cohorts was given by the Confidentiality Advisory Group (CAG 3-02(a)/2014).

ORCID

Clare Gilham https://orcid.org/0000-0002-9477-6090
Belinda Nedjai https://orcid.org/0000-0001-8711-4577
Rawinder Banwait https://orcid.org/0009-0008-6413-3933
Adam R. Brentnall https://orcid.org/0000-0001-6327-4357
Jack Cuzick https://orcid.org/0000-0001-7420-7512
Attila T. Lorincz https://orcid.org/0000-0001-7617-4355

REFERENCES

1. Bouvard V, Wentzensen N, Mackie A, et al. The IARC perspective on cervical cancer screening. *N Engl J Med*. 2021;385(20):1908-1918.
2. Ronco G, Dillner J, Elfstrom KM, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. *Lancet*. 2014;383(9916):524-532.
3. Tota JE, Bentley J, Blake J, et al. Introduction of molecular HPV testing as the primary technology in cervical cancer screening: acting on evidence to change the current paradigm. *Prev Med*. 2017;98:5-14.
4. Cuschieri K, Ronco G, Lorincz A, et al. Eurogin roadmap 2017: triage strategies for the management of HPV-positive women in cervical screening programs. *Int J Cancer*. 2018;143(4):735-745.
5. Kitchener H. Report to the National Screening Committee. 2015 Available from: https://view-health-screening-recommendations.service.gov.uk/document/320/download
6. Rebolj M, Rimmer J, Denton K, et al. Primary cervical screening with high risk human papillomavirus testing: observational study. *BMJ*. 2019;6(364):l240.
7. Loopik DL, Koenjer LM, Siebers AG, Melchers WJG, Bekkers RLM. Benefit and burden in the Dutch cytology-based vs high-risk human papillomavirus-based cervical cancer screening program. *Am J Obstet Gynecol*. 2020;224:200.e1-9.
8. Serrano B, Ibanez R, Robles C, Peremiquel-Trillas P, de Sanjose S, Bruni L. Worldwide use of HPV self-sampling for cervical cancer screening. *Prev Med*. 2022;154:106900.
9. Rebolj M, Sargent A, Njor SH, Cuschieri K. Widening the offer of human papillomavirus self-sampling to all women eligible for cervical screening: make haste slowly. *Int J Cancer*. 2023;153(1):8-19.
10. Australian_Government. National Cervical Screening Policy [01/06/2020]. Available from: http://www.cancerscreening.gov.au/internet/screening/publishing.nsf/Content/national-cervical-screening-policy
11. Kelly H, Benavente Y, Pavon MA, de Sanjose S, Mayaud P, Lorincz AT. Performance of DNA methylation assays for detection of high-grade cervical intraepithelial neoplasia (CIN2+): a systematic review and meta-analysis. *Br J Cancer*. 2019;121(11):954-965.
12. Barrett JE, Sundstrom K, Jones A, et al. The WID-CIN test identifies women with, and at risk of, cervical intraepithelial neoplasia grade 3 and invasive cervical cancer. *Genome Med*. 2022;14(1):116.
13. Vink FJ, Meijer C, Clifford GM, et al. FAM19A4/miR124-2 methylation in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Int J Cancer*. 2020;147(4):1215-1221.
14. Lehtinen M, Pimenoff VN, Nedjai B, et al. Assessing the risk of cervical neoplasia in the post-HPV vaccination era. *Int J Cancer*. 2023;152(6):1060-1068.
15. Nedjai B, Reuter C, Ahmad A, et al. Molecular progression to cervical precancer, epigenetic switch or sequential model? *Int J Cancer*. 2018;143(7):1720-1730.
16. Louvanto K, Aro K, Nedjai B, et al. Methylation in predicting progression of untreated high-grade cervical intraepithelial neoplasia. *Clin Infect Dis*. 2020;70(12):2582-2590.
17. Clarke MA, Wentzensen N, Mirabello L, et al. Human papillomavirus DNA methylation as a potential biomarker for cervical cancer. *Cancer Epidemiol Biomarkers Prev*. 2012;21(12):2125-2137.

18. Hernandez-Lopez R, Lorincz AT, Torres-Ibarra L, et al. Methylation estimates the risk of precancer in HPV-infected women with discrepant results between cytology and HPV16/18 genotyping. *Clin Epigenetics*. 2019;11(1):140.
19. Ramirez AT, Sanchez GI, Nedjai B, et al. Effective methylation triage of HPV positive women with abnormal cytology in a middle-income country. *Int J Cancer*. 2021;148(6):1383-1393.
20. Banila C, Lorincz AT, Scibior-Bentkowska D, et al. Clinical performance of methylation as a biomarker for cervical carcinoma in situ and cancer diagnosis: a worldwide study. *Int J Cancer*. 2022;150(2):290-302.
21. Dick S, Vink FJ, Heideman DAM, Lissenberg-Witte BI, Meijer C, Berkhof J. Risk-stratification of HPV-positive women with low-grade cytology by FAM19A4/miR124-2 methylation and HPV genotyping. *Br J Cancer*. 2022;126(2):259-264.
22. Zeng R, Liu Y, Jiang ZJ, et al. EPB41L3 is a potential tumor suppressor gene and prognostic indicator in esophageal squamous cell carcinoma. *Int J Oncol*. 2018;52(5):1443-1454.
23. Brentnall AR, Vasiljevic N, Scibior-Bentkowska D, et al. HPV33 DNA methylation measurement improves cervical pre-cancer risk estimation of an HPV16, HPV18, HPV31 and \textitEPB41L3 methylation classifier. *Cancer Biomark*. 2015;15(5):669-675.
24. Kitchener HC, Almonte M, Gilham C, et al. ARTISTIC: a randomised trial of human papillomavirus (HPV) testing in primary cervical screening. *Health Technol Assess*. 2009;13(51):1-150. iii-iv.
25. Lorincz AT, Brentnall AR, Vasiljevic N, et al. HPV16 L1 and L2 DNA methylation predicts high-grade cervical intraepithelial neoplasia in women with mildly abnormal cervical cytology. *Int J Cancer*. 2013;133(3):637-644.
26. Lorincz AT, Brentnall AR, Scibior-Bentkowska D, et al. Validation of a DNA methylation HPV triage classifier in a screening sample. *Int J Cancer*. 2016;138(11):2745-2751.
27. Gilham C, Sargent A, Kitchener HC, Peto J. HPV testing compared with routine cytology in cervical screening: long-term follow-up of ARTISTIC RCT. *Health Technol Assess*. 2019;23(28):1-44.
28. Cuzick J. A Wilcoxon-type test for trend. *Stat Med*. 1985;4(1):87-90.
29. Tukey JW. *Exploratory Data Analysis*. Addison-Wesley; 1977.
30. Adcock R, Nedjai B, Lorincz AT, et al. DNA methylation testing with S5 for triage of high-risk HPV positive women. *Int J Cancer*. 2022;151(7):993-1004.
31. Cook DA, Krajden M, Brentnall AR, et al. Evaluation of a validated methylation triage signature for human papillomavirus positive women in the HPV FOCAL cervical cancer screening trial. *Int J Cancer*. 2019;144(10):2587-2595.
32. Dick S, Kremer WW, de Strooper LMA, et al. Long-term CIN3+ risk of HPV positive women after triage with FAM19A4/miR124-2 methylation analysis. *Gynecol Oncol*. 2019;154(2):368-373.
33. Zhang L, Zhao X, Hu S, et al. Triage performance and predictive value of the human gene methylation panel among women positive on self-collected HPV test: results from a prospective cohort study. *Int J Cancer*. 2022;151(6):878-887.
34. de Strooper LMA, Berkhof J, Steenbergen RDM, et al. Cervical cancer risk in HPV-positive women after a negative FAM19A4/mir124-2 methylation test: a post hoc analysis in the POBASCAM trial with 14 year follow-up. *Int J Cancer*. 2018;143(6):1541-1548.
35. de Sanjose S, Quint WG, Alemany L, et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncol*. 2010;11(11):1048-1056.
36. Cuzick J, Adcock R, Wheeler CM. HPV genotype-specific risk for cervical cancer. *wwwHPVWorldcom*. 2021;181.
37. Gilham C, Sargent A, Peto J. Triaging women with human papillomavirus infection and normal cytology or low-grade dyskaryosis: evidence from 10-year follow up of the ARTISTIC trial cohort. *BJOG*. 2020;127(1):58-68.
38. Pimenoff VN, Gray P, Louvanto K, et al. Ecological diversity profiles of non-vaccine-targeted HPVs after gender-based community vaccination efforts. *Cell Host Microbe*. 2023;31(11):1921-1929.e3.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Gilham C, Nedjai B, Scibior-Bentkowska D, et al. Long-term prediction by DNA methylation of high-grade cervical intraepithelial neoplasia: Results of the ARTISTIC cohort. *Int J Cancer*. 2024;155(1):81-92. doi:10.1002/ijc.34913