Cervical cell lift: A novel triage method for the spatial mapping and grading of precancerous cervical lesions

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Summary

Background Primary HPV screening, due to its low specificity, requires an additional liquid-based cytology (LBC) triage test. However, even with LBC triage there has been a near doubling in the number of patients referred for colposcopy in recent years, the majority having low-grade disease.

Methods To counter this, a triage test that generates a spatial map of the cervical surface at a molecular level has been developed which removes the subjectivity associated with LBC by facilitating identification of lesions in their entirety. 50 patients attending colposcopy were recruited to participate in a pilot study to evaluate the test. For each patient, cells were lifted from the cervix onto a membrane (cervical cell lift, CCL) and immunostained with a biomarker of precancerous cells, generating molecular maps of the cervical surface. These maps were analysed to detect high-grade lesions, and the results compared to the final histological diagnosis.

Findings We demonstrated that spatial molecular mapping of the cervix has a sensitivity of 90% (95% CI 69-98) (positive predictive value 81% (95% CI 60-92)) for the detection of high-grade disease, and that AI-based analysis could aid disease detection through automated flagging of biomarker-positive cells.

Interpretation Spatial molecular mapping of the CCL improved the rate of detection of high-grade disease in comparison to LBC, suggesting that this method has the potential to decisively identify patients with clinically relevant disease that requires excisional treatment.

Introduction

Cervical cancer is the fourth commonest cancer in women globally, and nearly all cases are caused by persistent infection with high-risk human papillomaviruses (hrHPV).[1] In the past two decades improved interventions to identify and prevent this disease, namely primary HPV screening[2] and HPV vaccination,[3] have become standard practice in many high-income countries (HIC). Vaccination has been shown to significantly reduce the incidence of high-grade cervical disease,[3] however the global vaccine rollout has been hampered by inefficient national vaccine schemes and vaccine hesitancy being reported in many countries including the USA and France, with Japan reporting an uptake of only 1%.[4] For the foreseeable future, cervical cancer screening still has a role to play in detecting precancers.

It is well established that primary HPV screening detects more precancers and prevents more cancers

than conventional liquid-based cytology (LBC), with studies reporting sensitivities of 95%[2,5] to high-grade squamous intraepithelial lesions (HSIL). However, this high sensitivity comes at the expense of specificity/positive predictive value (PPV) for HSIL, with most DNA/

RNA screening studies demonstrating PPVs of <20%. Therefore, an additional triage step is necessary for all patients who are HPV positive. The current test of choice in HIC is LBC, with all HPV positive patients having reflex cytology, and if positive (regardless of grade of cytological abnormality), being referred for diagnostic colposcopy, while those that are solely HPV positive will have annual screening. However, LBC has low PPV to HSIL,[6] meaning that many patients with low-grade disease that the immune system will control over time being referred to colposcopy.[7,8]

While it is beyond dispute that conventional cytology screening has led to a remarkable decline in the incidence of cervical cancer,[9] the technology now exists for screening and triage to be more decisive. With the advent of better biomarkers to high- and low-grade disease,[10] together with a greater understanding of the molecular mechanisms that underpin a transforming HPV infection,[11] LBC and other technologies can evolve to identify patients that require treatment, and reassure patients that do not with a higher degree of confidence. This latter aspect is particularly relevant as we move towards offering conservative management to more patients with cervical intraepithelial neoplasia 2 (CIN2).[12,13] This would lead to fewer patients, the majority of whom are in their reproductive prime, having excisional treatment, and therefore preventing overtreatment and its associated consequences of preterm labour and neonatal morbidity.

Much work is being done to evaluate other triage methodologies that may have relatively superior sensitivities/PPVs to HSIL. The forerunners have been p16/Ki67 dual staining of cytology samples,[14] methylation analysis of cytology samples,[15] and visual inspection after acetic acid in low/middle income countries (LMIC).[16] While both p16 staining and methylation analysis have been shown to have better sensitivity and specificity for high-grade disease, the PPVs of these techniques are still poor and come at the expense of further referrals to colposcopy.

A significant drawback in using cytology samples and techniques such as methylation analysis for triage is that these samples lack the preserved topological information of tissue polarity and architecture seen in conventional histological samples, which aids in the interpretation and diagnosis of lesions.[6] To counteract this problem, here we describe a novel cervical cell lift (CCL) method for HPV triage where the surface cells of the cervix, which are the most important in grading disease as dysplasia extending to the surface is a hallmark of HSIL, are sampled in a manner that preserves their native position in the cervix and provides histology-level information without the need for a biopsy. Probing with biomarkers creates a molecular map of the cervical surface, allowing the identification of a lesion in its entirety. To aid high throughput identification of these abnormal areas, a mixed-scale dense convolutional

neural network (MSD-CNN)[17] was employed, which allows for rapid and reproducible identification of these abnormal biomarker-positive areas, enabling more decisive and efficient triage of HPV positive patients.

Materials and methods

Ethics

The cervical cell lift (CCL) trial was approved by an independent ethics committee (South Central Oxford B; 17/SC/0203). All clinical material used in this trial was collected in keeping with the Helsinki declaration of 1975.

Sampling protocol

Patients attending the colposcopy clinic at Cambridge University Hospitals NHS Foundation Trust with LBC abnormalities in 2019. Patients attending colposcopy who were HPV positive, cytology positive, and aged 25-50 years were recruited after giving written consent. Patients who were immunocompromised/suppressed or pregnant were excluded from the study. Each patient was set up for colposcopy in the standard manner. After visualisation of the cervix with a speculum, a pre-acetowhite photograph was taken. Next, the cervical cell lift (CCL) was performed with the application of the membrane (Amersham Protran Premium nitrocellulose blotting membrane, Merck) to the cervix for 15 seconds, ensuring that the 12 o'clock position marker on the membrane is aligned with the 12 o'clock position on the cervix (Supplementary Figure 1). The sample was fixed in Paraformaldehyde, 4% in PBS (Alfa Aesar), then transferred to the storage buffer, 0.05% ProClin 300 (Merck) in TBS, and stored at 4°C. The patient's colposcopy then proceeded as usual with the application of acetic acid, after which a further photograph was taken. We chose to perform the cell lift prior to colposcopy as acetic acid would alter the lifting characteristics of the membrane. To mitigate any risk of the cell lift impairing the final patient diagnosis, we assessed the impact of the cell lift on patients who underwent LLETZ subsequently. Here there was no detected difference in the diagnosis of patients who had undergone a cell lift prior to colposcopy.

Evaluation of the CCL

The aim was to recruit two low-grade LBC patients (<moderate dyskaryosis) for every HSIL LBC patient, with a total of 50 participants, which would be expected to provide 20 CIN2+ lesions for initial evaluation of the CCL method, assuming around 20% incidence of CIN2+ lesions in a population of HPV positive patients.[18] Within this group of 50 patients whose (Table 1), 19 had histologically proven HSIL (CIN2+ lesions), with the rest having low-grade histology or a normal colposcopy not needing a biopsy. When comparing these two groups, they were broadly similar in age (HSIL median age 31, interquartile range 7; LSIL/no lesion median age 31, interquartile range 14), smoking status, or comorbidity status. The final clinical diagnosis was blinded until the completion of interpretation of CCLs.

H&E staining of cell lift samples

H&E staining was performed following the manufacturer's instructions (H&E Staining Kit, Abcam). The cell lift membrane was washed in distilled water for 5 minutes. The membrane was completely covered in haematoxylin and incubated for 5 minutes at room temperature, then washed in two changes of tap water to remove excess stain. The membrane was destained with 10% glacial acetic acid/90% absolute ethanol for a few seconds and then rinsed in tap water. The membrane was then completely covered in Bluing Reagent for 10-15 seconds, then rinsed in two changes of tap water. The membrane was dipped in absolute ethanol, covered in Eosin Y Solution for 3 mins, then dipped again in absolute ethanol. The sample was dehydrated in three changes of absolute 2-propanol, before being cleared with xylene in a fume hood and mounted on a slide with VectaMount Permanent Mounting Medium (Vector Laboratories).

Immunohistochemical/fluorescent staining and image analysis

Immunofluorescence was performed as previously described.[10] For epitope retrieval of tissue sections, slides were incubated in Dako Target Retrieval Solution, pH 9 (Agilent) for 10 minutes at room temperature, autoclaved for 2 minutes at 121°C, and then equilibrated in PBS. For cells on the membrane or a slide chamber, after fixation cells were permeabilised with 0.2% Triton-X 100 (Sigma-Aldrich) in TBS for 10 minutes, and endogenous HRP was quenched with 3% hydrogen peroxide, twice. The tissue section or cells were blocked in 10% normal goat or horse serum (Sigma) in PBS for 1 hour.

A single biomarker (MCM) was used for the detection of HSIL. MCM is a licensing protein that is essential for the initiation of DNA replication and is positive only in cycling cells. MCM has been validated as a biomarker of HSIL and cancer by our group and others.[19] Primary antibodies used were anti-Cytokeratin 13 [EPR3671] (dilution 1:200, Abcam), anti-Cytokeratin 19 [A53-B/A2] (dilution 1:1000, Abcam), and anti-MCM7 [EP1974Y] (dilution 1:100, Abcam). For Cytokeratin 13 and Cytokeratin 19, proteins are detected using an anti-mouse Alexa 594-conjugated secondary antibody (dilution 1:150, Invitrogen) or anti-rabbit Alexa 488-conjugated secondary antibody (dilution 1:150, Invitrogen). For MCM, the signal was amplified using ImmPRESS HRP Horse Anti-Rabbit IgG Polymer Detection Kit

Patient ID	Age	Smoking status	Clinical diagnosis					Cell lift analysis	
			Cytology	Colposcopy impression	Treatment	Histology	CIN crade	MCM-positive fields	HSIL positive/negative
1	35	No	Mild	Mild	Biopsy	HSIL	2	6	Positive
2	32	Yes	Severe	Dense	LLETZ	HSIL	3	7	Positive
3	30	No	Moderate	Mild	Biopsy	HSIL	2	7	Positive
4	25	Yes	Severe	Dense	LLETZ	HSIL	3	13	Positive
5	31	Yes	Severe	Dense	LLETZ	HSIL	3	5	Positive
6	48	Yes	Severe	Dense	LLETZ	HSIL	3	13	Positive
7	32	No	Moderate	Dense	Biopsy	HSIL	3	20	Positive
8	28	No	Mild	Mild	Biopsy	HSIL	3	15	Positive
9*	29	No	Severe	Mild	Biopsy	HSIL	2	0	Negative
10	28	No	Severe	Dense	Biopsy	HSIL	3	3	Positive
11	41	No	Severe	Dense	LLETZ	HSIL	3	5	Positive
12	31	No	Severe	Mild	Biopsy	HSIL	3	12	Positive
13	31	Yes	Borderline	Dense	Biopsy	HSIL	3	13	Positive
14	27	Yes	Severe	Dense	Biopsy	HSIL	3	9	Positive
15	29	No	Severe	Mild	Biopsy	HSIL	3	16	Positive
16	36	No	Severe	Dense	LLETZ	HSIL	3	4	Positive
17	26	Yes	Severe	Dense	LLETZ	HSIL	3	10	Positive
18	25	No	Mild	Mild	Biopsy	HSIL	3	4	Positive
19*	44	No	Severe	Dense	LLETZ	HSIL	2	0	Negative
20	28	No	Mild	Mild	Biopsy	LSIL	1	1	Negative
21	47	No	Mild	Mild	Biopsy	LSIL	1	1	Negative
22	27	Yes	Mild	Mild	Biopsy	LSIL	1	0	Negative
23	41	No	Severe	Dense	LLETZ	LSIL	1	0	Negative
24	33	Yes	Mild	Mild	Biopsy	LSIL	1	0	Negative
25	44	No	Severe	Dense	Biopsy	LSIL	1	13	Positive
26	28	No	Moderate	Dense	Biopsy	LSIL	1	2	Negative
27	45	No	Mild	Dense	Biopsy	LSIL	1	0	Negative
28	36	No	Mild	Dense	Biopsy	LSIL	1	0	Negative
29	31	No	Moderate	Dense	Biopsy	LSIL	1	0	Negative
30	30	Yes	Mild	Dense	Biopsy	LSIL	1	0	Negative
31	27	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
32	28	Yes	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
33	28	No	Borderline	Mild	Watch & wait	Negative colposcopy	NA	2	Negative
34	33	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
35	37	No	Mild	Mild	Biopsy	LSIL	1	3	Positive
36	30	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	1	Negative
37	36	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	2	Negative
38	47	Yes	Mild	Mild	Watch & wait	Negative colposcopy	NA	1	Negative
39	57	Yes	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
40	42	No	Mild	Mild	Biopsy	LSIL	1	1	Negative
41	25	No	Borderline	Mild	Watch & wait	Negative colposcopy	NA	10	Positive
42	28	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
43	50	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
44	48	Yes	Severe	Dense	LLETZ	LSIL	1	2	Negative
45	25	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	13	Positive
46	25	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
47	28	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
48	27	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
49	35	No	Mild	Mild	Watch & wait	Negative colposcopy	NA	0	Negative
50	29	Yes	Borderline	Mild	Watch & wait	Negative colposcopy	NA	0	Negative

Table 1: Patient data, clinical diagnosis, and cell lift analysis results. Asterisks indicate two CIN2 cases that were misdiagnosed by the cell lift analysis in this study.

(Vector Laboratories) and TSA Fluorescein (Akoya Biosciences) according to the manufacturer's instructions. Nuclear counterstain was performed with 4'-6-diamidino-2- phenylindole (DAPI) (Sigma) before mounting in CitiFluor AF1 Mounting Medium (Electron Microscopy Sciences) for scanning. The whole membrane image was obtained using the ArrayScan XTI (Thermo Fisher Scientific) at 10x magnification. The membrane immunostaining method was validated and optimised on suitable controls − HeLa and HaCaT cells, and tongue lifts from healthy volunteers. The MCM signal was also developed using the DAB Substrate Kit, Peroxidase (HRP), with Nickel (Vector Laboratories) according to the manufacturer's instructions.

To make an HSIL diagnosis, we used a cut-off of ≥2 image fields containing an MCM positive lesion (an area defined as a cluster of >5 MCM positive cells) based on the ROC analysis of all patients which provided a balance between a high sensitivity and specificity. Samples not meeting these criteria were classified as LSIL/negative. These criteria were applied to all patient samples, and the results compared to the independent histological diagnosis (the best available clinical diagnosis) for the patient, or in the absence of a biopsy/LLETZ the cytology and colposcopy result. The images were assessed for MCM positive areas independently by three persons blinded to the initial referral cytology/colposcopic impression and histology results, and where disagreement occurred a majority consensus was used.

Detection of HPV DNA in sample storage buffer

The sample storage buffer was analysed for the presence of high-risk HPV DNA using the cobas 6800 System (Roche) by the East of England Cervical Screening Service, Norfolk & Norwich University Hospitals NHS Foundation Trust. The results are reported as invalid, hrHPV negative, or hrHPV positive with genotyping of HPV16/18/other hrHPVs.

Mixed-scale dense convolutional neural network (MSD-CNN) training and testing

To expedite and aid image analysis by differentiating between artefactual signal such as non-specific staining of granulocytes and abnormal cervical cells, a mixed-scale dense convolutional neural network (MSD-CNN)[17] was employed to identify and flag lesions in these large image sets (676 image fields per patient/cell lift).

A training set of 151 image fields (1104 × 1104 pixels per image field) was created using 38 MCM positive and 113 MCM negative fields. These image fields were selected from a group of 4 biopsy-confirmed patients. Each positive image field was manually annotated by indicating which cells were part of a lesion, resulting in a target image with a small disc in the centre of each

indicated cell and zeros everywhere else. The default configured MSD-CNN (previously described[17]) was trained to reproduce such target images using the raw MCM and DAPI images as input. 90% of the images (136 images) were used for training, while the remaining 10% (15 images) were used as a validation set to monitor performance during training. The MSD-CNN parameters that resulted in the best performance on the validation set were kept for further use.

Statistics

All statistics were performed using GraphPad Prism 9. Demographic differences between the HSIL and LSIL group were assessed using a t-test. Clinical utility of CCL was assessed firstly using a standard contingency table for sensitivity/specificity/PPV and negative predictive value (NPV). The PPV was also adjusted for HSIL prevalence using the following formula: (sensitivity x prevalence) / (sensitivity x prevalence + (1 − specificity) x (1 − prevalence)). The utility of the approach was also assessed using a Mann-Whitney U test to compare the difference in the number of biomarker-positive image fields between HSIL and LSIL, and this was used to generate a receiver operating characteristic (ROC) curve.

Role of funding source

Results

Sampling the transformation zone of the cervix

All triage methodologies should ideally sample the cervical transformation zone. This zone is made up of endocervical and ectocervical cell populations, with high levels of physiological metaplasia. The transformation zone is vulnerable to transforming HPV infection, and this susceptibility to dysplasia requires a sampling technique that can adequately visualise and assess the entire region.

To evaluate our method, cell coverage on the sampling membrane was assessed with DAPI nuclear stain. When the membrane was applied to the cervix (Supplementary Figure 1), there was a uniform sampling of

cells with no gaps in coverage (Figure 1a). The lifted cells had the expected nuclear-cytoplasmic ratio of normal cervical epithelium (Figure 1b) with the addition of granulocytic neutrophils in patients where cervical mucus was present during sampling. These observations were confirmed with haematoxylin and eosin staining (Figure 1c).

The cell lifts were then assessed using keratin markers for each cell type. The ectocervical/squamous cells were labelled with keratin 13, a marker of differentiating/stratified epithelial cells, and the endocervical/glandular cells were labelled with keratin 19, a marker of endocervical glandular epithelium.[20] A cross section through the cervical epithelium shows the mutually

(a)

(b)

(c)

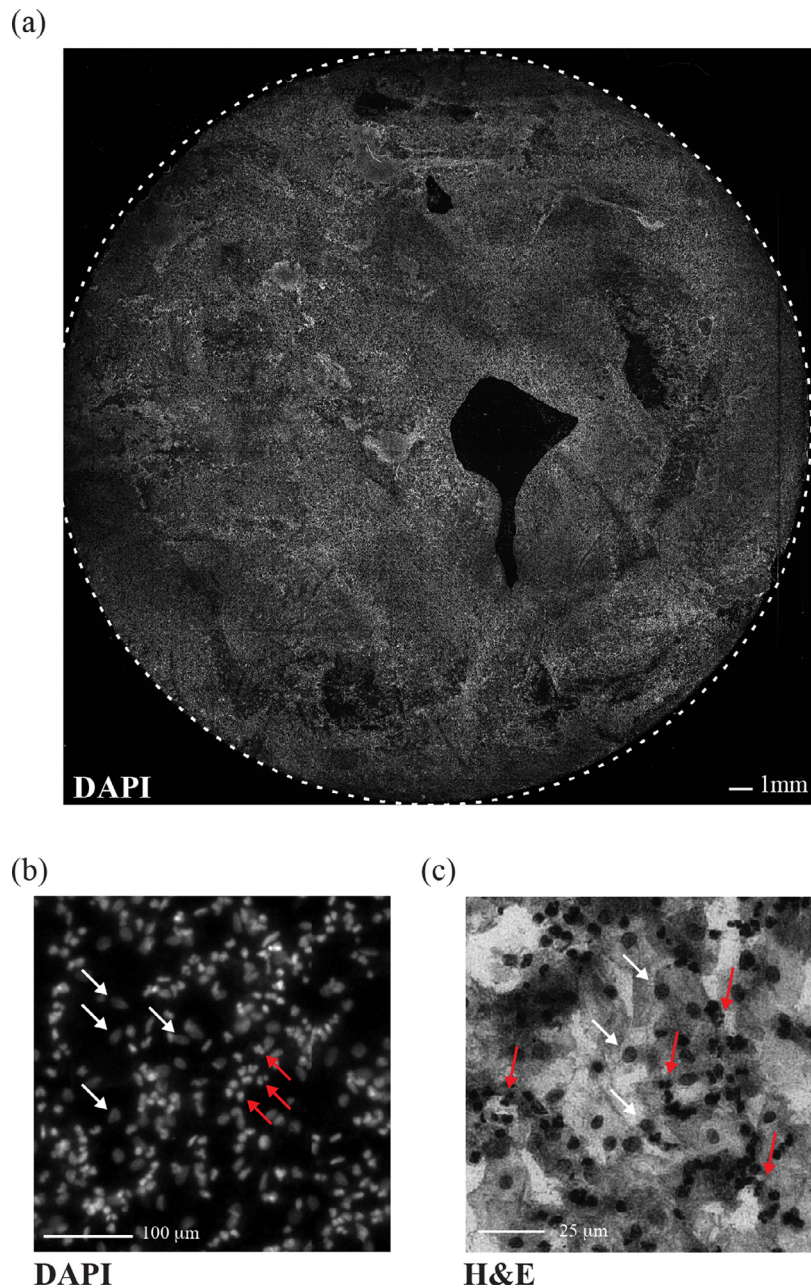**Figure 1. Scanned image of the cervical cell lift membrane.**
Cell lift membrane stained with DAPI. Near-uniform coverage of surface cervical keratinocytes. (b) Higher magnification image of cell lift membrane, highlighting cervical keratinocyte nuclei (white arrows) and multi-lobed neutrophil nuclei (red arrows). (c) H&E staining of normal keratinocytes (white arrow) and neutrophils (red arrow) on the membrane.

exclusive staining patterns of these markers in the cervix (Figure 2a). Keratin 13 is exclusively expressed in the ectocervical region (Figure 2c, arrows), whereas in the metaplastic transformation zone both markers are expressed (Figure 2d). In areas of metaplasia there were also cells co-expressing both markers (Figure 2d, arrows), further validating that our method samples the appropriate area of the cervix.

Detection of precancerous lesions using MCM

Patients with known CIN1 or CIN3 cases were identified from their final histology and the corresponding tissue sections were stained with MCM (Supplementary Figure 2). As expected, MCM was expressed across the entire cervical epithelium in the CIN3 cases, while the CIN1 cases showed MCM positivity in the basal and mid-layers of the cervical epithelium, in keeping with the genome amplification phase of the productive HPV lifecycle. MCM expression of the corresponding cell lifts of these patients were also assessed by immunofluorescence staining (see Materials and Methods). In the cell lifts of patients with CIN3 (Supplementary Figure 3), it was possible to identify HSIL/MCM positive lesions as clusters of MCM positive cells (Figure 3a and b). In contrast, there was no MCM expression or MCM positive lesions at the epithelial surface of the CIN1/LSIL cases as expected from our understanding of these marker
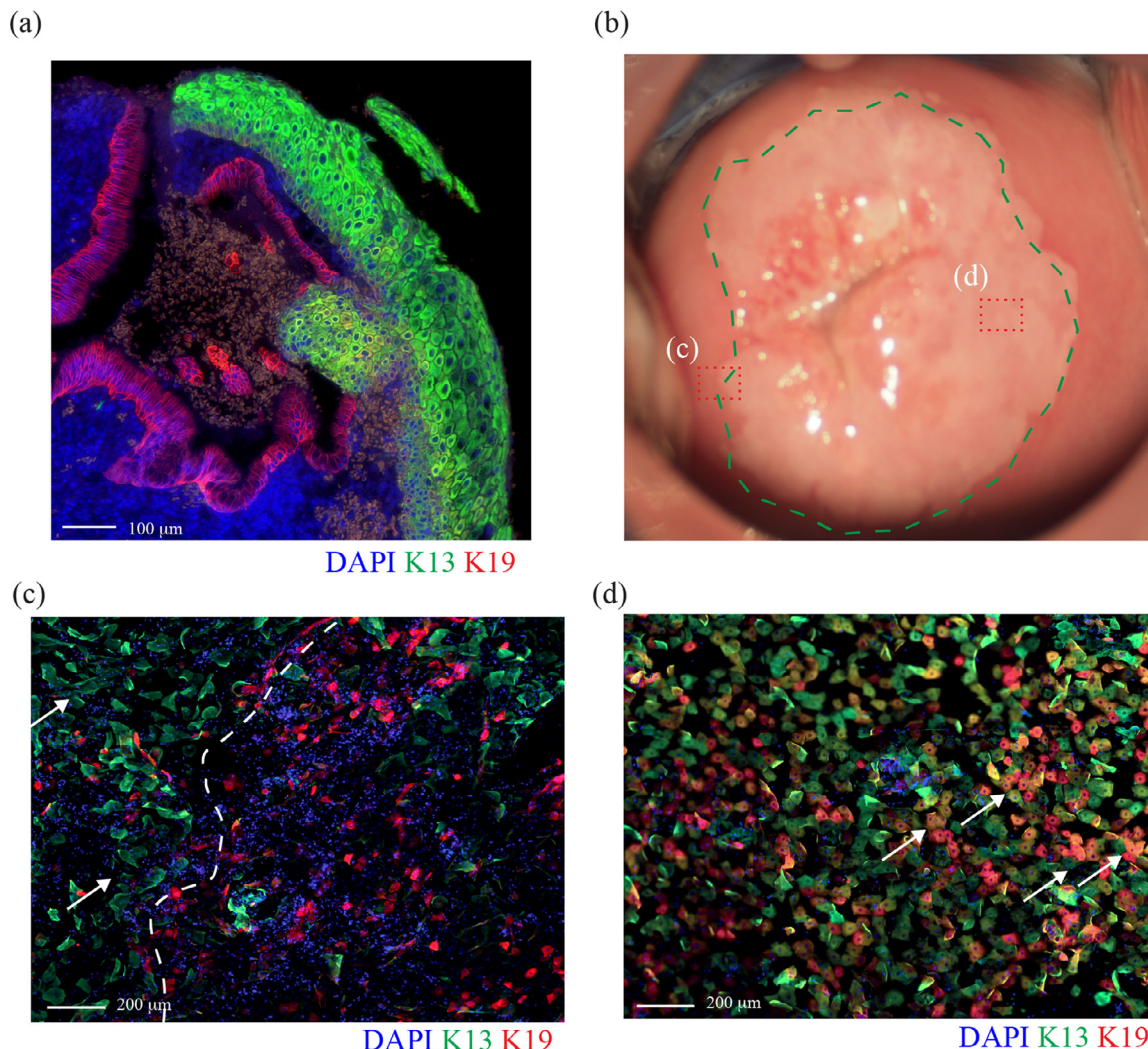
Figure 2. Immunostaining of the cervical epithelium with keratin markers to assess cell type.
Cross section of the cervical epithelium stained with K13 (green) and K19 (red). (b) Colposcopy photograph of the cervix following application of cell lift membrane. Acetowhite solution has been applied, revealing the large transformation zone (inside the green dotted line). (c) Demarcation of ectocervix (left) and transformation zone (right) boundary, K13 (green) and K19 (red) positive cells. (d) Cervical transformation zone. K13 (green) and K19 (red) positive cells. White arrows indicate K13/K19 dual positive cells.
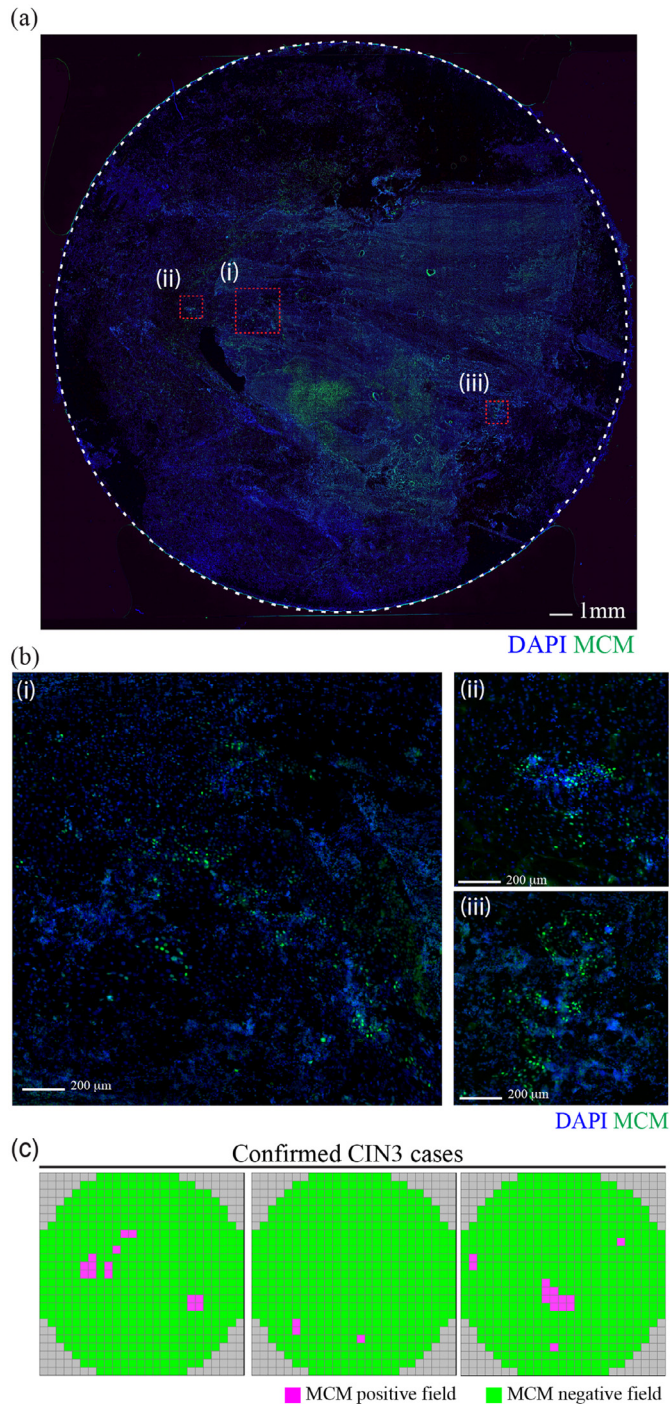
Figure 3. MCM biomarker staining of cervical cell lifts.

(a and b) Cell lift membrane from a patient with a confirmed CIN3 lesion, stained with MCM (green) and DAPI (blue). An entire cervical cell lift is shown (a). Three MCM positive HSIL/CIN3 lesions (red insets) are shown in (b). (c) Heat maps representing patient cell lift samples, showing MCM positive image fields (pink) and MCM negative image fields (green). The samples are three confirmed CIN3 cases.

patterns as these cells have exited the cell cycle. MCM positive image fields (pink) and MCM negative image fields (green) from cell lift samples of patients with confirmed CIN3 cases are shown as heat maps (Figure 3c).

In addition to immunofluorescence staining, a chromogenic staining method was also assessed. Chromogenic approaches are used frequently in histopathology, being less expensive to perform and analyse than immunofluorescence staining. Our results show that DAB staining is a feasible way of identifying MCM positive areas on the membrane (Supplementary Figure 4) and may prove especially useful in a LMIC setting. However, a significant disadvantage of chromogenic approaches is the difficulty in utilising multiple (3 or more) markers without problems interpreting the results.

Contamination of the cervical sample with different cell types such as granulocytes (Figure 1b and c) leads to difficulties in interpretation and unsatisfactory sampling, and was one of the main reasons for moving from conventional to liquid-based cytology.[21] Our novel approach of using a cell-cycle specific biomarker (MCM) together with endogenous peroxidase blocking and background subtraction allows these contaminant cells to be excluded from the interpretation. While this method leads to low 'noise', granulocytes are still visible, although this didn't hinder the HSIL identification due to the nuclear staining pattern of dysplastic cells vs. cytoplasmic/non-specific staining of the granulocytes (Figure 1b).

Cervical cell lift pilot study results

A total of 50 participants, who were HPV positive and cytology positive, were recruited for this pilot study to evaluate the potential of the CLL for the detection of HSIL patients. MCM expression on the anonymised cervical cell lifts was assessed for each patient manually and subsequently compared to the final patient clinical diagnosis after colposcopy examination with/without histopathological evaluation. For histologically proven HSIL cases, the median number of MCM positive image fields was 7, and for LSIL/negative samples the median number was 0 (Figure 4a, Table 1). This demonstrates that the cell lift significantly discriminates between a HSIL and a LSIL (Mann-Whitney U test; p <0.00001). Moreover, when a ROC curve (Figure 4b) was generated for this cohort of patients, the AUC was 0.87 (95% CI 0.75-0.98), further validating the accuracy of this approach.

All CIN3 cases and 1 CGIN case (n=15), and two out of four CIN2 cases, were correctly identified. This was made possible by the ability to sample all surface cervical cells, including the transformation zone, with the membrane and identify lesions in their entirety due to the preservation of cell topology.

The sensitivity of this method for the detection of histology proven HSIL in a patient is 90% (17/19; 95% CI 69-98), with a specificity of 87% (27/31; 95% CI 71-95), a crude PPV of 81% (95% CI 60-92), and a NPV of 93% (95% CI 78-99) (Table 2). This is a higher specificity and PPV in comparison to LBC, methylation analysis, and p16/Ki67 staining since high-grade lesions can be identified in their entirety, and akin to that of histology. When the PPV is adjusted to factor in the prevalence of HSIL in an HPV positive population, it drops to between 44% and 75%, with a corresponding NPV of 95-97% (Table 3), however these values are seemingly superior to other triage methodologies. If this high PPV is replicated in larger patient cohorts, the number of
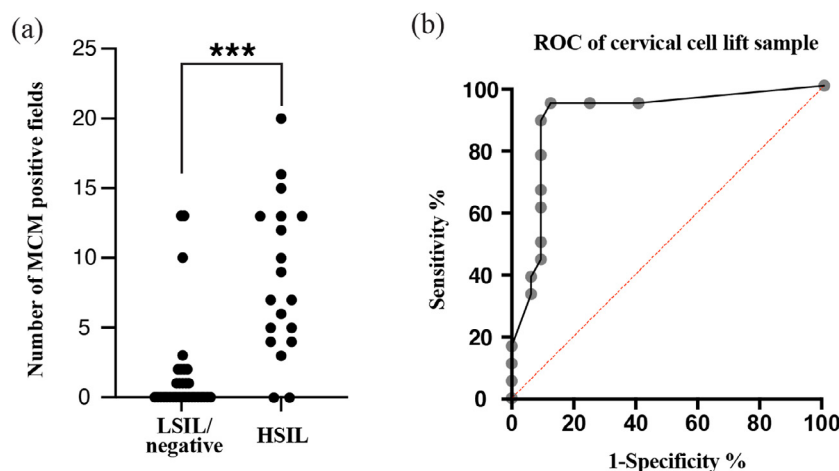

Figure 4. Cervical cell lift pilot study results.
Scatter plot of the number of MCM positive image fields in the HSIL vs. LSIL/negative cases (n=50). (b) ROC curve demonstrating an overall accuracy of this method of 88%, with a sensitivity of 89% to HSIL.

	%
Sensitivity	90 (95% CI 69 - 98)
Specificity	87 (95% CI 71 - 95)
PPV	81 (95% CI 60 - 92)
NPV	93 (95% CI 78 - 99)

Table 2: Sensitivity/specificity/PPV and NPV of cell lift sampling in the pilot study cohort.

patients referred for colposcopy with clinically irrelevant disease, which has nearly doubled since the introduction of primary HPV screening,[8] will decline with no apparent reduction in HSIL detection.

Automated high-throughput interpretation using machine learning to detect biomarker-positive cells

The MSD-CNN was trained as described in Materials and Methods. After training, the MSD-CNN was evaluated on 10 image fields that were manually annotated but not used during training, i.e. unseen by the algorithm, for comparison. The algorithm could identify these MCM-positive cells in a comparable manner to the human interpreter (Figure 5a-c).

The trained MSD-CNN analysed full patient data sets that hadn't been used for training and validation (*n*=35). With this data set the algorithm performed comparably to manual assessment, with the algorithm flagging a greater number of MCM positive cells compared to manual analysis, in keeping with the validation results. For the 35 patient samples (35 × 676 image fields), 113 MCM positive image fields were identified manually, and the algorithm correctly flagged each of these image fields. Furthermore, the MSD-CNN was able to analyse these large patient data sets of 676 image fields in under 5 minutes, flagging on average 20 areas of interest (true positives and artefacts) in a rapid, high throughput manner (Figure 6a-d, and Supplementary Figure 5a-c). The algorithm was able to correctly ignore the majority of artefactual staining (Figure 6d and e, and Supplementary Figure 5d) aiding the identification of lesions. However, while the algorithm was able to suppress most of these non-specific signals, within this data set the algorithm incorrectly called 162 image fields. This could be improved by including more artefactual areas in the training set.

Another important advantage of using this algorithmic approach is the ability to stitch the 676 image fields together to produce a single image of the entire cell lift membrane (Figure 6a and Supplementary Figure 5a), creating a map of the surface of the cervix. The precise location of a precancerous lesion can be identified, which not only aids diagnosis, but could also assist in targeted biopsies or treatment.

Detection of HPV DNA for primary screening

The ability to detect hrHPV DNA in the cell lift storage buffer would allow for both hrHPV screening and precancerous lesion detection from a single patient visit, as with conventional LBC. To explore this, storage buffers samples from 47 patients that had tested HPV positive during primary screening before being referred for colposcopy were tested for hrHPV DNA. 44/47 samples (Table 4) tested positive for a hrHPV, a detection rate of 94%. While being reasonably high, this detection rate would require further evaluation in a larger trial. To merit use in a clinical screening setting a detection rate close to 99% is required to ensure patients are not missed.

Discussion

Primary HPV screening will certainly lead to higher detection of cervical pre-cancer/cancer.[2] However, the current LBC triage methodology can miss a proportion of clinically relevant disease and lead to unnecessary colposcopy referrals that expose patients to further invasive procedures with the risk of excisional treatment and its consequences.[8,13] A number of techniques for HPV triage have been evaluated, such as p16/methylation status.[14,15] Indeed, p16/Ki67 staining of LBC samples does have a higher sensitivity to HSIL in comparison to LBC, however these techniques seem to offer no cost-effective alternative to LBC when the positive predictive values are compared.[6] Moreover, the current methodology for cell sampling disrupts topology, which makes correlation between a single abnormal cell and HSIL difficult to judge.

There is clearly a need for a superior triage test that samples the cervix in a manner that exploits our understanding of the HPV lifecycle. Here we described a novel cell lift sampling technique that preserves the

	HSIL prevalence		
	10%	**20%**	**30%**
PPV (%)	44 (95% CI: 23-66)	63 (95% CI: 41-81)	75 (95% CI: 54-88)
NPV (%)	99 (95% CI: 95-99)	97 (95% CI: 90-99)	95 (95% CI: 84-99)

Table 3: PPV and NPV is adjusted for prevalence of HSIL in an HPV positive population. 20-30% is accepted as the prevalence of HSIL in an HPV positive cohort in the UK.[14]
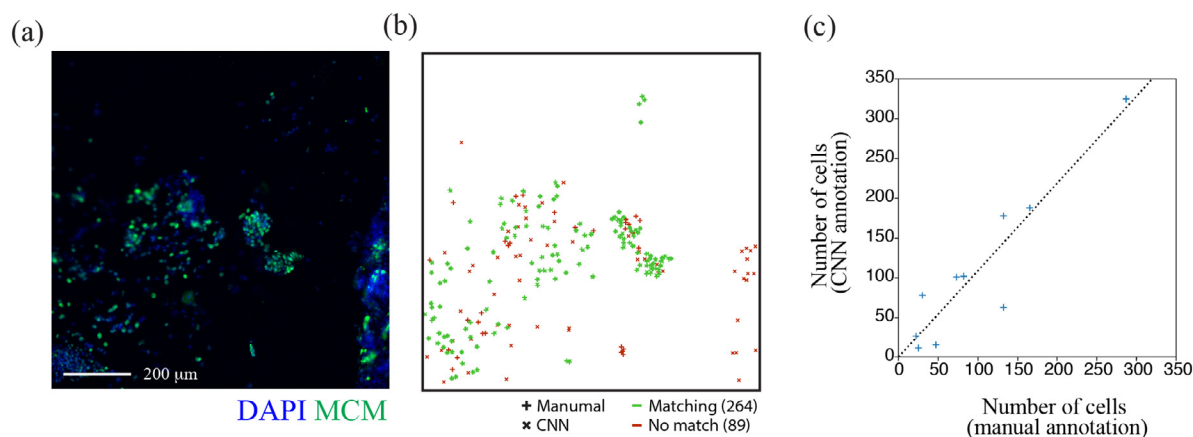
Figure 5. Evaluation of machine learning for MCM positive cells in patient samples.
(a) and (b) CNN analysis of an MCM positive image field. The scanned image (a) was manually annotated for MCM positive cells and analysed by the trained CNN (b). The CNN output was then compared to the manual annotation. The CNN detected most of the annotated cells (264 matches vs. 89 non-matches), and no abnormal cells were missed. The non-matches were artefacts that the CNN deemed positive. (c) The algorithm was further evaluated on a set of 10 new image fields. The graph compares CNN to manual annotation of MCM positive cells. A linear fit of the data points results in a line with a slope of 1.1, indicating close similarity between manual and CNN annotation.

surface layer of cervical cells, which are probed with biomarkers to precancerous lesions[19] to generate a molecular spatial map of the cervix. In this pilot study we found that this method is seemingly non-inferior to LBC and may have an advantage in the detection of HSIL/CIN3 lesions. This advantage is likely due to the preservation of the cervical cells in their native locations, where lesions can be identified in their entirety, in stark contrast to LBC where correlation to lesion status is based on the identification of individual or small clusters of morphologically abnormal cells that are observed out of context.

The ability to precisely locate lesions on the cervix provides invaluable information, especially to a colposcopist. The cell lift membrane has an orientation marker showing the position of its application on the cervix, allowing for precise location of lesions and facilitating targeted biopsies and more personalised treatment. Indeed, this advantage is akin to that of other colposcopic aids, such as spectroscopy, that are used to improve the sensitivity/specificity of colposcopy.[22] However, cell lift sampling is simpler to perform and by providing cellular level biomarker data akin to histology, provides an objective/quantitative result.

Our approach can also be adapted to facilitate multimarker analysis of the cervical epithelium. As knowledge of the HPV life cycle improves it is possible to employ several antibodies to identify and discriminate between lesions of low and high malignant potential. Other multimarker panels exist such as p16/Ki67 (Roche) or methylation markers such as S5,[15] and p16/MCM together with a marker of low-grade disease such as E4 may offer the maximum information on lesion status.[23]

The use of high content screening and image processing for the immunostained samples generates a large data set. Manual analysis is feasible in a trial setting, however when expanded to a population level machine learning can be used to improve time and cost efficiency, and can identify lesions that may be missed otherwise. The algorithm can flag up areas of interest on the cervix that will help the colposcopist to provide targeted biopsies/treatment. Machine learning is a feasible approach for the identification of abnormalities on the cervix,[24] however there is still resistance to the use of these techniques, much of which stems from many algorithms not demonstrating a clear logic for a given output (the black box phenomenon). In the case of our algorithm, the AI performs a biomarker-positive cell annotation task and not a final patient diagnosis. The algorithm has been trained to identify and flag areas of MCM positive cells, and these flagged regions are analysed and interpreted by an appropriately trained human who will provide the diagnosis. Thus, there is less ambiguity in regard to why the AI is providing a particular output.

Cervical cancer screening uptake is falling, so to improve uptake many groups are trialling urine and vaginal self-swab testing,[25,26] which if proven to be non-inferior to conventional primary HPV testing could lead to a significant increase in screening. A downside of self-testing is the requirement for triage testing at a separate date, which may lead to patients being lost to follow-up.[27] While our approach may not increase uptake, it may be more reassuring for patients to receive screening and triage results more quickly from a single sample. In this small pilot study, we demonstrate an HPV-
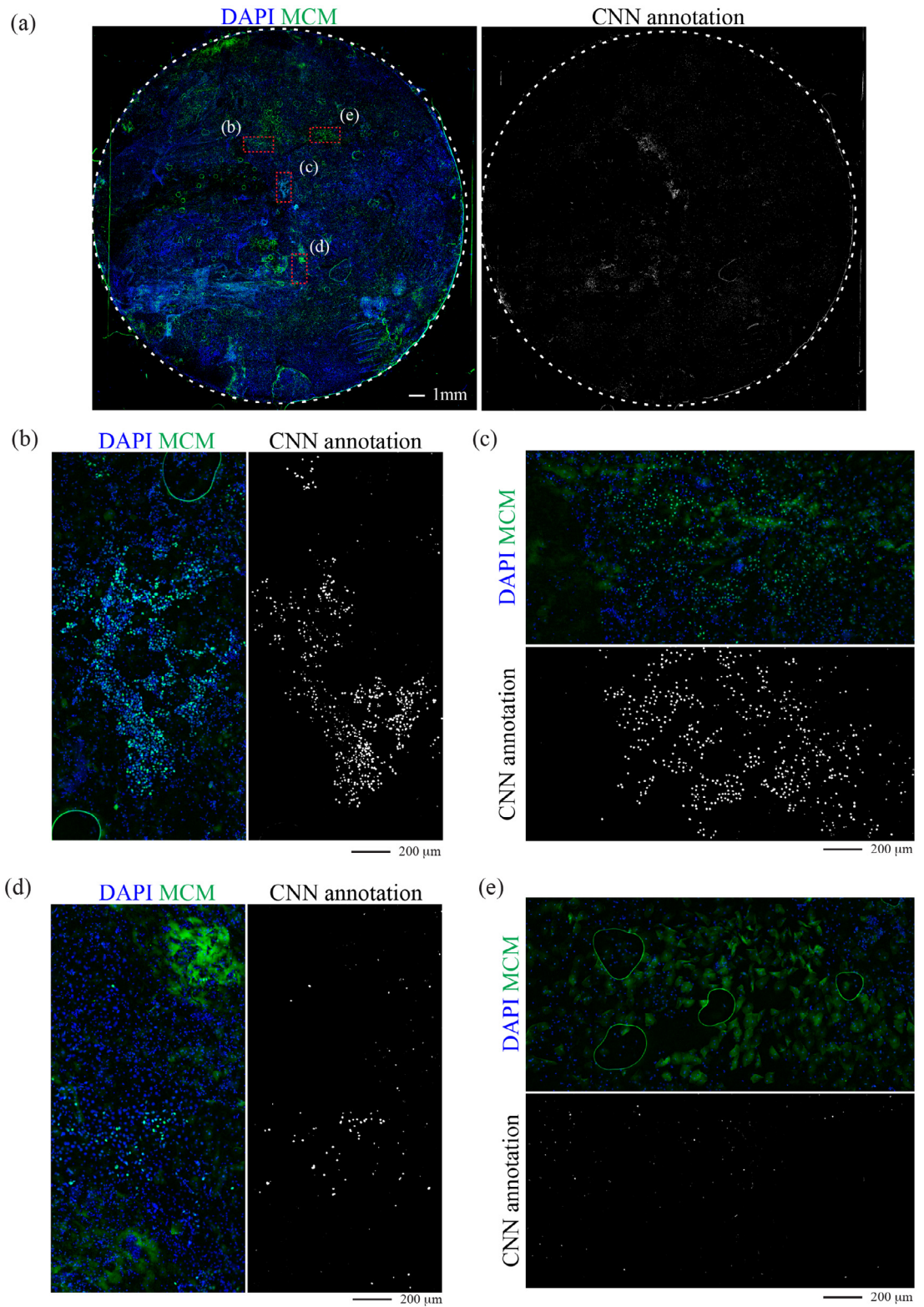
Figure 6. Evaluation of machine learning for cell lift image analysis.
(a) and (b) Cell lift membrane stained with MCM and DAPI (a) assessed by the algorithm (b). (c-e) Scanned image fields (red insets) and corresponding CNN output image showing a lesion (b-d) and non-specific staining (d and e).

Patient ID	HPV result			HPV TEST
	16	18	Other	
DNA-01	-	+	-	POSITIVE
DNA-02	-	+	-	POSITIVE
DNA-03	-	+	+	POSITIVE
DNA-04	+	+	-	POSITIVE
DNA-05	+	-	-	POSITIVE
DNA-06	-	+	+	POSITIVE
DNA-07	-	+	+	POSITIVE
DNA-08	+	+	-	POSITIVE
DNA-09	-	+	+	POSITIVE
DNA-10	-	+	+	POSITIVE
DNA-11	-	+	+	POSITIVE
DNA-12	-	-	+	POSITIVE
DNA-13	-	-	+	POSITIVE
DNA-14	-	-	+	POSITIVE
DNA-15	-	+	+	POSITIVE
DNA-16	-	-	+	POSITIVE
DNA-17	-	-	-	NEGATIVE
DNA-18	-	-	+	POSITIVE
DNA-19	-	-	+	POSITIVE
DNA-20	+	-	-	POSITIVE
DNA-21	-	-	-	NEGATIVE
DNA-22	-	-	+	POSITIVE
DNA-23	+	-	-	POSITIVE
DNA-24	+	+	+	POSITIVE
DNA-25	-	+	+	POSITIVE
DNA-26	+	-	+	POSITIVE
DNA-27	-	-	+	POSITIVE
DNA-28	-	-	+	POSITIVE
DNA-29	-	-	-	POSITIVE
DNA-30	+	-	+	POSITIVE
DNA-31	-	-	+	POSITIVE
DNA-32	+	+	-	POSITIVE
DNA-33	-	-	+	POSITIVE
DNA-34	-	-	+	POSITIVE
DNA-35	+	-	-	POSITIVE
DNA-36	-	-	+	POSITIVE
DNA-37	-	-	+	POSITIVE
DNA-38	+	-	-	POSITIVE
DNA-39	+	-	+	POSITIVE
DNA-40	-	-	-	NEGATIVE
DNA-41	+	-	+	POSITIVE
DNA-42	+	-	+	POSITIVE
DNA-43	+	-	-	POSITIVE
DNA-44	+	-	-	POSITIVE
DNA-45	+	-	-	POSITIVE
DNA-46	+	+	-	POSITIVE
DNA-47	-	-	+	POSITIVE

Table 4: **Results of hrHPV screening of 47 sample storage buffer samples.**

DNA detection rate of 94% from the cell lift sample buffer. Interestingly, a patient who tested HPV-positive at primary screening but had a negative sample buffer had no clinically relevant disease at colposcopy, so it's possible that this patient's HPV infection was being controlled by their immune system, hence the negative latter result.

These initial results will require further evaluation on a larger cohort of HPV positive patients, which is underway in the UK and also Japan, where vaccine hesitancy makes improved screening and triage even more important. All patients recruited to this pilot study had abnormal cytology as an entry requirement, which makes direct comparison between LBC and cell lift sampling difficult. A future study will be based in the community where cytology and cell lift sampling will be performed at the same time, allowing for a direct comparison.

CIN2 lesions form part of the spectrum of HSIL disease, and presently our method has a low sensitivity to CIN2. This may be because the top 2/3 cell layers of the cervical surface are evaluated for disease, and CIN2 abnormalities are confined to the lower epithelial layers. However, as CIN2 lesions progress to CIN3 and then cancer, and as all CIN3 cases were detected by the cell lift sampling, this method can flag the patients that need loop excisions while allowing patients with CIN2 lesions to be retested 12 months later. From the two CIN2 cases that were misdiagnosed in this study (Table 1, asterisks), the first patient had a LLETZ which subsequently only demonstrated CIN1, and the second patient was managed conservatively and 1 year later had cleared their HPV infection. Thus, perhaps using our approach we may be identifying more aggressive lesions that warrant expedited treatment. With the addition of an E4 biomarker there is the potential to monitor these patients non-invasively by obtaining information on lesion status without the need to take a biopsy. High E4 expression levels at the cervical surface indicates a non-transforming infection, adding a further level of reassurance.

It is important to note that the study also identified a case of glandular dysplasia, which is promising as such lesions are harder to detect given their localisation within the cervical canal. These lesions may not be caught by cell lift membrane unless the lesion is large, as it was in this case. The detection rate of such lesions will need to be stringently evaluated as we assess this approach on a larger population. Four patients were incorrectly identified as having high-grade disease. For two of these cases biopsies confirmed LSIL in the same quadrant where an MCM positive image field was noted, while no biopsy was taken for the other two patients and follow-up with repeat cytology found no HSIL. These four false positives may be the result of MCM expression in cases of inflammation or metaplasia and could be mitigated against by using an alternative panel of markers such as p16/MCM/E4 to provide more information on the clinical significance of disease.

While the current method of sampling using the cell lift membrane has not proven difficult from clinician's feedback, some optimisation is needed. During this trial, the cell lift membrane was applied using a pair of sterile forceps, which occasionally resulted in insufficient cell coverage, in some instances the cell lift membrane was damaged. To address these issues, the development of a sampling device which allows for standardised sampling in a larger trial is required.

We have described a novel triage method, using a cervical cell lift, which preserves cervical surface cells in their native topology for spatial mapping and grading of precancerous cervical lesions using biomarker immunostaining. The PPV of this cell lift method is seemingly superior to current triage methods, and the results of this pilot study support the concept that the cervical cell lift method could lead to better identification of patients with clinically relevant disease and more efficient allocation of healthcare resources.

Contributors

Dr Aslam Shiraz and Dr Nagayasu Egawa are joint first authors. They performed the experiments including study design, data collection, analysis, interpretation, verifying the underlying data and writing the manuscript. Dr Robin Crawford aided in project conceptualisation, study design, and data/sample collection from patients. Dr Daniël Pelt developed, trained and tested the machine learning algorithm. He also wrote sections of the manuscript pertaining to the machine learning and proofread it as well. Adeline Nicholas performed sample processing, image acquisition, image analysis, and assisted in the writing, editing, and proofreading of the manuscript. Veronika Romashova assisted in image acquisition. Dr Heather Griffin aided in project conceptualisation and established the use of biomarkers for this method. Prof Peter Sasieni in the statistical analysis of our data, aided in study design, reviewed and edited the manuscript. Prof John Doorbar was involved in all aspects of the study including manuscript preparation. All authors read and approved the final version of the manuscript.

Data sharing statement

All data generated or analysed during this study are included in this report. All materials are commercially available.

Declaration of interests

Dr Egawa, Dr Shiraz and Prof Doorbar report grants from Janssen Pharmaceuticals/Advanced Sterilization Products (ASP). Dr Egawa was working for Maruho Co., Ltd as a consultant outside of this work. Ms Nicholas, Ms Romashova, Dr Griffin, Prof Sasieni and Dr Crawford have nothing to disclose pertaining to this project.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104157.

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