Chapter 7.1

Cervical cancer screening: on the way to a shift from cytology to full molecular screening

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ABSTRACT

Cytology-based nation-wide cervical screening has led to a substantial reduction of the incidence of cervical cancer in western countries. However, the sensitivity of cytology for detection of high-grade precursor lesions or cervical cancer is limited, therefore repeated testing is necessary to achieve program effectiveness. Additionally, adenocarcinomas and its precursors are often missed by cytology. Consequently, there is a need for a better screening test. The insight that infection with high-risk Human Papillomavirus (hrHPV) is the causal agent of cervical cancer and its precursors, has led to the development of molecular tests for the detection of hrHPV. Strong evidence now supports the use of hrHPV testing in the prevention of cervical cancer. In this review, we will discuss the arguments in favor of, and concerns on aspects of implementation of hrHPV testing in primary cervical cancer screening, such as the age to start hrHPV-based screening, ways to increase screening attendance, requirements for candidate hrHPV tests to be used, and triage algorithms for screen-positive women.
INTRODUCTION

Currently, cervical cancer is the fourth leading cause of cancer death in women worldwide, causing more than 275,000 deaths annually. The disease has a very uneven global distribution; over 85% of cases are found in low resource countries, with incidence and death rates being the highest in sub-Saharan Africa, Central America, South-Central Asia and Melanesia. This imbalance in disease burden can be explained by differences in background risk (exposure to hrHPV infection) and the fact that cervical cancer is preventable by an effective screening and intervention system. Therefore, the lowest incidence and mortality rates are recorded in countries where screening is available to women. The impact of population-based screening is reflected in a substantial reduction in incidence of cervical cancer over the past 50 years in countries with established cytology-based screening programs. Especially, quality-assured population-based programs have shown to be very effective. However, cytology-based cervical screening also has some limitations. The major problem is the low sensitivity of a single smear to detect high-grade precursor lesions (50-70%), which requires frequent testing. In addition, cytology has low reproducibility, leading to variable accuracy. Moreover, by repeating cytology, the number of false-positives increases substantially over time. Finally, the decrease in incidence of cervical cancer induced by cytology-based screening is mainly restricted to squamous cell carcinoma, whereas no change is observed in incidence of cervical adenocarcinoma, suggesting that cytology fails to detect adenocarcinomas and its precursors. Consequently, there is a need for a better primary screening test, and thus a new screening algorithm.

HUMAN PAPILLOMAVIRUS TESTING IN THE PREVENTION OF CERVICAL CANCER

Cross-sectional sensitivity

Infection with hrHPV is a necessary event in the multi-step process of cervical carcinogenesis. Thirteen hrHPV types have been identified, of which HPV16 and HPV18 are the most important types, causing about 70% of squamous cell carcinomas, and more than 90% of adenocarcinomas. Recently, two prophylactic vaccines against HPV16 and HPV18 (Cervarix GSK®, Gardasil Merck®) have shown good protection against vaccine type-related cervical intraepithelial neoplasia grade 2 or worse (CIN2+) precursor lesions. However, cervical screening is still required because the current vaccines do not protect against all carcinogenic HPV types. In addition, despite the high vaccine uptake among women in countries with a school-based program, the uptake remains sub-optimal in, e.g., the Netherlands, France, Germany and the USA.

The causal relationship between infection with hrHPV and cervical cancer has stimulated the application of hrHPV DNA testing, which has been proposed, either alone or in combination with cytology, as a means to improve existing cervical screening programs. In the past fifteen years, large randomized trials designed to evaluate the performance of hrHPV testing, have
provided important arguments for the implementation of this assay as a primary screening tool (Table 1). First, five of these trials showed, in cross-sectional studies, that hrHPV testing is about 30% more sensitive in detecting CIN2+, and four of these studies showed that it is also about 20% more sensitive in detecting CIN3+ (Figure 1). Most of these lesions are HPV16-associated. The higher cross-sectional sensitivity was confirmed by two more studies analyzing the baseline data of screening populations 26,27. The study published by Kitchener et al.26, showed somewhat different results; first it demonstrated similar cross-sectional sensitivity for hrHPV testing and liquid based cytology (LBC) in primary cervical screening, and secondly, the data suggest that LBC, as used in the first round has a lower specificity for CIN3+ than usually expected27. Possible reasons for these discrepancies could, firstly, be attributed to over-diagnosis of lesions, as LBC was just introduced at that time. Secondly, to incomplete follow-up of hrHPV positive women with normal cytology, resulting in lower detection rates of high-grade CIN in the hrHPV arm at baseline27,28.

*Long-term protection*

At present four randomized trials conducted in Europe, have published longitudinal data on CIN3+ diagnosed at subsequent screening rounds, which took place in 3 to 5 years (Figure 2) 26-29. 32. All trials reported an approximately 50% lower CIN3+ detection rate in the second screening round among women who were hrHPV negative at baseline, than among women who had normal cytology. The pooled detection rate ratio of CIN3+, of hrHPV testing versus cytology, was 0.42 (95%CI: 0.32-0.55). The consistency of results was reflected by absence of inter-study heterogeneity (p=0.68). Thus, a negative hrHPV test provides a better protection against CIN3+, than a negative Pap smear. Several cohort studies have also shown a consistently lower long-term cumulative incidence rate of CIN2+ among women negative for hrHPV than among those with normal cytology 25,33,34. In addition, in two trials, the detection rate of cervical cancer at the second screening round was significantly lower among women, who were hrHPV negative at baseline (OR: 0.19 (95%CI: 0.07-0.53) 30,35, Most importantly, in a pooled analysis of four European trials following women for at least two screening rounds, it was recently confirmed that hrHPV-based screening provides better protection against cervical cancer than cytology (Ronco et al., Lancet in press). Consequently, screening intervals might be extended when primary hrHPV testing has been introduced. Berkhof et al.36 have shown that, an extension to 6 or 8 years is possible without increasing the lifetime cancer risk, using a simulation model. However, it makes sense to extend the screening interval for hrHPV negatives only, as hrHPV positive women, even those with normal cytology, have a non-negligible CIN3+ risk (5.2%) 32,34. The risk is too high to delay follow-up to the next screening round (3-5 years) 37,38, but too low to refer these women for immediate colposcopy39. Therefore, hrHPV positive women with normal cytology at baseline require further triage testing and/or follow-up, which will be discussed below.
Table 1: Trials comparing cytology and hrHPV testing in cervical cancer screening

<table>
<thead>
<tr>
<th>Study</th>
<th>Description</th>
<th>Interval</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>POBASCAM</td>
<td>HPV (GP5+/6+-PCR) and Cytology versus Cytology alone</td>
<td>5 years</td>
<td>Bulkmans et al. 2004, 2007; Rijkaart et al. 2012</td>
</tr>
<tr>
<td>ARTISTIC</td>
<td>HPV (HC2) combined with Cytology (LBC) versus Cytology (LBC) alone</td>
<td>3 years</td>
<td>Kitchener et al. 2006, 2009</td>
</tr>
<tr>
<td>SwedeScreen</td>
<td>HPV (GP5+/6+-PCR) and Cytology versus Cytology alone</td>
<td>3-5 years (by age)</td>
<td>Naucler et al. 2007, 2009</td>
</tr>
<tr>
<td>NTCC</td>
<td>HPV (HC2) alone versus HPV (HC2) and Cytology (LBC) versus Cytology alone</td>
<td>3 years</td>
<td>Ronco et al. 2006, 2008, 2010</td>
</tr>
<tr>
<td>CCaST</td>
<td>HPV (HC2) and Cytology versus Cytology and HPV (HC2) (randomised order of collection)</td>
<td>1 year</td>
<td>Mayrand et al. 2007</td>
</tr>
<tr>
<td>Finnish Screening trial</td>
<td>HPV (HC2) and Cytology triage vs Cytology alone</td>
<td>5 years</td>
<td>Leinonen et al. 2009</td>
</tr>
<tr>
<td>India Screening trial</td>
<td>HPV (HC2) versus Cytology versus visual inspection with acetic acid (VIA) versus no screening</td>
<td>-</td>
<td>Shankaranarayanan et al. 2009</td>
</tr>
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</table>

Figure 1. Detection rate ratio in screening with hrHPV versus Cytology in the first screening round of randomized trials for outcome CIN3+.

*Ronco et al. is restricted to women of 35 years or older
Another important and consistent finding in the five trials with combined hrHPV and cytology co-testing, was that co-testing has virtually no additional value compared to single hrHPV screening. Although slightly more sensitive, no significant differences for CIN2+ or CIN3+ detection were found (sensitivity ratio cytology & hrHPV versus hrHPV alone: 1.06 (95%CI: 0.96-1.18) for CIN2+, and 1.03 (95%CI: 0.89-1.20) for CIN3+)27. Also, a recent study from the US showed that hrHPV and cytology co-testing has no advantage over sole cytology screening34. Collectively, these data show that sole hrHPV testing is sufficient for cervical screening, and argue against the use of combined hrHPV and cytology co-testing in women aged 30 to 60 years, as recently recommended by the American Cancer Society (ASC)40. Moreover, the data from the POBASCAM trial, in which hrHPV and cytology co-testing was used in both study arms in the second screening round, show that the total number of CIN3+ detected over two rounds is equal in both arms. These results indicate that, at least a part of the surplus of CIN2/3+ lesions detected in the first round by hrHPV testing represent non-regressing, clinically relevant lesions32.

Finally, an Indian cluster-randomized trial41 found that, in a low-resource setting, a single round of hrHPV screening was associated with a significant decline in the rate of advanced cervical cancers (FIGO stage II+), as compared with an unscreened control group (hazard ratio 0.47 (95%CI: 0.32-0.69). No significant reduction was observed in the study arms with cytology or VIA screening.

Collectively, the available evidence indicates that sole hrHPV testing should replace cytology as a primary screening tool in cervical screening.

**HRHPV DETECTION METHODS**

hrHPV detection methods include both HPV DNA assays and E6/E7 mRNA assays. The drawback of hrHPV testing is that it has an (apparently) unavoidable trade-off between sensitivity and specificity. Overall, hrHPV testing has a 3-4% lower specificity than cytology (at cut-off atypical squamous cells of undetermined significance or worse (ASC-US+))29 due to its inability to
distinguish between persistent hrHPV infections associated with (precursor lesions of) cervical cancer and transient hrHPV infections. The specificity could even be lowered more than 25% when an ultra-sensitive hrHPV test were applied \(^42\). From a clinical point of view, testing for hrHPV is only useful when a positive hrHPV test result is informative about the presence or absence of CIN2+ (clinical sensitivity and specificity). Thus, in order to prevent excessive follow-up procedures for women with transient hrHPV infections or hrHPV positive women without cervical lesions, candidate hrHPV tests to be used for cervical screening should be clinically validated. Two hrHPV DNA tests, i.e. HC2 and GPS+/6+, have shown, in large clinical trials, to perform better in reducing the incidence of CIN3+, and are thus considered as clinically validated prototype assays. Guidelines for hrHPV test requirements and clinical validation were developed, based on the available data from large prospective screening studies \(^43\). These guidelines can be used to assess the clinical performance of a candidate HPV test relative to one of two prototype hrHPV tests with proven good clinical performance (i.e., HC2 or GPS+/6+-PCR) by a cross-sectional clinical equivalence analysis in a screening setting \(^44\). In short, the candidate test should have a clinical sensitivity for CIN2+ not less than 90%, and a clinical specificity not less than 98% of that of the reference assays.

Thus far, three additional hrHPV DNA tests i.e., Cobas 4800 Roche\(^\text{a}\), RealTime (RT) PCR Abbott Molecular\(^\text{b}\), and Papillocheck Bio-Greiner\(^\text{c}\) (when only 14 hrHPV types are considered), have fulfilled the criteria provided in these guidelines, with sensitivities ranging between 100% and 95.8%, and specificities from 96.7% to 92.3\% \(^45-51\). Thus these assays can be considered as clinically validated for primary hrHPV-based cervical cancer screening.

In contrast to the HPV DNA tests, the APTIMA HPV Assay (GenProbe) relies on aggregate detection of mRNA of 14 hrHPV types \(^52\). Two studies \(^53,54\) have evaluated the performance of this assay for primary screening compared to the HC2 test, and the results were summarized in a recent review \(^39\); the relative sensitivity ratio (outcome CIN2+) of APTIMA versus HC2 was 1.02 (95\%CI: 0.86-1.20), and the relative specificity ratio was 1.07 (95\%CI; 1.05-1.08). Thus, the APTIMA assay seems to perform with sensitivity closely to that of the DNA test and with a possible slightly higher specificity. A recent study comparing the APTIMA HPV Assay with GPS+/6+ PCR showed similar results, and indicated that also the APTIMA HPV Assay is clinically non-inferior to GPS+/6+ PCR with respect to sensitivity and specificity for CIN2+ (Heideman et al., JCM in press). Another approach in trying to validate new hrHPV tests was used by Cuzick et al.\(^55\) in the predictors-study: the sensitivity was primarily analyzed in a cytology-based referral population \(^56\), and specificity in a cytology-based screening population \(^55\). This study evaluated six HPV tests and established that four tests (Roche Cobas, Gen-probe APTIMA, Abott Realtime PCR and BD HPV assay), achieved the required sensitivity and specificity compared with HC2. This would also qualify the BD test as clinically validated. However, the predictors screening-study only included cytology driven CIN2+ lesion, which made the sensitivity criterion too soft to consider it in line with the guideline described earlier\(^43\). In addition, no details of any previous screening history of participants were available. The use of validated HPV test assays alone, however, is not sufficient to reduce the number of false positive tests among healthy women; triage testing of hrHPV positive women is necessary to keep the number of invasive follow-up examinations, and thus the costs within acceptable limits \(^57\).
MANAGEMENT OF HRHPV POSITIVE WOMEN

Cytology and hrHPV genotyping

Epidemiological studies have estimated that HPV16 and HPV18 cause approximately 70% of cervical cancers worldwide, and that the cumulative 10-year risk of CIN3+ in HPV16/18 positive women ranges from 10% to 20% \(^{58,59}\). These facts have led to several studies evaluating the value of hrHPV genotyping, with or without cytology, to triage hrHPV positive women.

In the VUSA-screen study, evaluating 14 triage strategies \(^{57}\), cytology triage at baseline and repeat cytology testing at 12 months emerged as the optimal strategy; it showed the highest positive predictive value (PPV 37.5%; (95%CI:32.6-42.6)) in combination with a high negative predictive value (NPV) for CIN3+ (99.3% (95%CI: 89.1-99.8)). A CIN3+ risk of <2% (corresponding with a NPV for CIN3+ of >98%), was considered to be acceptable for dismissal from further follow-up. This threshold was based on the five-year CIN3+ risk of women with BMD (borderline or mild dyskaryosis) cytology at baseline, and normal cytology at six and 18 months follow-up (1.2%), which is presently accepted in the Netherlands \(^{37}\).

In addition, we have recently performed a post-hoc analysis of data from the POBASCAM trial \(^{38}\) to evaluate useful triage strategies, including HPV16/18 genotyping and cytology. Three triage strategies met the criteria for NPV (≥98%) and PPV (≥20%) that were set to minimize risk of over investigation and excessively aggressive management; i.e., the NPVs ranged between 98.1% and 99.6%, while the PPVs ranged between 25.6% and 34.0%. The eligible triage strategies were: 1. cytology and HPV16/18 genotyping at baseline without repeat testing, 2. cytology at baseline with repeat cytology testing after six months, and 3. cytology and HPV16/18 genotyping at baseline followed by repeat cytology examination at 6 months \(^{38}\). Further, a nested evaluation of the Swedish SWEGDESCREEN study, suggested to follow-up hrHPV positive women with normal cytology at baseline, by one repeat hrHPV test \(^{60}\). This strategy showed comparable results in terms of sensitivity for CIN3+ (96.0%), however, with somewhat lower PPV (22.0%) than obtained with the preferred strategies in the POBASCAM sub study. Moreover, implementation of this strategy leads to a substantial increase in colposcopy referral rate, and thus possible overtreatment \(^{38,57}\).

Finally, Castle et al., evaluated the performance of Cobas HPV testing (Roche Molecular systems, USA) as a primary screening test among women aged 25 years and older \(^{61}\). The preferred strategy in this sub-analysis of the ATHENA study, was cytology triage (threshold LSIL or worse) in combination with detection of HPV16, HPV18, or both types, resulting in a sensitivity for CIN3+ of 72.2% (95%CI: 66.4-77.4) and a PPV of 13.9% (95%CI:12.8-15.0). Though, the authors recommended re-testing of screen-negatives after 1 year, because the sensitivity for CIN3+ was less than 80% and the NPV lower than 98%.

Thus, several triage strategies seem feasible, also because the results of the different studies indicate that it does not appear that the exact triage protocol has any effect on the outcome. However, as preferences and the quality of cytology will vary between countries, policymakers
will have to weigh the pros and cons of the different triage strategies when making a choice. Especially, the balance between the safety of a triage strategy (NPV) and the burden of screening on patients and clinicians (PPV and referral rate) is important.

**HPV mRNA, p16\textsuperscript{INK4a} IHC and methylation markers**

In future cervical screening, it might be expected that the role of cytology, as a triage test, will become more limited, as the hrHPV test result may influence the subjective reading of cytology. Promising biomarkers involved in cervical carcinogenesis have already emerged. Especially, biomarkers that indicate a shift from the productive phase of hrHPV infection, to the transforming phase are valuable. hrHPV E6/E7 oncoproteins are highly expressed in (para)basal cells of high-grade CIN, and interact with p53 and pRB respectively; in this way, they interfere with cell cycle control. As a consequence, uncontrolled proliferation and chromosomal instability occur, resulting in additional (epi) genetic changes. Therefore, detection of elevated E6/E7 mRNA levels in cervical smears has been suggested to be an attractive biomarker.

Two commercial hrHPV mRNA assays can be used for triage testing, i.e., the PreTect HPV-Proofer (NorChip) and the NucliS eNS easy Q HPV (bioMérieux). Both are based on the same technology, and are marketed under different brand names in different countries. These assays detect HPV E6/E7 mRNA from the five most prevalent hrHPV types in cervical cancer (HPV16, 18, 31, 33, 45). A recent study showed that the HPV Proofer assay is particularly of value to triage hrHPV DNA positive women with normal cytology, given their markedly increased risk of CIN2+ in case of a positive mRNA result (55% (95%CI 34-76%). However, this study also revealed that hrHPV positive, cytological normal women who are mRNA test negative would still need follow-up, as their CIN2+ risk was 20% (95%CI 7-33%). Thus, as a primary screening test, the clinical applicability of these HPV mRNA tests is insufficient.

An alternative is detection of cellular host genes that are specifically up-regulated and overexpressed, or silenced in cells that have undergone the shift into the transforming phase of hrHPV infection. Cyclin dependent kinase inhibitor p16\textsuperscript{INK4a} is up-regulated as a consequence of hrHPV E7 expression in proliferating cells, and several studies have shown that p16\textsuperscript{INK4a}-based cytology, either alone or in combination with hrHPV-testing, can detect underlying CIN2+ with high sensitivity. However, the results for p16\textsuperscript{INK4a} were less favourable in the Predictors studies, comparing the sensitivity and specificity of several tests for the detection of high-grade CIN in a cytology-based referral population. In addition, even after p16\textsuperscript{INK4a} immunostaining, still morphological interpretation necessary to differentiate hrHPV-transformed cells from endometrial cells with non-hrHPV induced p16\textsuperscript{INK4a} expression. To overcome this limitation, a double-staining kit for p16\textsuperscript{INK4a} and Ki-67 has been developed that allows simultaneous detection of p16\textsuperscript{INK4a} and nuclear Ki-67 expression in dividing cervical cells (CINtec\textsuperscript{C} Plus, MTM laboratories). The potential of this double staining kit to identify women at risk for underlying high-grade disease was shown in women with abnormal cytology, as well as in hrHPV positives with normal cytology, when used as a reflex test. At present,
these good results will need to be confirmed in prospective studies. Furthermore, a recently published retrospective study evaluating different triage strategies in a cytology-based referral population, suggested that further testing for p16\(^{INK4a}\) and HPV16 genotyping may also be an important strategy to determine which women are in need of referral. They advocate this strategy, especially for hrHPV screen positives with normal cytology \(^{88}\). Now, these strategies should be evaluated in prospective studies.

Although, a hrHPV infection can induce immortalization and trigger chromosomal instability, additional changes in oncogene expression and loss of function of tumor suppressor genes are necessary, to obtain a full blown invasive cancer cell. Methylation of Cpg islands is an epigenetic modifier of gene expression. In many cancers, tumor suppressor genes were found to be inactivated by hypermethylation of their promoter region. Therefore, detection of hypermethylation of tumor suppressor genes involved in cervical cancer genesis may provide powerful biomarkers for cancer detection \(^{84,85}\), especially as methylation has been detected already at precancerous stages \(^{86,87}\). In fact, a recent study of Bierkens et al., showed that methylation levels of two genes (i.e. CADM1 and MAL) increased with the grade of underlying CIN and were highest in carcinomas. Moreover, cervical scrapes of women with CIN 2/3 lesions with long-lasting hrHPV infections (≥ 5 years) had higher methylation levels than those with a shorter duration of preceding hrHPV infection (< 5 years) \(^{88}\). These findings indicate that, at least lesions with a longer duration of existence are detected by such methylation markers, further strengthening their value for triage testing of hrHPV positive cervical scrapes. Recently, a study on scrapes from women participating in screening was performed to evaluate the value of an objective real time PCR assays that assesses the methylation status of the promoter regions of CADM1 and MAL to triage hrHPV positive women for CIN3+ \(^{89}\). Because of the design of the study, the results were compared to CIN3+ sensitivity and specificity of cytology only, and of cytology combined with HPV16/18 genotyping. An optimal threshold resulted in a sensitivity of 84.2%, and corresponding specificity of 52.5% for the methylation assay; for cytology these were 65.8% and 78.8%, and for cytology with HPV16/18 genotyping these were 84.2% and 54.0%, respectively. Consequently, the authors concluded that, in hrHPV positive women, this methylation marker panel was at least equally discriminatory for high-grade CIN as cytology, or as cytology with detection of HPV16/18. These results indicate that complete cervical screening by objective, non-morphological molecular methods seems feasible. However, further validation is needed before any of these tests can be considered for screening. Currently, a large randomized controlled trial to validate methylation markers to triage hrHPV positive women, is being performed in the Netherlands.

**PRIMARY HRHPV TESTING: AT WHAT AGE SHOULD WE START?**

Another important issue is the age at which hrHPV testing should be offered for primary screening. Most experts agree that in women younger than 25 years of age hrHPV testing is not recommended, because the prevalence is very high, and at this age hrHPV infections are commonly transient.
Ronco and colleagues 30, reported that hrHPV testing in women aged 25–34 years could lead to substantial overdiagnosis of regressive CIN2+ lesions, particularly when hrHPV positives in this age group are directly referred for colposcopy, without further triage testing. The results from the POBASCAM trial 32, showed that hrHPV screening does not have to be postponed until age 36 years or older, but can be started at age 30 years. The long-term follow-up results of this study showed that the cumulative detection of CIN2+ and CIN3+ lesions over two screening rounds did not differ between women aged 29-33 years, and women of 34 years of age and older 32. Thus, hrHPV screening does not result in excessive diagnosis of lesions destined to regress, even in women in the 30-34 years age category. These findings indicate that primary hrHPV screening should be recommended for women aged 30 to 60 years. Additional follow-up after the age of 60, is indicated for women with a positive hrHPV test at the last screening round. The residual risk of CIN3+ is too high to dismiss them from follow-up, even when triage testing in hrHPV positive women is negative (Dijkstra et al. submitted). These women can only be discharged from further hrHPV testing after they have cleared the virus. Future studies should investigate whether methylation markers might be used as a more specific triage test for hrHPV positive women in the younger age-range (below 30 years) to detect clinically relevant lesions, as high methylation levels seem to be related to the degree and duration of underlying CIN and highest levels are found in carcinomas. 88.

**INCREASING THE SCREENING COVERAGE**

One other problem concerning the effectiveness of current cervical screening programs remains non-attendance 90,91. Especially non-participating women are at increased risk of cancer 3,92, as half of the cervical carcinomas are found in non-attending women. Therefore, targeting non-attendees is important in achieving optimal protection from screening programs.

Self-sampling is a less costly and less invasive collection method 93, and several studies have shown that non-attendees actually do take part in self-sampling studies 90,94-97. Thus, there is a basis for self-sampling in cervical cancer screening. In addition, self-collection makes cervical screening accessible to women in medium- and low-income countries 98-100. That is why, in recent years several studies have focused on the use of self-collected samples for hrHPV testing.

In most studies a moderate to good agreement between hrHPV test positivity in self-collected and physician-taken samples, was found. The data have been summarized in (systematic) reviews and meta-analyses 92,101, in which, study parameters included concordance in hrHPV detection rates between self- and physician-collected samples through kappa values, which varied between 0.45 and 1.00 (Table 2). Though, the hrHPV test positivity rates varied across the studies, most likely due to differences in study populations (age range and country of origin), the use of different collection devices for self- and physician-sampling, as well as differences in hrHPV tests and protocols. For example, in studies using the HC2 method, a higher hrHPV detection rate in self- compared to physician-collected samples was observed 92,96,98,103. The HC2 assay, however, is known to show some cross-reactivity to low-risk HPV types 104, and these types
tend to more commonly affect vaginal than cervical mucosa. In other studies, a higher hrHPV detection rate was found in the physician-collected cervical samples. Yet, overall the data show that self-sampling is concordant with physician-sampling in detecting hrHPV DNA.

Table 2. Systematic reviews and meta-analyses of the literature comparing self-collected and physician-taken samples for the detection of hrHPV DNA

<table>
<thead>
<tr>
<th>Reference</th>
<th>Nr. of studies included</th>
<th>Kappa value for agreement (range)</th>
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<tbody>
<tr>
<td>Ogilvie et al. 2005</td>
<td>12 studies</td>
<td>0.45 - 1.00</td>
</tr>
<tr>
<td>Petignat et al. 2007</td>
<td>18 studies</td>
<td>0.50 - 0.82</td>
</tr>
<tr>
<td>Schmeink et al. 2011</td>
<td>19 studies</td>
<td>0.45 - 0.81</td>
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However, prior to its use in cervical cancer screening, it is most important to know how self-sampling performs with regard to relevant disease outcomes. In other words, hrHPV self-sampling procedures should be clinically validated in terms of sensitivity and specificity to detect CIN2+ lesions. Various cross-sectional studies have been performed comparing the value of hrHPV testing on self- versus physician-collected samples to detect CIN2+, and the results have recently been summarized by Snijders et al. This review showed that hrHPV testing on self-sampled specimen is as sensitive for CIN2+ in several studies as hrHPV detection on physician-taken cervical samples, though sometimes less specific. However, the authors also reported that, both the type of self-sampling device and the type of hrHPV test seem to influence the clinical performance of hrHPV testing on self-samples. Thus, for reaching clinical equivalence, in terms of detecting high-grade CIN, between self-sampling and physician-sampling, a certified combination of self-sampling device and validated hrHPV test is important. Overall, the data show that self-sampling for primary hrHPV testing offers possibilities to increase screening coverage by reaching non-responders, and in future might even be offered as a safe alternative screening method to regular attendees.

Additionally, hrHPV-oriented cervical screening programs will increase public awareness of the link between hrHPV and cervical cancer, which may in turn lead to a higher uptake of the prophylactic HPV vaccines and consequently a further reduction of cervical cancer incidence.

CONCLUSION

All the evidence collected so far, suggest that the time has come for the implementation of hrHPV testing as a primary screening test, as it provides a superior protection against cervical (pre-)cancerous lesions compared to cytology. hrHPV testing detects 30% more CIN2+, and 20% more CIN3+ lesions in women over 30 years of age. Though, any hrHPV test used, should be clinically validated, and hrHPV-based primary screening should be implemented preferably within a population-based screening program with a call and recall system. The somewhat lower specificity, which may cause excess false positive tests among healthy women, can be overcome
by triage of hrHPV positive women. At this time, triaging these women by cytology at baseline and repeat cytology testing after 6 months, possibly in combination with baseline HPV16/18 genotyping, seems very suitable for this purpose. Though, as preferences and the quality of cytology will vary between countries, policymakers will have to weigh the pros and cons of the different triage strategies when making a choice. In future screening, however, it is likely that the role of cytology becomes more limited and validated (molecular) biomarkers gain attention; among these, p16\textsuperscript{INK4a}/Ki-67 double staining and host genome or viral DNA methylation markers appear to be promising.

As for the age to commence screening, we advocate the introduction of primary hrHPV testing in women from the age of 30, to ensure that mostly clinically relevant, non-regressing high-grade lesions are detected. Furthermore, since a negative hrHPV test offers a better protection (50%) against CIN3+ than normal cytology, hrHPV testing can be implemented together with an extension of the screening interval for hrHPV screen negative women. In addition, to achieve optimal protection and screening coverage, self-sampling might be offered to non-attendees, and in future possibly as an alternative method to regular attendees, under the condition that a validated combination of self-sampling device and hrHPV assay is used.

Collectively, these data show that the time has come to implement primary hrHPV testing in population-based cervical screening, thereby offering women maximum protection against high-grade CIN with less uncertainty and fewer screening rounds.
REFERENCE LIST


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Chapter 7.2

Discussion, key-messages and future perspectives

M.G. Dijkstra
KEY-MESSAGES & FUTURE PERSPECTIVES

Increasing the screening coverage

In the Netherlands about 35% of the invitees do not respond to a screening invitation 1. Targeting these non-attendees is important to achieve optimal protection from screening programs, as half of all cervical carcinomas are found in non-attending women. Self-sampling seems an effective method to improve screening participation, as up to 40% of the non-attendees respond by returning their self-collected sample 2-4. Furthermore, the yield of high-grade lesions was higher in this group compared to screening participants 3,5,6. Several studies have shown, that hrHPV testing on self-collected samples is just as sensitive for detecting CIN2+ as hrHPV testing on physician-collected samples, however a certified combination of self-sampling device and validated hrHPV test is important 7. Thus, self-sampling for primary hrHPV testing offers possibilities to increase screening coverage by reaching non-responders. Indeed, in addition to implementing hrHPV testing as primary test, the Health Council of the Netherlands has recommended to send a self-sampling device for hrHPV testing on request, to women who do not want to attend regular screening 8. In addition, self-sampling may facilitate access to cervical screening in developing regions 9,10.

Key-messages

- Self-sampling for primary hrHPV testing offers possibilities to increase screening coverage by reaching non-responders, and in future, might even be offered as a safe alternative screening method to regular attendees.

Primary hrHPV-based screening and management of hrHPV positives

In the past 15 years, numerous cross-sectional studies and several large longitudinal randomised controlled trials designed to evaluate the performance of hrHPV testing, in comparison to cytology, have provided important arguments for the implementation of a clinically validated HPV test as primary cervical screening tool. These trials showed that hrHPV testing is approximately 30% more sensitive in detecting CIN2+, and approximately 20% more sensitive in detecting CIN3+ in women over 30 years of age 11-16. More importantly, a reduction of 50% of CIN3 and cervical carcinoma was found in the second screening round (3-5 years) among women after HPV-based screening compared to women after cytology screening 16-20. Thus, hrHPV-based screening provides superior long-term protection against cervical disease than cytology. Consequently, screening intervals might be extended when primary hrHPV testing has been introduced, without a rise in high-grade cervical lesions in the meantime 13,18,21-24. However, it makes sense to extend the screening interval (presently 5 years in the Netherlands) for hrHPV screen negative women only, as hrHPV positive women, even those with normal cytology, have a non-negligible CIN3+ risk (5.2%) 16,19. The risk is too high to delay follow-up to the next screening round (3–5 years) 25,26, but too low to refer these women for immediate colposcopy 27 (Dijkstra et al. submitted).
Thus, primary hrHPV testing can detect cervical precursor lesions earlier than cytology, but also results in a higher number of abnormal test results. In the Netherlands, about 5% of women between 30-60 years of age are hrHPV positive, and approximately 13% of them have underlying CIN3+ and about 22% a CIN2+ lesion. Therefore, hrHPV positive women require further triage testing, to prevent over-referral and overtreatment.

Several suggestions for strategies to triage hrHPV positive women have been made in the literature. At this time, triaging these women by cytology at baseline and repeat cytology testing after 6 to 12 months, possibly in combination with baseline HPV16/18 genotyping, seems to be very suitable for this purpose. Still, as preferences and the quality of cytology will vary between countries, policy-makers will have to weigh the pros and cons of the different triage strategies when making a choice. Especially, the balance between the safety of a triage strategy (NPV) and the burden of screening on women and clinicians (PPV and referral rate) in relation to the resources available, is important.

The Health council of the Netherlands has recently advised to change from cytology-based screening to primary hrHPV testing in population-based screening to improve screening efficacy. This will be implemented in the Netherlands in 2016, and comprises primary hrHPV testing combined with cytology triage in 5 screening rounds, at ages of 30, 35, 40, 50 and 60 years. Women who are hrHPV positive, but triage negative (cytology at baseline and after 6 months) at the age of 40, 50 or 60 years, are re-screened after 5 years, because their CIN2+ risk exceeds 2%.

Key-messages

- Sole hrHPV testing should replace cytology as a primary screening tool in cervical screening.
- hrHPV-based screening provides superior long-term protection against CIN3 and cervical cancer than cytology by earlier detection of clinically relevant CIN2+ lesions.
- hrHPV screen negative women have a low long-term risk (0.011 (95%CI: 0.0068 - 0.015) to develop cervical disease. Therefore, an extension of the screening interval beyond 5 years is justified.
- hrHPV positive women require triage testing to prevent over-referral for colposcopy, and should be screened every 5 years, as the long-term risk to develop cervical disease is too high to delay re-screening, even after negative triage testing.

Future perspectives

In future cervical screening, it might be expected that the role of cytology, as a triage test, will become more limited as knowledge of the hrHPV test result may influence the subjective reading of cytology. Therefore, there is a need for objective triage markers. Promising biomarkers involved in cervical carcinogenesis have already emerged (Table 1). For example, a dual staining test for p16INK4a and Ki-67 has been developed that allows simultaneous detection of p16INK4a and nuclear Ki-67 expression in dividing cervical cells (CINtec® Plus, MTM laboratories). The potential
of this dual staining test to identify women at risk for underlying high-grade disease was shown in women with abnormal and equivocal cytology (Uijterwaal et al. In press, BJC), as well as in hrHPV positives with normal cytology, when used as a reflex test. Since these promising results have been obtained with reviewed cytology, they need to be confirmed in prospective studies without review cytology.

Table 1. promising triage tests for future cervical screening

<table>
<thead>
<tr>
<th>Test</th>
<th>Manufact. / Distrib.</th>
<th>Strenght</th>
<th>Weakness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology (LBC)</td>
<td>Hologic, BD</td>
<td>Standard of care</td>
<td>Low sensitivity and reproducibility; slide-based (not applicable to selfsamples)</td>
</tr>
<tr>
<td>CINtec Plus (p16 / ki67)</td>
<td>Roche</td>
<td>Good sensitivity and specificity</td>
<td>Slide-based, interpretation not uniform</td>
</tr>
<tr>
<td>ProEx C proliferation markers (TOP2A, MCM2)</td>
<td>BD</td>
<td>Improvement of Pap cytology</td>
<td>Not linked to transformation event; morphology-based</td>
</tr>
<tr>
<td>OncoFISH cervical test (FISH 3q)</td>
<td>Ikonysis</td>
<td>Associated with carcinogenesis</td>
<td>Difficult standardization; Slide-based</td>
</tr>
<tr>
<td>Terc FISH</td>
<td>Quest diagnostics</td>
<td>Associated with carcinogenesis</td>
<td>Difficult standardization; Slide-based</td>
</tr>
<tr>
<td>GynTect (methylation markers)</td>
<td>Oncognostics</td>
<td>Simple, objective evaluation</td>
<td>Clinical performance in screening population</td>
</tr>
<tr>
<td>Precursor-M Test (methylation markers CADM1, Mal, miR-142)</td>
<td>Self-Screen</td>
<td>Tested in clinical trials; No fixative needed; Use on smears, LBC, and self-collected lavage specimen</td>
<td>Some CIN2/3 lesions not detected</td>
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Another application of p16INK4a immunostaining is to improve objective cervical lesion grading in histology. Histomorphological diagnosis of CIN by interpretation of H&E stained slides is subjective and prone to large variability. This is reflected in poor inter-observer agreement between pathologists. However it was shown that, the conjunctive interpretation of p16INK4a immunostains significantly improves the accuracy and reproducibility of grading cervical lesions on biopsy samples. Moreover, the accuracy of CIN lesion grading by a single pathologist with the additional use of p16INK4a stains is comparable with the consensus diagnosis of an expert pathology panel.

Further, in many cancers, tumor suppressor genes were found to be inactivated by hypermethylation of their promoter region. Therefore, detection of hypermethylation of tumor suppressor genes involved in cervical cancer genesis may provide powerful biomarkers for cancer detection, especially as methylation has been detected already at precancerous stages.
In fact, a recent study of Bierkens et al. showed that methylation levels of two genes (i.e. CADM1 and MAL) increased with the grade of underlying CIN and were highest in carcinomas. Moreover, cervical scrapes of women with CIN 2/3 lesions with long-lasting hrHPV infections (≥5 years) had higher methylation levels than those with a shorter duration of preceding hrHPV infection (<5 years). These findings indicate that at least lesions with a longer duration of existence are detected by such methylation markers. Moreover, methylation markers detect nearly all carcinomas (De Strooper et al. submitted), further strengthening their value for triage testing of hrHPV positive cervical scrapes. In addition, in contrast to cytology examination, methylation markers are directly applicable to self-collected samples. This will make cervical screening more accessible and also available to medium and low resource countries. In these countries, lacking medical services, complete molecular objective, non-morphological cervical screening would be ideal to select hrHPV positive women in need of colposcopy. Even women in remote areas could receive a self-sample device to collect a sample for primary hrHPV testing and, if screen-positive, triage testing with methylation markers. Only "double test positive" women require direct referral to a gynaecologist. This strategy could increase screening coverage, lower the burden on the colposcopy capacity of gynaecologists, and eventually could extend the lead-time to cervical cancer by decreasing the incidence of advanced precancerous lesions. The latter would justify a longer screening interval (3-5 years), which again reduces the burden on the health care system of these low resource countries.

Cytology triage and triage testing with methylation markers seem to be complementary to each other, as cytology has high specificity, while methylation markers detect nearly all carcinomas. Therefore, in high income countries with efficient cytology screening, we imagine that testing for methylation markers in combination with cytology examination could be an effective strategy to triage hrHPV positive women. However, further research on the potential of the different (combinations of) biomarkers for triage of hrHPV screen positives is needed. Recently, a large randomized, controlled trial, in which cytology-triage was compared to triage by the bi-marker MAL/miR124 methylation test on a self-collected sample, showed similar sensitivities for CIN2+, indicating that triage by methylation analysis was feasible. Thus, full molecular cervical screening by HPV self-sampling in combination with molecular triage by methylation markers will be possible in the near future.

In summary, the time has come to implement primary hrHPV testing in population-based cervical screening, thereby offering women maximum protection against high-grade CIN with less uncertainty and fewer screening rounds. The somewhat lower specificity, which may cause excess false-positive tests among healthy women, can be overcome by triage of hrHPV positive women. At this time, triaging these women by cytology at baseline and repeat cytology testing after 6 months, possibly in combination with baseline HPV16/18 genotyping, seems to be very suitable for this purpose. Although, as preferences and the quality of cytology will vary between countries, policymakers will have to weigh the pros and cons of the different triage strategies when making a choice. In future screening, however, it is likely that the role of cytology becomes more limited and validated (molecular) biomarkers gain attention; among these, p16INK4a/Ki67 double staining and host genome or viral DNA methylation markers appear to be promising.
REFERENCE LIST


