Triage of high-risk HPV positive women in cervical cancer screening

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ABSTRACT

Introduction: High-risk human papillomavirus (hrHPV) testing is expected to replace cytology as primary screening method for cervical cancer screening in an increasing number of countries. The high sensitivity of hrHPV testing is combined with a limited specificity which makes triaging of hrHPV positive women necessary. As an ideal triage method does not yet exist, an optimal triage strategy for hrHPV positive women based on current knowledge should be obtained. The aim of this article is to present an overview of available options for triage of hrHPV positive women, with their strengths and limitations and possible future opportunities.

Areas covered: Current knowledge on morphological biomarkers, molecular biomarkers and combined triage strategies will be discussed to give an overview of the state-of-the-art on triaging hrHPV positive women. The literature search was limited to studies on triage strategies for hrHPV positive women.

Expert commentary: Experience with morphology-based biomarkers makes these a valuable triage method. However, they lack the ability of differentiating productive from transforming infections. Molecular biomarkers are objective, highly reproducible, can be used in high throughput testing, and show promising results. With more extensive knowledge on these molecular markers, cervical cancer screening may transform to a full molecular screening in the future.

1. Introduction

With an estimated 527,600 new cancer cases and 265,700 deaths in 2012, cervical cancer is the fourth most diagnosed cancer and fourth cause of cancer death in females worldwide. Developing countries account for almost 90% of all cervical cancer deaths, and in some countries in Melanesia, eastern, middle, and southern Africa, it even is the leading cause of cancer death amongst females. Cervical cancer incidence and mortality rates are lowest in Europe, eastern and western Asia, northern Africa, northern America, Australia, and New Zealand [1]. The availability of screening and differences in human papillomavirus (HPV) prevalence are the cause of these major geographic variations [2].

The role of HPV in development of cervical cancer was first described by zur Hausen in 1977, for which he received the Nobel Prize in Physiology or Medicine in 2008 [3]. Approximately two decades later, Walboomers described the necessity of a persistent infection with high-risk HPV (hrHPV) for the development of precancerous or cancerous lesions of the cervix [4]. Since then, over 200 HPV genotypes have been identified and clinically relevant types are grouped by the innate risk of causing cervical cancer (Table 1) [5]. Low-risk types generally only induce benign warts, whereas hrHPV types have the ability to induce cervical premalignancies and malignancies, of which approximately 70% is caused by types 16 and 18. The lifetime risk of an infection with hrHPV is high; however, only a minority of infections develop into cervical cancer. After 12 months, two-thirds of all infections can be cleared by the host immune system, and after 24 months, over 90% can be cleared [6,7]. Infections that are not cleared may develop into ‘productive’ infections, cytologically and histologically known as low-grade squamous intraepithelial lesion (LSIL), or histologically known as low-grade cervical intraepithelial neoplasia (CIN; CIN grade 1). These infections morphologically show dysplastic features overlapping with those in progressive precancers. However, such infections show no signs of cellular transformation and the majority of productive infections still clear quickly. Only a minority of all persistent hrHPV infections results in altered E6 and E7 viral gene expression, thereby becoming a ‘transforming’ infection. In transforming infections the normal viral life cycle is aborted and the viral early genes E6 and E7 are overexpressed in proliferating cells, leading to altered expression of cell cycle and DNA repair regulators (Figure 1) [8–10]. Transforming infections are cytologically and histologically known as high-grade squamous intraepithelial lesion (HSIL) or histologically known as high-grade CIN (CIN grade 3), which may finally result in cancer if left untreated (Figure 2). These premalignant stages preceding cervical cancer allow for detection and treatment of these lesions before they progress to cervical cancer.

Screening has been very successful in decreasing cervical cancer incidence and mortality in Western countries [12].

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Screening programs worldwide differ regarding age, frequency, participation rate, and screening modality [13]. Nowadays, cervical cytology is used as primary screening test in the majority of programs [14]. However, many Western countries are on the verge of replacing cytology as primary screening by testing for the presence of hrHPV. The Netherlands and Australia will be among the first countries to initiate full hrHPV-based organized screening in 2017.

Advantages of primary hrHPV testing are the objectivity of the assay and high-throughput testing, and its high sensitivity of 90% for CIN2 or worse (≥CIN2) and 95% for CIN3 or worse (≥CIN3), compared to moderate sensitivity of 30–87% for cytology [15,16]. Additionally, HPV screening holds the possibility to analyze self-sampled material of brush- or lavage-based samples, which may improve the efficacy of cervical cancer screening by increasing participation of non-responders [17–20]. The major limitation of hrHPV testing is its inability to distinguish transient infections from clinically relevant infections, resulting in limited specificity compared to cytology. This limited specificity would result in higher numbers of unnecessary colposcopy referrals compared to cytology screening [21,22]. Effective triage of hrHPV-positive women is therefore essential.

An ideal triage strategy meets two important requirements: (1) The strategy gives a highly sensitive and specific result, differentiating between cervical cancer and CIN lesions that need treatment, and abnormalities with a risk that is low enough to safely return a woman to the next screening round. Ideally, low-risk groups would need no follow-up. (2) No additional sample or additional visit is needed to perform the triage, thus minimizing the efforts for the screened women, and to limit loss to follow-up. The ideal triage method for hrHPV-positive women is not yet available; therefore the most optimal triage strategy using current knowledge should be obtained. Current triage methods all have advantages and disadvantages and some may possibly be improved or combined to measure up to an ideal strategy as far as possible.

This review outlines current knowledge and future opportunities for triage of hrHPV-positive women, which is especially important in the transition from cytology-based cervical

<table>
<thead>
<tr>
<th>IARC group</th>
<th>Risk estimate</th>
<th>HPV types</th>
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<tbody>
<tr>
<td>1</td>
<td>High-risk</td>
<td>16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59</td>
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<tr>
<td>2A</td>
<td>Probable high-risk</td>
<td>68</td>
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<tr>
<td>2B</td>
<td>Possible high-risk</td>
<td>26, 53, 66, 67, 70, 73, 82</td>
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<td>3</td>
<td>Low-risk</td>
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Figure 1. Molecular mechanisms by which the human papillomavirus induces cervical carcinogenesis.
(a) Genome organization of the human papillomavirus (HPV). HPV are DNA viruses coding for a long control region (LCR) several early functional genes (E1-E7) and two late structural genes (L1-L2). (b) The INK4A/ARF locus at chromosome 9p21 encodes proteins p16\textsuperscript{INK4a} (p16) and p14\textsuperscript{ARF} (p14) that ultimately link the Retinoblastoma (Rb) and p53 tumour suppressor pathways. p16 is a cyclin-dependent kinase (CDK) inhibitor that prohibits progression from G1 phase to S phase and slowing down the cell cycle. In a normal cell, p16 acts as a tumour suppressor by binding to CDK4/6 and preventing its interaction with cyclin D resulting in arrest of cell proliferation. p14 inhibits mdm2, therefore promotes p53, which promotes p21 activation. p21 binds and inactivates certain cyclin-CDK complexes which otherwise would promote transcription of genes that would carry the cell through G1/S checkpoint of the cell cycle resulting in S-phase induced p16 stimulation. When hrHPV types integrate into the host genome, loss of negative feedback control will result in increased expression of viral E6 and E7 oncoproteins. HPV protein E6 binds tumour suppressor gene p53 and promotes its destruction, resulting in inhibition of apoptosis and loss of inhibition of cyclin-CDK complexes via loss of p21 stimulation. CDK4/6 binds cyclin D and forms an active protein complex that phosphorylates Rb which dissociates from transcription factor E2F1. Liberated E2F1 enters the nucleus and promotes transcription of target genes essential for transition from G1 to S phase. HPV protein E7 dissociates the E2F-Rb complex and binds and inactivates Rb. This also causes release of transcriptionally E2F1 dependent genes necessary for DNA replication, resulting in stop of growth arrest and therefore progression the cell cycle. HPV E7 oncoprotein expression also induces KDM6B histone demethylase expression, which triggers the p16 promoter, also resulting in upregulation of p16 expression. Stimulation of progression of the cell cycle, combined with loss of apoptosis results in immortalization, genomic instability and finally in increased risk of transformation and malignant progression [8–11].

Table 1. Human papillomavirus types grouped by the innate risk of causing cervical cancer.
cancer screening to primary hrHPV screening. A variety of triage options are discussed: morphological biomarkers such as Papanicolaou (Pap)-stained cytology or cytology with different types of immunocytochemistry, and molecular biomarkers such as HPV genotyping, RNA-based biomarkers, and methylation. We focus on the advantages and disadvantages of these strategies and their value as triage method of hrHPV-positive women. Finally, a 5-year view on cervical cancer screening in a postvaccination era will be discussed.

2. Morphological biomarkers

2.1. Cytology

Cytological examination of exfoliated cervical cells stained with the multichromatic Pap staining was introduced by Papanicolaou in the 1940s [14]. Triage of hrHPV-positive women with cytology is a common choice because of the worldwide experience with this technique. This widely evaluated triage method has shown to improve the initially limited specificity of hrHPV testing and is known to reduce colposcopy referrals and follow-up testing [23–28]. However, cytological assessment in these studies is generally performed without knowledge on hrHPV-positive status, which is different to primary hrHPV based screening. The knowledge on positive hrHPV status is known to affect the interpretation of cytology. In hrHPV-based screening with knowledge on hrHPV-positive status, this might result in slightly higher sensitivity and a lower specificity compared to these previously published result [29–31]. The inability of high-throughput testing and subjectivity of the examination are limitations of this technique as triage method for hrHPV-positive women. The performance of cytological examination highly depends on training and experience of cytotechnologists, resulting in variations in performance and quality. Quality management and benchmarking are therefore needed to obtain and maintain high quality of cytological examination. With the introduction of primary hrHPV-based screening, the number of samples for cytological examination will decrease and maintaining highly trained cytotechnologists might become more difficult.

For cytological examination of Pap stained cells, a slide can be obtained from a primary hrHPV-positive clinician-taken cervical sample. In case of a hrHPV-positive self-sample, an additional clinician-taken sample will be necessary as cytology cannot be reliably performed on self-sampled materials [32]. Cytological triage has limited sensitivity, and therefore still needs follow-up for cases with a hrHPV-positive result combined with normal cytology, also additional follow-up is warranted for cytology-positive cases which show no abnormalities during colposcopy or in a biopsy. The worldwide experience with cytological assessment of cervical samples makes cytology an interesting triage method for hrHPV-positive women; however, limitations as average sensitivity, subjectivity of the analysis, and inability to perform on self-sampled material are major disadvantages.

2.2. P16 staining

Expression of the HPV E7 oncprotein induces KDM6B histone demethylase expression and causes epigenetic reprogramming. Through KDM6B, de-methylation of the p16\textsuperscript{INK4a} (p16) promoter is triggered, with upregulation of p16 expression as a result [11]. The upregulation of p16 in transforming infections might therefore be used as biomarker for differentiating between productive and transforming infections. HRHPV testing with p16-staining triage produces a significant increase in sensitivity compared with conventional cytology, with no
substantial increase in referral to colposcopy [33]. The longitudinal sensitivity of p16-staining as triage method for hrHPV-positive women is 77.8% for ≥CIN3, with a 95% confidence interval (CI) ranging from 63.9 to 91.6%. The relative sensitivity of p16 triage of hrHPV-positive women aged 35–60, compared to primary conventional cytology is 2.08 (95% CI 1.13–3.56) [34]. Positive p16 staining distinguishes hrHPV-positive women in need of immediate colposcopy referral, from hrHPV positive and p16 staining negative women who can safely be managed with repeat screening after a 2–3-year interval [34]. Expression of p16 is not limited to dysplastic cells but can also be found in normal cervical cells such as squamous metaplastic and endocervical cervical cells. This is not problematic in histological samples, but makes morphological assessment necessary in cytological samples. Assessment is therefore more subjective and limits reproducibility [35]. This can be partially overcome by combining p16 with the proliferation marker Ki-67.

2.3. P16/Ki-67 dual-staining

In normal cells, expression of proliferation marker Ki-67 can be found in the nucleus during all proliferative cell cycle phases, and its expression is limited to basal or parabasal layers. In CIN lesions its expression extends above the first one-third of the epithelium. Expression of both p16 and Ki-67 within the same cell is a sign of neoplastic transformation, independent from morphological criteria [36]. The clinical performance of p16/Ki-67 has been studied extensively as triage method for low-grade cytology; however, also studies in hrHPV-positive women were performed. In triage of hrHPV-positive women with a negative cytology result, sensitivity for ≥CIN2 varies between 67% and 92%, with a specificity of 73–82% [36,37]. In triage of women with hrHPV-positive low-grade cytology, sensitivity and specificity of ≥CIN2 vary from 75–94% to 51–88%, respectively [38–43]. Limited studies have been performed with immediate p16/Ki-67 triage of hrHPV-positive women, without additional cytological interference. In a study with 1509 hrHPV-positive women aged 30 and older, dual-stain cytology shows similar sensitivity but a higher specificity for ≥CIN2 detection, when compared with Pap-stained cytology with a threshold of atypical squamous cells of undetermined significance (ASC-US). For women with a positive dual-stain, immediate referral to colposcopy was justified. Dual-stain negative women had a risk of a high-grade lesion that was lower than the risk for hrHPV-positive women with normal cytology, which is considered low enough to re-examine after 1 year according to US management guidelines [44,45]. In another study performing p16/Ki-67 dual-stained triage of 446 hrHPV-positive women, sensitivity similar to cytology, and cytology combined with HPV genotyping has been achieved. Specificity for ≥CIN3 was significantly higher than with cytology, either or not combined with HPV genotyping [46]. In a prospective population-based study in over 6000 women, 396 women aged 35–64 were hrHPV positive. Triage of these hrHPV-positive women by p16/Ki-67 dual-stain yielded a sensitivity of 87.6%, compared to 77.6% for cytology with ASC-US threshold. Specificity and colposcopy referral rate were similar in both groups. Combined triage with cytology (threshold ASC-US or worse) and p16/Ki67 yielded an increased sensitivity but at the expense of a decreased specificity. With an adjusted threshold of HSIL for cytology, the sensitivity and specificity were similar to triage with p16/Ki-67 alone [47].

Combined p16/Ki-67 dual-staining shows promising results in triaging hrHPV-positive women, as well as in triaging hrHPV-positive women with negative or low-grade cytology. However, the subjectivity of the examination, the inability for high-throughput testing and its inability to be used on self-sampled material are shortcomings. Also a negative dual-stain result still needs follow-up, as well as a positive dual-stain result without colposcopic or histologic abnormalities.

2.4. MCM2/TOP2A dual-staining

Minichromosome maintenance protein 2 (MCM2) and topoisomerase II-a (TOP2A) are both indicative for the formation of the origin recognition, and indicate an aberrant S phase induction when detected in suprabasal cells of the epidermis. Aberrant S phase induction is the premature and prolonged entry in the S-phase of the cell cycle, which is induced by HPV E6 and E7 oncoproteins, resulting in G1/S cell-cycle checkpoint malfunction. Both MCM2 and TOP2A have been shown to be overexpressed in high-grade CIN and cancer. The performance of the MCM2/TOP2A dual-stain has been examined as triage method of low-grade cytology showing varying sensitivity for the detection of ≥CIN2, when compared to cytology. In triage of hrHPV-positive women in one study, this dual-stain yielded a relative sensitivity of 1.30 (95% CI 1.20–1.41) and relative positive predictive value (PPV) of 2.89 (95% CI 2.58–3.15), when compared to cytology alone [48]. Knowledge on clinical utility of the assay in triage of hrHPV-positive women is limited and further studies on this morphology-dependent assay are needed to determine and validate its clinical value.

3. Molecular biomarkers

3.1. HPV genotyping

Cervical infections with HPV16 and HPV18 have demonstrated the highest risk of developing precursor cancer and cancer. HPV16 is found in 50–60% of all cervical cancers, and HPV18 in 10–15% of cervical cancer cases [49]. HPV18 and HPV45 are especially known for their association with the less prevalent adenocarcinoma of the cervix [50,51]. Risk estimates for individual genotypes or the combination of different genotypes can be used for triage of hrHPV-positive women.

In the guideline of the American Society for Colposcopy and Cervical Pathology (ASCCP), HPV16/18 genotyping is already recommended for hrHPV-positive women with normal cytology results; all HPV16 or HPV18-positive women are immediately referred for colposcopy, women positive for other hrHPV types without morphological changes are advised follow-up after 1 year with hrHPV and cytology co-testing [52]. The additional value of genotyping with immediate referral of HPV16/18 positives was confirmed in the HPV FOCAL trial which included over 6000 women screened by hybrid capture
2 (HC2) and Cobas hrHPV testing at baseline and liquid-based cytology (LBC) 24 months later. hrHPV-positive women were tested with HPV genotyping and reflex LBC for colposcopy triage. Of the $\geq$CIN2 lesions identified in the first round of screening, which includes baseline and 12-month follow-up, 55% had abnormal cytology and an additional 17% with normal cytology was HPV16/18 positive. Concluding that immediate referral of HPV16/18-positive women with normal cytology may allow for earlier detection of $\geq$CIN2 lesions [53].

Multiple studies have been performed on HPV16/18 genotyping triage of hrHPV-positive women, of which the ATHENA trial is one of the largest. hrHPV testing with HPV genotyping was compared with LBC for cervical cancer screening in women over 21 years old. In a subgroup analysis of women aged over 25, the sensitivity and PPV for $\geq$CIN3 in triaging hrHPV-positive women with HPV16 and/or HPV18 was equivalent to detecting ASC-US or worse. Detection of HPV16 and/or HPV18 or cytology with LSIL or HSIL threshold showed increased sensitivity or similar or increased PPV for $\geq$CIN3 detection than single cytology triage with an ASC-US threshold [54]. Other studies have also shown promising results with high negative predictive values (NPV) by combining an ASC-US threshold with HPV16/18 or HPV16/18/33/35/39/68. The clinical and analytical performance of this test identifies an additional three groups of 33/58, 56/59/66, and 35/39/68. In a recent European study, HPV16/18 genotyping performance was explored in a setting with prior knowledge on hrHPV-positive status during cytology assessment, similar to hrHPV-based screening. This study concludes that by adjusting the threshold of cytology and combining it with HPV16/18 genotyping, specificity for $\geq$CIN3 increases with similar sensitivity and no increase in colposcopy referrals. However follow-up is still needed for hrHPV-positive women with low-grade cervical cytology results [31]. A cost-effectiveness study on triage strategies of primary hrHPV screening in women over 30 years old, with screening sensitivity and specificity based on the ATHENA trial, indicates that incorporating HPV16/18 genotyping in primary hrHPV screening with reflex cytology with an ASC-US threshold is cost-effective [55].

Compared to partial genotyping, extended genotyping methods are also available. The Onclarity HPV test offers extended genotyping of types 16, 19, 31, 45, 51, and 52 and identifies an additional three groups of 33/58, 56/59/66, and 35/39/68. The clinical and analytical performance of this test was recently assessed according to the VALGENT framework concluding that this assay offers applications for clinical workflows [56]. Also other techniques as the GP5+/6+–LMNX, MALDI-TOF, qPCR test, and PapilloCheck offer extended genotyping. Extended genotyping methods for immediate triage of hrHPV-positive women have however not been studied as extensively as the previously mentioned partial genotyping tests. These studies might be performed in the near future.

A variety of hrHPV tests have the ability of immediate and combined genotyping, which makes this triage strategy easy to use. The promising results of previous studies, combined with the ability of immediate triage of both clinician-taken and self-sampled specimens, makes hrHPV genotyping an interesting triage option for hrHPV-positive women. However, adding Pap-stained cytology to HPV16/18 genotyping increases clinical value of the triage, with the disadvantage of the need of a clinician-taken sample for cytology. Current knowledge indicates that HPV16/18 genotyping may improve triage of hrHPV-positive women; however, for management of hrHPV-positive women with infections other than HPV16 and HPV18, other techniques might still be necessary.

3.2. RNA-based biomarkers

RNAs can play an active role within cells by communicating responses to cellular signals, catalyzing biological reactions, and controlling gene expression in form of small interfering (siRNA) or micro RNA (miRNA). Cellular organisms use messenger RNA (mRNA) to direct synthesis of specific proteins, and miRNA to regulate the function of mRNA. RNA-based tests are able to detect differences or changes in gene expression related to cancer development, while DNA-based tests detect the presence or absence of the HPV genome.

3.2.1. E6/E7 mRNA-based biomarkers

A limitation of the DNA-based hrHPV test is its inability to distinguish transient from persistent hrHPV infections resulting in low specificity. Cellular transformation of hrHPV infected cells begins with upregulation of E6 and E7 mRNA, and progression from hrHPV infection to cancer is dependable of E6/E7 integration. Overexpression of E6/E7 mRNA could therefore be used to detect only active infections which could result in high-grade lesions. E6/E7 mRNA-based test are widely studied as substitute for DNA HPV-based tests, indicating significance as diagnostic tool. Already three commercial E6/E7 mRNA-based tests are known [57]. A recent review of the clinical performance of the E6/E7 mRNA-based Aptima HPV assay indicates stable similar sensitivities of the E6/E7 mRNA hrHPV assay for detection of CIN2/3+, independent from study design, compared to the HC2 and GP5+/6+DNA tests. This stable sensitivity was combined with a higher specificity of the mRNA-based HPV test [58]. In a second review the Aptima assay also showed consistently similar study-specific and pooled sensitivity and superior specificity for CIN2+ compared to HC2. The pooled relative sensitivity for the Aptima assay was 0.98 (90% CI 0.95–1.01) with a pooled relative specificity of 1.04 (90% CI 1.02–1.07) [59]. In a study comparing multiple triage algorithms for hrHPV-positive women, an E7 mRNA test with HPV16/18/33/35/45/52/58 genotyping achieved similar performance to HPV16/18 genotyping and cytology in $\geq$CIN2 detection [60]. Triage of hrHPV-positive women with ASC-US cytology in the CLEAR HPV study was performed with the Aptima HPV 16 18/45 genotype assay. This study demonstrated that the assay has utility in stratifying low and high risk of $\geq$CIN2 and CIN3 among women with hrHPV-positive ASC-US [61]. These E6/E7 mRNA-based tests could possibly combine primary mRNA-based hrHPV testing with HPV genotyping. However, follow-up of hrHPV-positive women with genotypes other then 16, 18, or 45 would still be needed, and additional longitudinal studies and economic evaluations must be conducted before more solid conclusions regarding clinical applicability can be made.
3.2.2. Other mRNA-based biomarkers

It has been previously demonstrated that HPV infection alone is insufficient for development of cervical cancer; abnormal host genes play an important role in carcinogenesis [62]. The mRNA-based expression profile of normal cervical tissue is known to change during carcinogenesis, and expression of single genes or gene profiles might be used as molecular biomarker to distinguish between different stages of carcinogenesis or indicate response to particular treatment. The expression status of thousands of genes can be studied at once to create a profile of cellular function. DNA microarray, which measures expression of previously identified target genes, and the newer sequence-based techniques can be used to obtain gene expression profiles [62]. Also bioinformatics tools can be used to identify key genes and potential biomarkers, by analyzing gene expression profiles and differentially expressed genes between cervical samples of different stages in carcinogenesis [63].

Previous studies compared expression profiles of cervical cancer with normal cervical tissue, early-stage with late-stage cervical cancer, and squamous cell carcinoma with adenocarcinoma of the cervix [64–66]. Also studies on therapy response and resistance were performed [67,68]. Differences between CIN lesions and cervical cancer or normal tissue have also been studied in a small number of studies, finally resulting in proposed genes for further research, with special attention to HOXC10, BPA1, HIF-1a, PTP, HME1, HNTH1, and PHGDH [69–72]. In a recent feasibility study, the detection of mRNA-based biomarkers in cervical samples obtained by LBC was studied, showing promising results. Single markers and combinations of markers CDKN2A/p16, BIRC5, MMP9, TOP2A, MCM5, and MKI67 were studied. TOP2α was most sensitive, with a sensitivity of 97% for detection of HSIL, and CDKN2A/p16 was most specific with a specificity of 78%. The combination of TOP2α and CDKN2A/p16 was highly sensitive 96% (95% CI 88–99) with a specificity of 71% (95% CI 55–82) [73].

Identified differences in these genomic expression profiles may in the future be used to distinguish productive from transforming hrHPV infections, and may be able to identify prognostic markers and targets for molecular therapy. Knowledge on these biomarkers is however still at an early stage, and at current state knowledge on these markers is far too limited for implementing these in clinical practice.

3.2.3. miRNA-based biomarkers

miRNA are more recently discovered and are noncoding parts of RNA that regulate mRNA function by modulating mRNA stability and the translation of mRNA into proteins. miRNAs can be upregulated or downregulated and are thought to play an important role in processes as cell proliferation, metabolism, and apoptosis, with a possible role in onset or progression of cervical cancer. An important feature of miRNAs is that one miRNA often interacts with more than one mRNA and one transcript can be targeted by multiple miRNAs, indicating a variety of interactions for one miRNA.

In a meta-analysis comparing 27 studies, including 1132 cancer samples and 943 normal samples, frequency of upregulation and downregulation of miRNAs was scored. Upregulation of miRNAs was most consistently reported for miR-20a and miR-21. Downregulation was shown most frequently for miR-143, miR-03, and miR-145 [74]. In a large systematic study on deregulated miRNAs in cervical cancer development, 85 published reports with 3922 cases and 2099 noncancerous control tissue samples were analyzed. Expression of miRNAs in cervical cancer, as well as in different CIN lesions was reviewed. A meta signature of miRNAs reflecting the cervical carcinogenesis from CIN1, CIN2, and CIN3 to cervical cancer was made, reporting 42 upregulated and 21 downregulated miRNAs with a trend of increasing numbers of deregulated miRNAs during progression of CIN to carcinoma. The meta-analysis shows a selection of five upregulated and seven downregulated miRNAs in CIN1 compared to noncancerous tissue, which are also visible in more severe CIN lesions and cervical cancer, indicating a possible role in development of cervical cancer. CIN2 and CIN3 lesions showed an increased number of deregulated miRNAs with an additional 35 and 36 deregulated genes compared to CIN1. In cervical cancer, another five downregulated and ten upregulated miRNAs genes were reported [75]. Knowledge on these miRNA expression profiles may be used for disease classification or monitoring. To our knowledge, this profile has however not yet been studied in large prospective studies or on cervical samples, so its value for triage of hrHPV-positive women is yet unknown.

3.3. HPV E6 protein biomarkers

Expression of E6 and E7 genes is integral to hrHPV-induced malignant transformation, indicating that HPV E6/E7 protein markers could potentially serve as markers for identifying high-grade CIN. Most diagnostic E6/E7 protein markers are, as previously described, based on mRNA testing and therefore susceptible to degradation. Development of the whole-cell enzyme-linked immunoabsorbent assay (ELISA) using an HPV16, 18 and 45 anti E6 monoclonal antibody to detect HPV E6 protein in cervical samples, could tackle this problem. In the first pilot-study as well as the first clinical trial and follow-up after 1 year, increased specificity with a considerable lower sensitivity for ≥CIN3 was found, compared to a hrHPV DNA test [76–78]. Clinical evaluation of the assay in triage of HC2 hrHPV-positive clinician-taken samples showed a high specificity for ≥CIN3 of 93.8% (95% CI 92.1–95.2), with again limited sensitivity of 54.2% (95% CI 43.7–64.4) [79]. An explanation for this limited sensitivity could be the fact that this E6 test only covers HPV types 16, 18, and 45. Future research to possibly increase the number of covered hrHPV types in this ELISA-based test should reveal if sensitivity can be improved to increase its value as triage marker for hrHPV-positive women. Also, this marker has not yet been tested on self-sampled specimens, so its value in triaging hrHPV-positive self-sampled specimens is yet unknown.

3.4. Methylation markers

Methylation of CpG islands is a normal epigenetic event where functionally relevant changes to the genome are made without changing the nucleotide sequence. Abnormal DNA
methylations in host or viral DNA promoter regions during carcinogenesis may result in inactivation of tumor suppressor genes and silencing of gene expression. These changes in the DNA sequence can be detected and may possibly be used as biomarkers to distinguish productivity from transforming CIN lesions and cervical cancer. DNA methylation is easily detectable in clinician-taken, self-sampled, and histological cervical specimens [13]. Understanding the role of epigenetic events in host and viral genes is an important and promising area of investigation and is expected to result in novel risk stratifying strategies for triage of hrHPV-positive women.

3.4.1. Viral gene methylation markers

Methylation of the HPV DNA genome shows type-specific variation within the viral life cycle and differs during carcinogenesis. Methylation of HPV DNA may be a host response to foreign intracellular agents, a method of evading immune recognition, or a signaling event indicating viral integration into the host genome. From studies using different HPV-positive cell lines it is known that methylation of the late region is indicative for integration of the viral genome [80,81]. Viral gene methylation may also be indicative of the likelihood of persistence or clearance of the infection, therefore possibly holding a strong diagnostic or prognostic value in triaging hrHPV-positive women [82].

HPV genome sequence methylation is most widely studied in HPV16; hypermethylation of the HPV16 L1, L2, E2, and E4 regions is associated with an increased risk of CIN3 and HPV persistence, and hypermethylation of the E6 gene is associated with a lower likelihood of ≥CIN2. Some of the hypermethylated CpG sites also showed significant higher methylation levels in pre-diagnostic ≥CIN2 specimens with a median time of 3 years before diagnosis, compared to a control group, indicating a positive predictive property for high-grade lesions of these markers [83-86]. Conclusion of these studies is that elevated levels of methylation in the HPV16 genome may be useful in predicting concurrent or even future development of ≥CIN2. Methylation of other hrHPV types shows similar results to HPV16; hypermethylation of the L1, L2 regions of HPV18, 31, 33, and 45 was associated with high-grade CIN lesions, and increased methylation of the E2 region was found in HPV18, 31, and 45 induced high-grade CIN lesions [87,88]. HPV DNA methylation of HPV16, 18, 31, 33, and 45 may be useful in differentiating transforming from productive hrHPV infections. However, validation studies in large cohorts are necessary before these biomarkers could be used in clinical practice.

3.4.2. Host gene methylation markers

Methylation markers based on host DNA methylation have been studied extensively and have been summarized in reviews indicating a promising role in triage of hrHPV-positive women [8,89]. Studies in this field still continue, as for most genes no highly consistent result has yet been found. Combinations of markers most widely studied in hrHPV-positive women include the marker panels JAM3/EPB41L3/TERT/C13ORF18 and JAM3/C13ORF18/ANKRD18CP, and various combinations of the markers CADM1, MAL, miR-124-2, and FAM19A4. Many other individual markers and marker panels have been studied less extensively and most of these studies did not include hrHPV status. Some small studies did include the HPV status when testing markers. Bi-marker panels, DLX4/SIM1 [90]; tri-marker panel DAPK1/RARB/MGMT [91]; and single markers JAM3-M4 [92], EPB41L3 [93], hsa-miR-203 [94], PAX1 [95], and POU4F3 [96], show promising results; however, to our knowledge these are not yet confirmed by large prospective follow-up studies.

Triage of hrHPV-positive clinician-taken samples by methylation panel JAM3/EPB41L3/TERT/C13ORF18 yielded a ≥CIN3 detection rate of 65%. The panel is also shown feasible to use on self-sampled brush and lavage samples [97,98]. An adjusted panel of JAM3/C13ORF18/ANKRD18CP has only been studied in women with positive cervical cytology [99]. Further validation in population-based cohorts and large prospective studies is the next step for these panels. Methylation panels CADM1/MAL and MAL/miR-124-2 were the first panels validated in a population-based screening setting. CADM1/MAL methylation levels are related to the degree of the cervical disease and the duration of preceding hrHPV infection and the methylation status in cervical scrapes appears to be representative for the worst underlying lesion [100,101]. The CADM1/MAL bi-marker panel was equally discriminatory for ≥CIN3 as cytology at similar specificity in the triage of hrHPV-positive women [102,103]. When combined with cytology, this panel showed a high sensitivity with a slight drop in specificity in triage of hrHPV-positive women [103,104]. In triaging 79 hrHPV-positive women, the marker panel showed a sensitivity of 70% and specificity of 78% for ≥CIN3 [105]. In triaging women for colposcopy, the bi-marker panel MAL/miR-124-2 yielded a sensitivity of 64.9–71.6% at a specificity of 70% for ≥CIN3, which was significantly higher than the sensitivity for HPV16/18 genotyping in this specific study cohort [106]. In a large prospective study with 1038 hrHPV-positive nonresponders who were randomized between MAL/miR-124-2 and cytology triage, the DNA methylation panel was at least as sensitive as cytology at a threshold of borderline or mild dyskaryosis or worse for ≥CIN2 detection. The methylation panel showed a decreased mean time to diagnosis; however, at the cost of more colposcopy referrals [107]. In a recent pilot study, FAM19A4 methylation in clinician-taken samples showed to be an attractive triage marker for hrHPV-positive women [108]. Validation of bi-marker FAM19A4/miR124-2 in lavage- and brush-based self-samples resulted in a ≥CIN3 sensitivity of 69.4–70.5% with a specificity of 67.8–76.4% [109].

By adding HPV16/18 genotyping to the MAL/miR-124-2 methylation panel with an adjusted threshold, similar sensitivity with increased specificity for ≥CIN3 can be achieved, when compared to the methylation panel alone [110]. By adding HPV16/18 genotyping to FAM19A4 mutation, sensitivity increased, with decreased specificity, when compared to cytology alone, FAM19A4 mutation alone and HPV16/18 genotyping alone [111]. Combined FAM19A4/miR124-2 and HPV16/18 genotyping showed a ≥CIN3 sensitivity of 84.7% and specificity of 54.9% [109]. This indicates that combining host methylation markers and HPV16/18 genotyping may increase the clinical value of both techniques separately in triaging hrHPV-positive women.

3.4.3. Combined methylation marker panels

Recently, also studies combining HPV viral DNA genome methylation and host DNA methylation have been performed.

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A study with methylation of DAPK, L1 and L2 of HPV16, 18, and 54 shows lowest methylation in asymptomatic infections and increased methylation in progression to cancer [112]. Another combined methylation panel of L1 and L2 regions of HPV16, 18 and 31, and human gene EPB41L3 was tested in 1493 hrHPV-positive exfoliated cervical specimens from a colposcopy referral cohort and showed a sensitivity of 90%, combined with specificity of 36% and a PPV of 46% [113]. By adding HPV33 to the panel, specificity increased to 49% with a stable 90% sensitivity [114]. Validation of this assay in exfoliated cervical specimens of 710 women attending routine screening, of which 341 hrHPV positive, yielded a similar specificity of 65% with better sensitivity than HPV16/18 genotyping (47% vs. 54%) in identifying ≥CIN2 [115].

In summary, currently studied methylation markers show great potential as triage marker for hrHPV-positive women and could be the key to full molecular screening, possibly even with predictive value. Marker panels CADM1/MAL, MAL/mir124-2, and FAM19A/mir124-2, and combined HPV methylation and host methylation panels currently show the most potential. However, previously studied markers generally do not detect all ≥CIN3 lesions and detect less CIN2 lesions than cytology [8]. Also high referral rates have been described with host methylation triage, resulting in an increase of unnecessary colposcopies. Therefore, at the moment, we do not have methylation markers that merit clinical use yet. Future research with large population-based studies will prove whether methylation marker panels, either or not combined with other triage strategies, will eventually result in a triage strategy with high sensitivity and specificity and limited referral rates, to play a role in the triage of hrHPV-positive women.

3.5. Chromosomal biomarkers

Cellular genomic alterations are needed for progression of HPV-induced premalignant lesions, which could make chromosomal biomarkers a valuable triage method. The chromosomal regions most frequently altered in cervical squamous cell carcinoma are a loss at 3p and 11q, and gains at 3q, especially in HPV16-positive carcinomas [116]. The human telomerase RNA gene (hTERC) plays a role in maintenance of chromosome length and stability, and is located in chromosome 3q26 region. Gain of 3q26 shows a strong association with severity of dysplasia [117], and several small studies triaging LSIL and ASC-US report a high NPV, with a possible role in triage of women with low-grade CIN [118–120]. Most of the studies on genomic alterations are small and retrospective. Before these markers could be considered as triage method further research with prospective large studies with long-term follow-up is needed.

3.6. Other molecular biomarkers

Several other potential triage methods for hrHPV-positive women have been proposed; cellular proliferation-associated proteins, viral markers as HPV L1 capsid protein and E4 markers, the cervical microbiome and its cytokine profile, and proteomics based on differences in expressed proteins, all in a limited number of small studies [121–125]. Further prospective research is needed to determine the utility of these molecular biomarkers in triage of hrHPV-positive women.

4. Expert commentary

With the introduction of hrHPV-based screening with high sensitivity but limited specificity, effective triaging of hrHPV-positive women is essential. None of the triage strategies discussed in this review currently meets the criteria for an ideal triage method; however, several show great potential each with their own advantages and limitations.

The worldwide experience with morphology-based Pap-stained cytology makes this a common triage method for hrHPV-positive women. To improve sensitivity, different immunohistochemistry stains, as p16 staining, or p16/Ki-67 dual-stain can be used as biomarker. However, these triage strategies are still more or less based on morphological criteria and cannot differentiate between productive and transforming infections or predict the development of high-grade lesions. Besides they are not applicable on self-sampled specimens, which may play an important future role in hrHPV-based screening. An additional clinician-taken sample would therefore still be needed for further triage of hrHPV-positive women.

Molecular biomarkers have been extensively studied with a consistent increase of knowledge in this area. Molecular triage of hrHPV-positive women could in theory differentiate productive from transforming infections and some studies have already shown to be able to predict developing high-grade lesions. Molecular biomarkers are objective and highly reproducible, and can be used in high-throughput testing. They do not need high cellularity, and some have already been shown applicable on lavage- and brush-based self-samples. Studies on these molecular techniques as single biomarker or as a combination of biomarkers show promising results. Yet, no molecular triage method can differentiate all women with a high risk from women with a low risk for high-grade CIN. However, with further increasing knowledge on these molecular markers, cervical cancer screening may transform to full molecular screening in the future.

5. Five-year view

It is expected that in the next 5 years primary hrHPV testing, due to its high sensitivity, is increasingly incorporated in programs for cervical cancer screening in many Western countries. Results from the first years of primary hrHPV screening in some countries will then already be available. With the increased number of countries incorporating hrHPV screening, improving triage of hrHPV-positive women becomes more and more important. As knowledge on the molecular genesis of cervical precancer and cancer is expanding; triage tests other than cytology could fulfill the role of an additional triage test for HPV-positive women. In the next 5 years p16/Ki-67 dual-stain may replace or be added to morphology dependent Pap-stained cytology as triage method for hrHPV-positive women. However, it is expected that triage of hrHPV-positive women by morphological biomarkers will finally be taken over by molecular triage techniques with advantages as objective
evaluation and high-throughput triage. HPV genotyping has been widely studied and could be used as triage method for hrHPV-positive women on short notice. Methylation of host DNA is being widely studied and may also be used as triage method for hrHPV-positive women in the near future. The number of studies on predictive value of biomarkers is expected to increase and may finally result in biomarkers with predictive characteristics to detect high-grade abnormalities even earlier in the process of carcinogenesis. To improve attendance to the screening program in the Netherlands, self-sampling will be offered to non-responders of the new hrHPV-based screening program. If this approach turns out successful, other countries may also consider implementing self-sampling in their screening programs in the future. Cervical cancer screening is expected to gradually transform into a more woman-friendly program with more objective screening and triage methods with higher clinical accuracy.

Approximately 10 years ago, vaccines for HPV types 16 and 18 first became available and were followed-up by the quadrivalent and nonavalent vaccines. Vaccination programs based on vaccinating girls in their pre-sexarche for high-risk types 16 and 18 and possibly also other types are introduced. Recently published large studies show great promise of these vaccines with strong herd protection and no sign of type-replacement yet. It will however take many years before the vaccination program will affect the incidence of cervical cancer, and it is not yet fully known how this will affect the effectiveness of screening programs. Screening will therefore remain necessary for the next decades. Vaccination will however decrease the prevalence of high-grade CIN and therefore test characteristics of screening must show high sensitivity and specificity. In time, screening programs might need to be re-evaluated and adjusted again.

Key issues

- High-risk human papillomavirus (hrHPV) testing is expected to replace cytology as primary screening method for cervical cancer screening in an increasing number of countries. The high sensitivity of hrHPV testing combined with a limited specificity makes triaging of hrHPV positive women necessary.
- An ideal triage strategy consists of a biomarker that can be applied on the primary screening sample, resulting in a highly sensitive and specific result differentiating women with cervical cancer or high-grade CIN lesions from women with a low risk for these lesions who can safely return to the next screening round. An ideal triage strategy does not yet exist. Therefore the most optimal triage strategy should be obtained based on current knowledge.
- Multiple options for triaging hrHPV positive women are available. Previous experience with morphologically based methods makes them a logical first choice as triage method for hrHPV positive women. However, these morphological markers lack properties that make molecular biomarkers more attractive as triage method such as: objectivity, option for using high-throughput systems, the capacity to distinguish productive from transforming infections, predict developing high grade CIN lesions, and the opportunity to be performed on self-sampled material.
- At the moment, most biomarkers lack sufficient evidence to introduce them as triage method for hrHPV positive women in screening programs. Improved sensitivity and specificity, and more evidence from large prospective studies is needed before introducing these biomarkers as triage test into standard of care in cervical cancer screening programs. Different triage techniques may also be combined to improve diagnostic value as triage method for hrHPV positive women.
- In the near future, cervical cancer screening programs are expected to be based on full molecular screening with primary hrHPV testing and molecular triage of hrHPV positive women.
- The number of studies on predictive value of biomarkers is expected to increase and may finally result in biomarkers with predictive characteristics to detect high-grade abnormalities even earlier in the process of carcinogenesis.
- Self-sampling may attain a role in hrHPV based screening programs, to finally result in a more women-friendly screening programme with less loss to follow-up.
- It will take many years before vaccination programs will affect the incidence of cervical cancer, and it is not yet known how this will affect the effectiveness of screening programs. In time screening programs might need to be re-evaluated and adjusted again.

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References

Papers of special note have been highlighted as either of interest (i) or of considerable interest (ii) to readers.

19. • Systematic review and meta-analysis assessing hHPV testing on self-collected samples compared to HPV testing of clinician-taken samples.
• Large study evaluating p16/Ki-67 dual-stain in triaging hrHPV-positive women, showing similar sensitivity and higher specificity than ASC-US threshold.


• Subanalysis of women aged over 25 from the ATHENA clinical trial on the performance of hrHPV testing and HPV16/18 genotyping, compared to LBC.


• Systematic review on which hrHPV tests fulfill the criteria defined by an international expert team in 2009, for use in primary cervical cancer screening.


