The early detection of cervical cancer. The current and changing landscape of cervical disease detection

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Abstract
Cervical cancer prevention has undergone dramatic changes over the past decade. With the introduction of human papillomavirus (HPV) vaccination, some countries have seen a dramatic decline in HPV-mediated cervical disease. However, widespread implementation has been limited by economic considerations and the varying healthcare priorities of different countries, as well as by vaccine availability and, in some instances, vaccine hesitancy amongst the population/government. In this environment, it is clear that cervical screening will retain a critical role in the prevention of cervical cancer and will in due course need to adapt to the changing incidence of HPV-associated neoplasia. Cervical screening has, for many years, been performed using Papanicolaou staining of cytology samples. As our understanding of the role of HPV in cervical cancer progression has advanced, and with the availability of sensitive detection systems, cervical screening now incorporates HPV testing. Although such tests improve disease detection, they are not specific, and cannot discriminate high-grade from low-grade disease. This has necessitated the development of effective triage approaches to stratify HPV-positive women according to their risk of cancer progression. Although cytology triage remains the mainstay of screening, novel strategies under evaluation include DNA methylation, biomarker detection and the incorporation of artificial intelligence systems to detect cervical abnormalities. These tests, which can be partially anchored in a molecular understanding of HPV pathogenesis, will enhance the sensitivity of disease detection and improve patient outcomes. This review will provide insight on these innovative methodologies while explaining their scientific basis drawing from our understanding of HPV tumour biology.

KEYWORDS
biomarkers, cervical cancer, cervical screening, human papillomavirus, human papillomavirus testing, high-grade squamous intraepithelial lesion
INTRODUCTION: CERVICAL NEOPLASIA, CERVICAL CANCER AND ITS CONTROL

Human papillomaviruses (HPVs) are causally involved in the development of almost all cases of cervical cancer,1 a disease which currently affects over half a million women each year worldwide.2 Over the past 50 years, the incidence of cervical cancer has declined in high-income countries (HIC), with much of this reduction attributed to the availability of screening programmes such as those that exist in the UK.3 Indeed, in the UK, cervical screening is thought to have prevented a dramatic rise in the incidence of cervical cancer in the cohort of women born in the 1940s and early 1950s who were involved in the sexual revolution following the introduction of the contraceptive pill.4 With the introduction of HPV vaccination over the past 10 years in HIC that already have good cervical screening infrastructures, there is now the potential to further reduce cervical cancer incidence.4

Unfortunately, the impact seen in HIC, has not yet been emulated in low and middle-income countries (LMIC). In such settings, there are a multitude of confounding factors that limit screening success, which are related partly to cost, but also more generally to the poorer healthcare infrastructure, which hampers diagnosis and effective treatment within the community. Thus, the vast majority of afflicted individuals now live in LMIC,5,6 and although initiatives such as the GAVI (https://www.gavi.org) vaccine programme are improving this in some settings (eg, Rwanda), cervical cancer remains a disease of the poor. Even in HIC, however, where both screening and vaccination programmes exist, poor uptake of screening, and poor vaccination coverage remain a problem. In the UK, for instance, there has been a steady decline in screening attendance over the last 10 years, and although vaccination coverage remains high (>70%), other HIC suffer from low coverage (USA/France), or very low uptake (Japan).7,8

There remains therefore, a role for straightforward and cost-effective detection strategies that are able to decisively identify women who are at risk of developing cervical cancer. The diagnostic approaches used should address the issues (see below) that restrict the widespread uptake of our current systems. Linking disease detection to effective treatment is an additional but necessary requirement when designing management strategies, and these should be tailored to the available resources in LMIC and HIC settings. While vaccination remains the best primary control measure, it is very unlikely that vaccination programmes will achieve adequate herd immunity in the near future in the most vulnerable communities. In this review, we provide insight from our current understanding of viral pathogenesis, that we hope will guide the development and future use of approaches for cervical screening and cervical disease stratification.

HUMAN PAPILLOMAVIRUS INFECTION AND THE SELECTION OF DIAGNOSTIC BIOMARKERS

Papillomaviruses have evolved over many hundreds of millions of years and comprise a group of viruses that have become specialists in infecting and propagating themselves in epithelial tissue.3 To date, over 200 HPV types have been identified, which cause a variety of epithelial lesions ranging from benign skin warts to invasive cancers. The majority of HPV infections are adequately controlled by the host’s immune system, as evidenced from the numerous asymptomatic HPV infections that occur in immuno-competent individuals.9 The cancer-causing high-risk HPV (hrHPV) types are members of the Alpha genus of papillomaviruses, a group that also contains a plethora of low-risk types that cause genital warts and respiratory papillomas (types 6 and 11). The Alpha HPV types have been extensively studied, and we have a reasonable understanding how viral gene expression facilitates virus synthesis, and how deregulated viral gene expression predisposes to the development of neoplasia and cancer.10,11 Despite this accumulated knowledge, our understanding of the cervical transformation zone, and the reasons for its particular vulnerability to hrHPV-driven neoplasia remains incomplete.12 Similarly, we cannot say with confidence why some individuals are able to immunologically control or clear their infections, while others are prone to persistence and neoplastic progression.

Like all viruses, HPVs are obligate intracellular parasites that subvert the host cell regulatory mechanisms to replicate and produce infectious progeny. In most cases, the target cells for infection are the epithelial basal cells, that reside in the lowest layers of stratified squamous epithelium. To access these cells, HPV requires a wound-inducing event, such as the epithelial trauma and/or inflammation that can occur at the cervix due to the exposure of the columnar epithelium to the acidic environment of the vagina (after puberty) or during sexual intercourse. Access to vulnerable epithelial basal cells is facilitated at the cervical transformation zone and, for some HPV types, hair follicles and eccrine glands have also been considered as points of entry.1 After infection, papillomavirus genomes can persist in cells of the epithelial basal layer for months or years, with such infected cells providing a reservoir of HPV infection in the body, and virus synthesis occurring only when the epithelial cell leaves the epithelial basal layer and differentiates. Only very limited viral gene expression is normally tolerated in the basal cells, to allow the virus to maintain its genome and to control differentiation. The molecular processes that papillomaviruses require for persistence can, however, become misdirected towards the development of cancer. The HPV E6 protein for instance, normally modulates pathways that control how the epithelial basal cell becomes committed to differentiation and, when deregulated, can lead to the accumulation of undifferentiated epithelial cells at sites of infection.13 The E7 protein has a key role in
benign lesions in driving cell cycle re-entry to allow virus replication in the superficial post-mitotic cells. However, the deregulation of E7 expression can stimulate uncontrolled cell proliferation throughout the epithelium and, when considered alongside E6, it is easy to appreciate how the expression of the hrHPV E6/E7 genes may drive a neoplastic phenotype. Indeed, the deregulated expression of hrHPV E6/E7 can result in low and intermediate grades of cervical neoplasia, but it is the persistent expression of these genes and in particular the accumulation of additional genetic changes in the infected host cell that eventually drive the development of cancer. As we will see later, many of the most promising biomarkers of disease severity and disease prognosis reflect the molecular changes that persistent E6/E7 expression can impart on the cell.

Although both high- and low-risk E6 and E7 proteins share many functions that are essential for papillomavirus lesion formation and virus synthesis, the proteins of the hrHPV types have some well-defined additional characteristics that play a key role in the development of neoplasia. In the most problematic high-risk types, these functions are at their most extreme. For example, the HPV16 and 18 E6 proteins bind and functionally disrupt a diverse range of cellular PDZ domain proteins involved in the regulation of cell polarity and cell contact, a characteristic not present in the low-risk HPV types, and that is present in a more limited form in high-risk and intermediate-risk types. Similarly, the high-risk E6 proteins are able to bind and to degrade the cellular p53 protein, a specific activity which has evolved from the E6AP binding-ability shared more widely amongst the HPV E6 proteins.14,15 Such interactions with host cellular targets, which are thought to delicately modulate normal epithelial homeostasis during productive hrHPV infection, are important drivers of the neoplastic phenotype at epithelial sites such as the cervix, where viral gene expression can become deregulated. Indeed, biomarkers that highlight these important molecular differences between high and low-risk HPV types, are particularly useful in establishing hrHPV disease causality in the clinic. p16, DNA methylation and the detection of viral proteins such as E4, harness some of this discriminatory power.

### 3 | TRANSFORMING INFECTIONS: DISCRIMINATORY BIOMARKERS OF DISEASE SEVERITY

Given that deregulated hrHPV gene expression is considered a necessary first step in the progression from infection to cancer, it makes some sense that the detection of high-grade squamous intraepithelial lesion (HSIL) has been regarded as the gold standard for cervical diagnostics. Setting a standard that is based on the morphological characteristics of a haematoxylin and eosin stained tissue section sets its own limitations, however, especially as our molecular understanding of disease progression continues to move forward. Clearly, the consequences of hrHPV gene expression, and the molecular changes that are imparted on the infected cell,11 have the potential to offer a higher level of diagnostic precision, especially when assessing the likelihood that an infection will either regress as it is brought under control by the host immune system, or progress to cancer if persistent deregulated expression is tolerated. Lesions associated with deregulated HPV gene expression are often referred to as transforming infections, but other terms are used by virologists, including abortive or non-permissive infection, to recognise the fact that the lesion is no longer productive, or permissive for virus synthesis. Just as transforming infections have their own morphological characteristics, including the presence of mitotic figures, and cells with a basal-like nuclear to cytoplasmic ratio in the suprabasal epithelial layers, productive infections are characterised by the presence of koilocytosis in the mid and upper epithelial layer, which is often regarded as a cytopathic effect of vegetative viral DNA replication and virus synthesis.1,11 At a molecular level, these phenotypic changes mark completion of the early phase of the papillomavirus life cycle, and a progression into the late phase where viral genomes or episomes are amplified, packaged and eventually shed from the epithelial surface. Interestingly, genome amplification is absolutely dependent on E6 and E7 to drive cell cycle re-entry, but in contrast to the proliferating cells of the basal and parabasal cell layers, these cells become stalled in the G2 phase of the cell cycle and begin to accumulate the HPV E4 protein at very high levels.16 Although these productively infected cells go on to express the major and minor virus coat proteins L1 and L2, it is E4 that is most prominent, accumulating to levels as high as 30% of total cell protein, due to its ability to multimerise and to assemble into amyloid-like fibres. E4 is thought to play a role in virus release and virus transmission, by disrupting the normal desmosomal contacts that tie epithelial cells together, and by protecting released virus particles from desiccation in the environment.16,17 The presence of E4, and its accumulation during productive infection, has led to its use as a biomarker of low-grade neoplasia, where it appears useful in separating cervical intraepithelial neoplasia grade 2 (CIN2), a subgroup of HSIL, into those that may be more appropriately categorised as low-grade squamous intraepithelial lesions (LSIL).18

Finally, when considering discriminatory markers of disease severity, it is necessary to consider the unique characteristics of the cervix that make it a hotspot for the development of HPV-driven cancer. The cervix consists of the stratified ectocervix, the columnar epithelium of the endocervix, and the transformation zone in between these two cell populations. This transformation zone is a site where the normal process of metaplasia leads to the formation of a multi-layered squamous epithelium, where previously there was columnar epithelium.12 This process occurs throughout a woman’s life in response to cervical irritation, (eg, tissue damage/inflammation following exposure to the acidic vaginal environment during puberty) and, as hrHPV types are sexually transmitted, they are able to gain access to this diverse spectrum of epithelial tissue.19 Unlike the stratified epithelium of the ectocervix, the transformation zone and endocervix are unable to support the productive papillomavirus lifecycle, a situation that has led to the evaluation of epithelial site-specific disease markers such as simple epithelial keratins, as adjuncts to the HPV-encoded gene products and their surrogates outlined above19,20 (Figure 1).
It has become clear that hrHPV infection of vulnerable epithelial sites such as the cervical transformation zone can facilitate the deregulated expression of E6 and E7, and that this poses a carcinogenic risk for the infected host. Based on the molecular functions of the viral gene products, and on the disease progression path that follows infection, we are able to screen and diagnose HPV-mediated disease at its precursor stages, and indeed our understanding of the HPV lifecycle can provide us with new opportunities for the triage of HPV-positive women as outlined below.

### FIGURE 1
Organisation of the cervix, and the consequences of human papillomavirus (HPV) infection. (A) Schematic representation showing the different epithelial sites of the cervix that are prone to infection by HPVs. The transformation zone and endocervix are high-risk sites for HPV-mediated carcinogenesis and are thought to be maintained by a specialised stem-like cell (the reserve cells shown in blue) that are involved in the normal process of metaplasia. (B) Possible consequences of infection by HPV. The ectocervix is able to support the productive HPV life cycle, which is manifest as low-grade squamous intraepithelial lesions (LSIL), with associated high levels of the late viral protein E4. Endocervical abortive infections are characterised by low levels of E4 and high levels of p16/MCM. The transformation zone appears to have a range of HPV gene expression patterns which are manifest as LSIL to high-grade squamous intraepithelial lesions (HSIL), which may be dependent on the cell types that becomes infected and the duration of viral persistence, which allows the accumulation of genetic errors which lead to cancer. SCJ, squamocolumnar junction.

| (A) | Ectocervix | Transformation Zone | Endocervix | Endometrium |
|-----|-------------|---------------------|------------|-------------|
| Original SCJ | Current SCJ | Active Metaplasia |

Site of HPV infection leads to an increased risk of abortive infection

![Diagram of HPV infection outcomes](https://example.com/diagram.png)

| (B) | Productive Infection | Productive Infection | Abortive Infection | Non-permissive |
|-----|-----------------------|----------------------|-------------------|---------------|
| LSIL | LSIL | HSIL | HSiL |
| High E4 Low p16 Low MCM | High E4 Low p16 Low MCM | Low E4 High p16 High MCM | Low E4 High p16 High MCM |

4 | **THE CURRENT CERVICAL SCREENING ENVIRONMENT: MERITS AND LIMITATIONS**

Screening for cervical cancer began with the Papanicolaou stain in the 1950s, which involves the collection of exfoliating cells from the transformation zone of the cervix. After Papanicolaou staining (using a mixture of haematoxylin, orange G, eosin Y, light green SF yellow and Bismarck brown Y), these are examined microscopically for changes for cancerous or precancerous changes, such as nuclear enlargement, irregular nuclear membrane and hyperchromasia. These approaches were in use well before the link between HPV infection and cervical cancer was established, and in its original form, involved the smearing of exfoliated cells onto a slide. A more robust liquid-based cytology approach is now used, which also allows co-testing for the presence of HPV DNA.

The screening programme in England has undergone considerable change over the last 2 decades (https://www.gov.uk/government/publications/cervical-screening-programme-and-colposcopy-management), and is underpinned by the classification of cytological abnormalities as indicators of the presence of clinically relevant disease. The Bethesda system is widely used, and allows...
grading either as LSIL or HSIL, the latter being broadly equivalent to CIN2+. On the basis of such classification, women are invited for colposcopy, where the cervix is visualised under low power magnification to detect the presence of lesions that stain white with acetic acid. This subjective assessment of acetowhite change currently determines whether a biopsy is collected for further analysis, or if excisional treatment is required. Such screening has been effective in reducing the disease burden associated with cervical cancer and indeed, the introduction of organised cervical screening in 1988 averted an impending epidemic of cervical cancer. Cervical cytology, however, has a sensitivity of only 59%-70% for the detection of HSIL, which means that a proportion of high-grade disease will be missed, even with three yearly follow-ups. This limitation stems from the fact that the cytological classification is morphology-based and requires the identification of subjective HPV-mediated changes that are prone to interpreter bias. Cytology screening is further complicated by the lack of in situ positional information, which typically facilitates pathology-based diagnosis of stained tissue sections. Inadequate sampling may also hamper test-sensitivity and require additional smears, with the inevitable risk that some patients will be lost to follow-up.

To address the limitations of cytology screening, primary DNA testing has been introduced as an alternative to cytology, on the basis that 99.7% of cervical cancers are caused by HPV. HPV DNA testing allows women who are HPV-negative, and therefore at negligible risk of developing cervical cancer, to be identified and be re-tested less frequently. Primary HPV testing also offers an objective result (ie, positive or negative), as well as cost benefits without imposing any significant change to the screening infrastructure. The superior sensitivity of HPV testing in comparison to cytology for the detection of CIN2+ has been demonstrated in multiple studies. The utility of the approach is, however, confounded by the high prevalence of HPV infections in the general population and many HPV-positive women have only asymptomatic or transient low-grade infections, which must be distinguished from high-grade disease prior to treatment. As a result, HPV DNA tests are most useful for older women (ie, age >30 years) who may harbour a persistent HPV infection and are less useful for women in their late teens and 20s, who are more likely to have a transient low-grade disease that will resolve spontaneously. In the short term, the lower specificity of HPV testing for the detection of clinically relevant disease (Figure 3), if used as an alternative to cytology, will increase secondary referrals, and present an additional burden on the healthcare system. This increase will in due course decline in countries where effective HPV vaccination has been implemented, but it is an important consideration in LMIC, where the increased infrastructure and cost requirements cannot be accommodated. To overcome these problems, effective triage strategies to detect women who are persistently rather than transiently infected are paramount.

Triage of HPV-positive women by combining cytology and hrHPV typing yields the highest negative predictive values (NPV) of 98.9%. An alternative is HPV testing combined solely with baseline cytology triage, followed by repeat cytology testing after 12 months for women who are HPV-positive. This has a lower NPV (95.1%) but is advantageous due to its lower colposcopy referral rate vs that of genotyping and cytology. Both approaches are, however, associated with a significant risk of loss to follow-up, which in some studies has been as high as 40%. This risk has to be given serious consideration in the context of declining screening uptake. Moreover, this increased requirement for colposcopic evaluation is important as colposcopy, like cytology, is also subjective, as evidenced by the diverse sensitivities (60%-70%) and specificities (30%-60%) quoted in the literature. Thus, there is a risk that HSIL may be under diagnosed or that women with LSIL may be over treated by excisional techniques such as loop excision. Moreover, in over 50% of women it must be remembered that LSIL will regress as infections are immune controlled/eliminated. Thus, treating these women with excisional techniques is thought to be potentially detrimental especially due to the loss of cervical volume increasing the risk of pre-term deliveries. This inability of current tests to prognosticate on which women will progress or regress is something that many groups are now trying to address (see below).

Although routine HPV testing is generally not advocated below the age of 30 years, the ability of HPV testing to identify older women who may be persistently infected is one of the primary merits of the approach. Indeed, persistent deregulated HPV gene expression is essential for the accumulation of genetic errors in the infected cell that eventually lead to cancer. Interestingly, a recent study has proposed that routine primary HPV screening intervals can safely be extended, from 3 to 5 years, which is currently the case for cytology-based screening for older women (age >50 years).

Many studies have also examined whether HPV DNA abundance offers any additional useful diagnostic information, and in particular, whether this may correlate with HSIL. Importantly, low-grade disease where viral genome amplification occurs, typically has much higher levels of virus and viral DNA than non-productive HSIL, where neither virus genome amplification or virus production are supported. In addition, integration of the HPV genome into the host cell chromosome, which can occur in HSIL and cancer, can occur at between one or two copies per cell, up to many hundred copies per cell, with infections classified as HSIL on the basis of the most severe pathology, often being surrounded by adjacent LSIL and/or uninfected epithelium (Figure 2) with very different HPV DNA abundance. Not surprisingly, the considerable effort that has been devoted to the analysis of HPV DNA copy number, which is often erroneously referred to as virus load, achieved very little. As anticipated, the crude analysis of HPV DNA content in heterogeneous lesions provides little insight as to the severity of disease.

Thus, although HPV DNA detection and its use in primary HPV screening has clear advantages in the detection of HSIL, the associated low specificity for clinically important disease can lead to overdiagnosis and overtreatment. A recent paper underscores this limitation and suggests from a quality of life and cost-benefit perspective that the current UK strategy of cytology screening with HPV triage every 3 years, may be more appropriate than a primary HPV screening approach. Such findings reiterate our view that not
only are better triage methods required for HPV-positive women, but also that primary HPV testing may not be the best screening strategy in its current form. Given our growing knowledge of HPV tumourigenesis, and the general value of molecular diagnostic methods, our aim should be towards the use of disease stratification and/or prognostic markers from the outset, thus enabling effective triage of these HPV-positive women.

5 | THE DETECTION OF HPV mRNA AND GENE EXPRESSION AS AN ALTERNATIVE TO HPV DNA

As HPV mRNA is an indicator of viral gene expression, and as the extent of viral gene expression determines disease pathology, its detection as a triage/screening marker is conceptually sound. The detection of viral mRNA, and in particular transcripts spanning the HPV E6/E7 region, would only be present in women with active productive or transforming infections, and would be generally absent in women who are HPV DNA positive because of a recent deposition of virus from a partner. In addition, there is evidence suggesting an association between levels of E6/E7 RNA and severity of pathology, which fits well with our general concept that increased expression of these genes is important in maintaining the precancerous phenotype. Historically, the detection of mRNA, which is more fragile than DNA and therefore susceptible to damage during storage/processing, has posed problems for its use in a screening/triage test, with past studies being hampered by poor detection sensitivities when compared to DNA. In recent years, however, advances in RNA detection technology have allowed patterns of HPV transcription to be visualised both in routinely formalin-fixed paraffin-embedded tissue, as well as in cytology specimens.

Many groups initially assessed the role of RNA testing as a triage technique for DNA positive women. Meijer et al used an E7 mRNA (seven hrHPV types) test for the triage of HPV DNA positive women and showed a slight increase in sensitivity (to HSIL in HPV-positive women) when compared to HPV genotyping (sensitivity 66.9% vs 60.9%, respectively). This marginal difference in sensitivity may reflect that fact that E7 expression occurs not only in abortive or transforming infections, but also in productive low-grade disease, where it is required for viral genome amplification. However, a real problem with these first-generation RNA tests was their incomplete coverage of relevant HPV types, and as a triage of DNA positive women, RNA tests were not significantly superior in comparison to cytology. As a result, RNA tests did not initially receive wide attention.

More recently, the APTIMA test (Hologic, San Diego, CA, USA), which detects the E6/E7 mRNA of 14 clinically relevant hrHPV types has been the subject of extensive research. These studies demonstrated a sensitivity similar to that of DNA tests, but more importantly that the specificity (vs DNA testing) was markedly improved. This superior specificity with no decrease in sensitivity suggests that perhaps the real advantage of RNA testing is its use as a primary screening test. Current tests (such as

FIGURE 2  Histology of infected cervix reveals a diversity of human papillomavirus-associated pathologies. Insets B and C are diagnosed as cervical intraepithelial neoplasia grade 1 (CIN1) and CIN2, while inset A contains both pathologies. This is a typical example of lesion-heterogeneity and highlights the problem of using viral load measurements to delineate lesion status. The presence of productive and abortive infections in close proximity confounds simple interpretation.
APTIMA) can be carried out on liquid-based cytology samples, similar to DNA testing, thus requiring no significant change in screening infrastructure. Moreover, in the context of a vaccinated population, such as that seen in the UK, RNA tests would appear conceptually superior for the detection of clinically established infections, as compared to DNA tests, which can also detect recent virus deposition and/or virus particles produced at adjacent non-cervical epithelial sites. These perceived merits have meant that RNA testing is now gaining momentum as the primary HPV screening test in much of the UK (from 2020), with RNA testing used exclusively at present in Scotland and Wales. Like DNA tests, however, RNA-based tests still do not adequately discriminate between low-grade disease, which in time may regress, and precancerous high-grade disease, prompting the further development of adequate triage approaches.

6 | EPIGENETICS, METHYLATION AND PATTERNS OF GENE EXPRESSION

Epigenetics is the study of DNA modifications such as methylation, which involves the addition or removal of a methyl group on to the aromatic ring of a cytosine (C) residues lying adjacent to a guanine (G) nucleotide. Such changes at CpG sites can be mediated by HPV gene products, leading to gene expression changes in the infected cell. The addition of even a small number of methyl groups in the vicinity of a regulatory DNA sequence, can lead to DNA condensation, which acts to block transcription. Aberrant methylation can inhibit tumour suppressor gene expression and is now known to play an important role in carcinogenesis.

Much work has been done evaluating the role of methylation in the development of cervical cancer, and, at the time of writing, over 20 gene methylation signatures have been proposed as disease markers. In many cases, these methylation markers have been identified by high-throughput screening, and the mechanistic basis for their link with neoplastic progression and cancer is not well understood. Conceptually, epigenetic changes are thought to play a significant role in the accumulation of secondary genetic mutations that are necessary for carcinogenesis. With hrHPV persistence and aberrant expression of E6 and E7, there is a significant increase in hypermethylation, due to E6/E7-mediated modulation of DNA methyltransferases, of the host genome, and indeed the level of hypermethylation can be correlated to disease state and thus provides valuable prognostic information.

One of the most well studied methylation panels targets the promoters of the MAL (T-lymphocyte maturation associated protein) and CADM1 (cell adhesion molecule 1) genes using a quantitative methylation-specific polymerase chain reaction approach and has shown an association with HSIL. Combining this test with primary HPV screening has a superior sensitivity when compared to HPV screening plus cytology, with the approach also proving useful for the detection of recurrent HSIL post treatment. Other panels that have been examined include MAL/micro-RNA (miRNA)-124, which showed a sensitivity (to HSIL) in HPV-positive women of 70.5%, compared to 70.8% for cytology. Comparable results were also obtained using a multi-marker methylation panel (JAM3, EPB41L3, TERT and C13ORF18), which showed a HSIL detection sensitivity of 71%, as compared to 70% for cytology. It is apparent from these studies, that methylation testing could provide a practical triage strategy for women who are HPV-positive. These approaches would, however, still lead to increased referrals to secondary care, and this may in part be related to the use of polymerase chain reaction-based methodologies on total DNA extracted from a diverse population of mostly normal exfoliated cells but may also be linked to our limited understanding of the biological role that the target genes play during cancer progression. Properly understanding the functional relevance of genes and gene combinations such as MAL and CADM1 during productive HPV infection, as well as during the development of neoplasia and cancer, is likely to help us to improve both the sensitivity and specificity of such biomarker-based approaches (Figure 3).

Although research on methylation and cervical cancer has mainly focused on human genes, a link has been demonstrated between viral genome methylation and neoplastic progression. Hypermethylation of CpG sites in the L1, L2 and E2 genes increases during persistent infection, which may be related to the role of E6 and E7 in modulating the function of DNA methyltransferases. Work such as this has led to the development of the S5 methylation panel, which focuses on DNA methylation of the late regions of HPV16, 18, 31 and 33 in combination with the promoter region of the human gene EPB41L3. This panel has been evaluated in multiple trials and appears a superior triage methodology to HPV 16/18 genotyping, and indeed cytology for the detection of CIN3/cancer (although similar for CIN2+ lesions). However, as with other methylation panels, S5 still suffers from low positive predictive values, and as it focuses on methylation of only certain HPV types, there is the potential of missing disease caused by other high-risk hrHPV types.

An additional and potentially important application of methylation may lie in the development of self-sampling approaches for cervical screening. Self-sampling can decrease the number of women lost to follow-up, associated with traditional two-step screening and triage approaches. One study that looked at this, used the MAL/miR-124 panel on women who had provided self-collected samples for HPV testing. This study demonstrated that methylation triage was non-inferior to cytology, and more importantly, fewer women were lost to follow-up due to the ability to perform methylation analysis on the same self-collected sample for HPV testing, while the cytology triage group required a second visit. The ability to carry out both layers of screening using a single self-collected sample demonstrates a unique advantage of methylation analysis over cytology.

Thus, it is reasonable to state that methylation signatures certainly have merit as an HPV triage strategy. Unfortunately in its current form methylation analysis still suffers from the same problems that afflict HPV nucleic acid/cytology tests, in that all of the morphological and gene expression detail present in individual cells is lost, and we are left analysing the average methylation patterns across hundreds of thousands of cells collected from the surface of
the cervix, where only a small fraction may be abnormal. Ideally, it would be attractive to overlay methylation patterns onto standard cytology images, as can be done for protein biomarker detection. Maintaining the spatial arrangements of cells collected from the surface of the cervix in this way would not only improve the identification of high-grade lesions but may also provide prognostic information relating to the risk of disease progression. Although this would appear a very promising avenue of research, reproducible in situ methylation-detection methodologies remain to be developed.

6.1 miRNA: REGULATOR OF VIRAL AND CELLULAR GENE EXPRESSION AND CANCER

In addition to epigenetic modifications, other regulatory molecules that control viral and cellular gene expression can also be used as biomarkers of disease. miRNAs for instance, are short single stranded RNA stem-loop structures that can regulate gene expression by binding to complimentary mRNA sequences in a tissue and/or differentiation-specific manner.63 Their ability to control gene expression is linked to changes in mRNA stability or transcription, with miRNA over- or under-expression seen in many tumours, including both ovarian and breast cancer. Indeed, miRNAs are often regulated by chromosomal regions that are mutated or epigenetically silenced in tumours, with hrHPV E6/E7 expression stimulating miRNA epigenetic silencing through the modulation of cellular DNA methyltransferases.62 Interestingly, fragile sites, which are hotspots for HPV genome integration, are often proximal to sequences that encode miRNAs. The potential value of miRNAs as markers of cervical cancer, was first examined by Pereira et al.,64 who used an array of 281 miRNAs to identify 21 that were dysregulated in cervical pre-cancers and cancers. Of these 21, five miRNAs (miRNA-10a, 132, 148a, 196a, 302b) showed an elevated expression as lesion severity increased. Other miRNAs examined in this study were also elevated during CIN3 progression, but decreased in invasive cancer, and although the expression patterns were not fully explained, this work did demonstrate the utility of miRNA panels as triage tools for HPV-positive women. Indeed, follow-up studies have generated broadly concordant results with regard to many of these miRNAs, with additional candidates such as miRNA-196a and miRNA-21 being added.65 miRNA-21 in particular is over-expressed in many cancers and has been referred to as an oncomir due to its effect on the PTEN tumour suppressor gene. For methylation triage, however, miRNA-21 levels alone are insufficiently sensitive for the detection of HSIL and require combination with other miRNAs or biomarkers.

Interestingly, while most groups have focused on the use of miRNA biomarkers for disease identification, one group66 has grouped miRNAs as: early-transient, which were present in HSIL but not SCC: late, which had distinct cancer-related expression patterns; and early continuous, which were found in both HSIL and SCC, with
the aim of providing insight into lesion age and persistence, which might ultimately provide prognostic information on lesions. This ability to provide prognostic information, as suggested also for methylation, should be the goal of such miRNA-based triage tests and as with methylation tests, adapting the approach for in situ use may improve disease detection.

7 | PROTEIN BIOMARKERS AND DISEASE STRATIFICATION OF HPV-ASSOCIATED NEOPLASIA

As the products of viral and cellular genes are the direct drivers of disease pathology, their detection as biomarkers would appear to offer advantages over more indirect markers of viral gene expression such as miRNAs and patterns of methylation. Indeed, the detection of an appropriate combination of virus and cell-encoded driver gene products using antibodies, may in due course render HPV testing unnecessary, especially given the inability of HPV tests to effectively stratify disease. As viral and cellular gene products are objective indicators of neoplastic phenotype, this category of biomarker has perhaps the most promise with regard to improving diagnostic specificity without loss of sensitivity. The most obvious markers of this type are the hrHPV E6/E7 oncoproteins themselves, and although a number of research publications have managed to demonstrate proof of principle, translation into routine practice has proved difficult because of the low abundance of these proteins in cervical lesions. A single commercial test (OncoE6, Arbor Vita) currently exists, that uses a genotype-specific mouse monoclonal antibody to detect the E6 protein of a small number of hrHPV types in cytology cell extracts using a dipstick approach. Despite a positive predictive value of 41%, the approach has a sensitivity of only 53.5% for CIN3, which is lower than both hrHPV testing, and indeed cytology testing. Furthermore, test coverage currently extends to only the two most important HPV types (16 and 18), with the precursor lesions that contribute to the 30% of cervical cancers caused by other HPV types not being detected. The simplicity of the approach, and its ability to detect HPV-associated disease rather than just HPV DNA are important considerations with the approach having particular value in developing countries, where self-collection and a need for simple processing and interpretation are important.

An alternative to this is the detection of surrogate markers of HPV E6/E7 expression, with p16INK4a being the most well studied example of a number of such cellular biomarker proteins, including the cellular DNA replication factors, MCM, DNA Topoisomerase IIa and CDC6, which accumulate in the cell as a consequence of high-risk E7 expression. p16INK4A is a cell cycle inhibitor, that is often repressed, or over-expressed in a mutant form in many tumours. In cancers caused by HPV, as well as in cells expressing high-risk E7 in both LSIL and HSIL, p16INK4A expression results from the induction of E7-mediated epigenetic modifications at the p16INK4A promoter, and through the E7-mediated stimulation of cell cycle entry driven by association with Rb. Because proliferation is E7-driven, rather than being regulated by growth factor-mediated cyclin D activation, the ability of p16INK4A to suppress cell proliferation is subverted. Importantly, p16 does not specifically mark high-risk HPV-associated HSIL and cancer, but the change in the distribution of this marker in lesions of different grade make it useful in disease stratification. In the PALMS trial, p16 staining of cytology was compared to that of cytology alone in the triage of HPV-positive women. p16 staining was more sensitive for HSIL than conventional cytology (86.7% vs 68.5%) and was more specific than primary HPV testing across women of all ages. As with other biomarkers, p16INK4A appears particularly useful when used in combination, with dual positivity for Ki67 or MCM acting as a specific marker of HPV-driven cell proliferation. This has been taken further in the analysis of tissue biopsies, where the spatial distribution of these marker combinations, when used alongside detection of the abundant HPV E4 protein, has allowed an understanding of how molecular biomarker patterns could eventually replace standard haematoxylin and eosin staining. In these studies, p16INK4A staining facilitated greater concordance amongst pathologists with regard to CIN2+ classification, a conclusion that was also supported by Wentzensen et al., who used p16/Ki67 in a cytology-based triage approach for HPV-positive women. The approach reduced unnecessary colposcopy referrals, but the positive predictive value at 24% (see Figure 3) was only marginally higher than cytology (20%). The CIN2+ detection sensitivity obtained with cell cycle entry biomarkers such as MCM and Ki67 was similar to that obtained using p16, even though cell cycle proteins are stimulated during inflammation, wound healing and cervical metaplasia. In general, however, the combined use of more than one biomarker type, allows greater discrimination than can be achieved using biomarkers individually. As an example, LSIL are characterised by an abundant surface expression of the HPV-encoded E4 protein, with only low-level expression of p16INK4A and MCM. HSIL, by contrast, is characterised by the surface expression of p16 and MCM, with very little or no E4 expression. The use of E4 as a distinct class of biomarker alongside p16 and MCM, can not only facilitate the detection and subsequent monitoring of LSIL, but also help discriminate HPV-associated neoplasia from lesions with similar pathologies such as metaplasia and/or inflammation. The combined use of p16INK4A and E4 has in fact been used in a number of recent studies to stratify both cervical and anal precancerous tissue biopsies as an adjunct to conventional pathology. In using these biomarkers in cytology, however, it would be desirable to retain spatial information relating to cell position, and the proximity of abnormal cells to neighbouring cells with similar phenotype. Moreover, the use of p16/MCM and E4 in combination using an in situ approach, should allow the identification of lesions that have low malignant potential le high E4/low p16 and vice versa. Such prognostic detail may facilitate the conservative management of women and also allow lesions to be monitored over time. To realise this goal, we have used a non-invasive sampling approach, that collects a layer of cells in their in situ positions, for subsequent biomarker staining and visualisation on a glass slide (Figure 4). We hope that such approaches will improve the positive predictive value of biomarker triage approaches, and that, in due course, it will be possible to generate heat maps of the cervix using a range of different biomarkers, including...
FIGURE 4  The preservation of cells in their in situ positions as a method for mapping cervical disease location and severity. Exfoliating cells were removed from the surface of the cervix using an adhesive matrix, prior to staining with a number of triage antibodies, including MCM, p16 and E4. A biomarker cervicogram using an MCM antibody is shown in (A), above an image of the acetowhite stained cervix. The areas highlighted in red in the cervicogram correspond to regions of high-grade squamous intraepithelial lesions, which may inform the collection of biopsies at colposcopy. The pattern of the MCM marker at the high-grade squamous intraepithelial lesion epithelial surface is shown in (B). In individuals with no evidence of cervical neoplasia (C), markers such as MCM are not apparent at the epithelial surface.

methylation and or miRNA probes, that would ultimately provide information regarding disease prognosis.

8 | MACHINE LEARNING AS AN AID IN THE DISEASE IDENTIFICATION PROCESS

Progress in the development and evaluation of biomarkers, has been accompanied by improvements in image analysis and computer processing power, and these approaches are now influencing the way that screening and diagnostic data are collected and processed. Indeed, even the basic disease information that can be derived from acetowhite staining at colposcopy, may be more diagnostically useful when rigorous and reproducible analysis criteria are used in its assessment. In a recent study, Schiffman et al used a conventional neural network (machine learning) to analyse colposcopy images and grade lesions. By expert annotation of colposcopic images, a training set of images was formed, which provided a basis for the preparation of a diagnostic algorithm that was subsequently evaluated on a large cohort. This machine learning approach was found to be superior for
the detection of HSIL when compared to human interpretation of these colposcopic images. Furthermore, this algorithmic approach was more accurate for HSIL detection than cytology, which may suggest a wider utility for the implementation of a see-and-treat approach in LMIC (if VIA is used as the primary screening approach). This approach is now being trialled through the incorporations of such algorithms in new mobile/hand-held colposcopes, as this would help to keep infrastructure costs associated with implementation to a minimum.80

Our initial results suggest that the machine learning and image analysis approach could also be used in the analysis of the biomarker heat maps discussed previously (see above and Figure 4), as the presence of underlying disease is related to high surface cell density, nuclear/cytoplasmic ratio and the presence of cells in clusters that have similar morphological appearance. These are characteristics that are straightforward to define as a part of an image analysis algorithm. Overcoming the barriers to the acceptance of these new approaches will be critical in ensuring uptake. A general limitation of machine learning is that the final algorithm is only as good as the training data it learns from, and although this may affect the early implementation of such approaches, it is unlikely to limit implementation in the long term. More important may be a reluctance of clinicians to accept a computer interpretation of an acetowhite or cytology image although, in time, it is likely that the confidence of the clinical community will be achieved during validation trials. Interestingly, a compromise is achieved by the DeepMind algorithm in the analysis of optical coherence tomography images, where an explanation of the decision-making process is incorporated into the software, in order to help the clinician to appreciate the underlying reasons for the diagnostic recommendation.81

9 | CONCLUSION

Cervical screening is lauded as one of the success stories for the early detection of cancers. This is demonstrated by the large decline in cervical cancer incidence and prevalence in countries such as the UK. Many HIC will implement primary HPV screening in the near future, which will lead not only to the earlier detection of cervical cancer but will allow the detection of pre-cancers and cancers that conventional cytology may have missed. This advantage of HPV detection is tempered by its poor predictive value, which now requires the development of better triage approaches. An understanding of the biology of HPV is expected to facilitate the development of better triage tests, which will make use of prognostic molecular markers and novel diagnostic strategies. Such approaches should ideally be quantitative and objective, which, in the long term, as automated methodologies improve, become cheaper and enable better health-care resource allocation.

Unfortunately, LMIC still lag behind in cervical cancer prevention. While there is a concerted effort to address the multitude of factors that contribute to this, it is generally understood that we will require more innovative approaches in these countries where advanced healthcare resources are scarce. While we advocate the implementation of universal vaccination and screening, newer machine learning-based colposcopic approaches (eg, see Ref79) may also allow the implementation of more successful see-and-treat strategies.

Ultimately, as we understand more about HPV and the unique vulnerabilities of the cervical transformation zone, there is a real potential to discover better screening and/or diagnostic markers, and as technology advances (eg, omics/sequencing) and becomes less expensive to implement, there is the potential that cervical cancer screening may once again revert to disease detection rather than HPV presence.

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CONFLICTS OF INTERESTS

The authors have no conflicts of interest to declare.

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