Novel biomarkers for cervical screening and surveillance of women treated for cervical precancer

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Chapter 8.1

Arguments in favor of HPV testing for cervical screening and post-treatment CIN2+ monitoring

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Abstract

Several studies have shown that the human papilloma virus (HPV) test is a more sensitive and objective primary cervical cancer screening tool than cytology. Therefore, conversion of cytology into HPV screening (as is planned in The Netherlands and some other European regions) will result in a better protection against cervical cancer and high-grade precursor lesions. Moreover, offering selfsampling for HPV testing will increase screening attendance by re-attracting former non-attendees. However, triage of HPV positive women is necessary because the specificity of HPV testing is 2–4% lower than of cytology. Several triage strategies have been evaluated, of which two, with cytology testing included, are feasible and were recently recommended. As an alternative for cytology triage, objective, non-morphological disease markers are upcoming and so far have shown promising results. Finally, HPV testing can also contribute to a more efficient monitoring of women treated for high-grade cervical precursor lesions, permitting fewer follow-up visits.

Background

Cervical cancer is the third most common cause of cancer-related death in women worldwide.[1] Nationwide, cytology-based cervical screening programs with a call and recall system have proven to be effective in decreasing the incidence of and mortality from cervical cancer.[2-3] However, the decrease of incidence of cervical cancer has leveled off and apparently a plateau has been reached for cytology-based screening. The main causes of this stabilization are the marked number of women (~30%) that fail to participate in the screening programme, and the relatively low sensitivity of cytological testing.[4]

Since it is known that an infection with high-risk human papillomavirus (hrHPV) is the main causative event for the multi-step process of cervical carcinogenesis, efforts to improve screening have focused on hrHPV testing as additive or primary screening tool. Unlike cytology, the hrHPV test is objective and has shown to be more sensitive for the detection of cervical intraepithelial neoplasia grade 2 or worse and grade 3 or worse (CIN2+/3+) compared with cytological testing (94% *vs* 65%).[5-6] The success of cytology-based screening programs can be attributed to relatively frequent repeat testing (in many countries every 1 to 3 years) to compensate for the low sensitivity of cytology. A meta-analysis of four randomised controlled trials[7-10] showed a 57% lower risk for CIN3+ amongst hrHPV test negative women compared with cytology test negative women.[6] In addition, a pooled analysis of these four trials revealed a 60-70% lower risk for cervical cancer in hrHPV test negative women compared to cytology test negative women.[11] Collectively, these data indicate that a hrHPV test is a more objective and more sensitive screening tool that better protects against cervical (pre)cancer than cytology. HPV testing permits extension of screening intervals resulting in less screening rounds and lower surveillance costs. Based on these data, the Ministry of Health in the Netherlands has decided to replace cytology testing by HPV testing as primary screening tool in 2016.[12] Moreover, five regions in Italy will introduce primary HPV screening in the forthcoming year, and several other developed countries such as Denmark and Sweden are considering to change their primary screening test as well. When hrHPV testing is proposed as primary screening test, it is important that a clinically validated HPV test should be used. Only HPV tests which have been proven to detect HPV infections associated with cervical cancer and its precursors (CIN2+) with low false positivity rates should be used to prevent follow-up of women with transient HPV infections resulting in unnecessary repeat tests, colposcopy referrals and overtreatment.

Another limitation of present cervical screening programs is the suboptimal participation rate. Since more than half of cervical cancers arise in women not attending cervical screening, it is important to target these non-attendees.[4] Offering self-sampling of cervico-vaginal material for hrHPV testing (HPV self-sampling) has shown to be effec-

tive to increase screening compliance: about one third of the non-attendees submit self-sampled material for HPV testing when HPV self-sampling is offered.[13] Also for HPV self-sampling, it is important to use a validated combination of self-sampling device and hrHPV test to achieve clinical equivalence in terms of CIN2+ detection of HPV self-sampling compared to physician-taken HPV testing.[14-15] Based on this statement, HPV-self-sampling could be introduced as an alternative for a physician-taken smear.

Triage of HPV-positive women

Even when a clinically validated HPV test is used, the specificity of HPV testing for CIN2+ is lower than that of cytology and the majority of women who are positive with such an HPV test will not have clinically relevant disease.[5-6] Therefore, a triage test is necessary to identify those HPV-positive women who have disease (CIN2+) and thus are in need for immediate colposcopy referral. It has been proposed that women with a >10% risk of CIN2+ should be referred for colposcopy, whereas those with a risk between 2% and 10% benefit from more intense follow-up by repeat testing within a year.[16] Women with a CIN2+ risk of < 2% can be dismissed from further follow-up until the next screening round after 3 to 5 years depending on the screening interval used. Moreover, to prevent overtreatment of HPV positive women, positive predictive values of 10% (as accepted in the US) to 20% (as accepted in the Netherlands) are used as a norm.[16, 18] Guided by the criteria listed above, several triage strategies for hrHPV positive women have been evaluated of which two were recently recommended. First, direct cytology triage at baseline with repeat cytology at 6 or 12 months, with or without HPV16/18 genotyping at baseline, was found to be the triage strategy of first choice in recent longitudinal studies.[17-19] This strategy resulted in the optimal balance between the safety of a triage strategy and the screening burden for patients and clinicians, because the low CIN3+ risk in women with two subsequent negative cytology test results was acceptable to refer them back to the national screening programme, while the colposcopy referral rate remained modest. The second strategy which was preferred in another study was HPV 16/18 genotyping in combination with cytology.[20] This strategy resulted in a relatively low CIN3+ risk (just beneath the CIN3+ threshold risk of 2%) for HPV-positive women who were tested negative for HPV16 and HPV18. However, retesting after 1 year was recommended in women with a negative triage test. Which of the above mentioned triage strategies will be used depends on the quality of cytology tests and the resources available in a specific country.[21]

Because cytology is a subjective test with a large inter- and intra-observer variability[5], there is a need for more objective and robust triage markers. Cytology can be made more objective when cells are dual stained for p16 and Ki-67 by immunohistochemistry. HPV infected cells in high-grade lesions show high p16 expression in proliferating (i.e. Ki-67 positive) cells due to viral oncoprotein E7 overexpression in these cells. Thus,

p16/Ki-67 double positive cells are indicative for the presence of CIN2+, because this combination of markers is not present in normal or reactive cervical cells. p16/Ki-67 dual-staining of cervical smears has shown promising, more objective results as triage test for hrHPV positive women.[22]

Non-morphological candidate markers include markers that detect DNA methylation of promoter regions of tumor suppressor genes involved in cervical carcinogenesis.[23] Recently, it has been shown that a molecular marker panel, targeting CADM1 and MAL genes, on physician-taken cervical smears, was equally discriminatory for the detection of CIN3+ as cytology, or cytology in combination with HPV 16/18 genotyping.[24] Moreover, methylation markers could also play an important role in triaging women with hrHPV positive self-sampled material.[25-26] Currently, a visit to the clinician is necessary for triage testing in women with a positive HPV self-sample, because cytology is not feasible on self-sampled material.[27] Since methylation marker testing is directly applicable on self-samples, this will open the possibility for full molecular screening, by offering a combination of primary HPV screening with methylation marker testing as triage tool. This could optimize screening especially among current non-attendees.

HPV testing in women treated for high-grade cervical disease

Women treated for high-grade disease retain an elevated post-treatment risk of CIN2+ for at least ten years.[28-29] Therefore, it is important to identify these women in order to repeat conservative treatment to reduce the risk of future invasive disease. Post-treatment surveillance should identify both women with residual disease as well as women with a persistent hrHPV infection who have an increased risk of progressive incident lesions.[30] In most European countries, treated women are followed-up by cervical cytology 6, 12 and 24 months after treatment. After three consecutive negative test results, women return to the screening programme where screening takes place at intervals of at maximum 5 years.

Current post-treatment protocols have several drawbacks, being the low compliance rate and the limited sensitivity of cytological screening. The current guidelines can be improved by implementing HPV testing since it is significantly more sensitive than cytology and has equal specificity compared to follow-up cytology in the detection of post-treatment CIN2+/3+.[6, 28] Thus, a positive HPV test may better identify women with an increased risk of post-treatment disease. The best results of detecting post-treatment disease have been reached by performing co-testing (both hrHPV and cytology). Women who test negative for both cytology and HPV 6 months after treatment have a low risk of developing post-treatment disease and may omit the 12-month visit. The 5-year CIN2+ risk in women with negative co-testing at 6 and 24 months post-treatment is 1.0%. This risk is similar to that of women with normal cytology in population based

screening, which indicates that these double negative tested women could safely return to regular screening.[31]

HPV testing in low- and middle resource countries

In low- and middle- resource countries cervical cancer remains largely uncontrolled because of ineffective or no screening possibilities. These countries have limited resources to provide for the costs and organisation of a screening program and, if present, cytology is of poor quality. As a result, a high incidence of and mortality from cervical cancer can be found. In addition, women in these countries are at best screened a few times per lifetime.[32] Therefore, a clinically validated and easy to perform test should be used.[33] Indeed, an Indian study showed a significant decrease of mortality from cervical cancer after one screening episode with HPV testing by HC2 compared to cytology and visual inspection with acetic acid.[34] Even more success, particularly with regard to the participation rate, might be expected when offering HPV self-sampling.[14, 35] In the Mexican Appraisal of Routine Cytology versus vaginal HPV screening (MARCH) study[36], HPV self-sampling revealed a higher sensitivity for CIN2+ compared with cytology testing. Moreover, a pooled analysis of HPV self-sampling in China concluded that 'Self-HPV testing may complement current screening programs by increasing population coverage in settings that do not have easy access to comprehensive cytology-based screening.[33] Therefore, the combination of the higher CIN2+ sensitivity in comparison with cytology and the expected higher participation rate make HPV self-sampling a good alternative to cytology in low and medium resource countries. However, implementation of HPV self-sampling in these countries should be preceded by education programs about the validity of self-collection that target general population to achieve maximum benefit.[37]

In conclusion, several studies have shown that HPV testing can replace cytology as primary screening tool in the detection of CIN2+/CIN3+ lesions. This replacement will increase the efficacy of current cervical screening programs by improving the sensitivity of regular screening. Moreover, HPV self-sampling could be used in cervical screening by increasing participation rate in developed countries and in low- and middle- resource countries lacking medical services. By the introduction of non-morphological markers, for example, methylation markers, as triage test in hrHPV positive women, full molecular screening might be feasible in the near future. Finally, the addition of HPV testing to cytology in women post-treatment, monitoring of high-grade cervical disease will result in a more efficient post-treatment follow-up, permitting fewer follow-up visits.

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Chapter 8.2

Key messages and future perspectives

PART 1: Screening

Triage of women with BMD cytology

Chapter 2 evaluates the performance of p16/Ki-67-dual-stained cytology in predicting the presence of CIN2+/3+ among women with borderline or mild dyskaryotic Pap smear results and compares the results with those obtained with HPV triage testing. Results indicate that p16/Ki-67 dual-stained cytology had a small but statistically significantly higher specificity for CIN3+ than HPV testing (64.4% and 57.6%;) and similar sensitivity (100% and 96.3%), resulting in a slightly lower colposcopy referral rate compared to HPV testing (43.6% and 49.1%). For CIN2+, there were no significant differences between triage with p16/Ki-67 dual-stained cytology and triage with HPV testing.[1]

The accuracy of p16Ki-67-dual-stained cytology to predict the presence of CIN2+/3+ among women with BMD cytology was consistent with previous studies.[2 3 4 5 6] Schmidt et al.[3] showed that p16/Ki-67 dual staining predicted CIN3+ in ASCUS cytology with a sensitivity 87.1% and specificity 85.5% and in LSIL cytology with a sensitivity 95.0% and specificity 70.7%. Taken together, present data indicate that p16/Ki-67 dual staining can be used as a triage tool to predict clinically relevant lesions among women diagnosed with BMD cytology.

Triage of HPV-positive women

In this thesis we also evaluated the effectiveness of p16/Ki-67 dual-stained cytology in HPV-positive women. HPV-positive women have a low risk of developing cervical precancer and cancer, since most HPV infections are transient. In order to avoid unnecessary referrals and over-treatment, triage testing is necessary. A number of triage strategies have been described in the literature. The presently recommended triage strategy for HPV-positive women is repeat cytology. HPV-positive women with abnormal cytology need to be directly referred to colposcopy. However, the optimal management for HPV-positive women with normal cytology is still not clear. These women have a 3-5 year CIN3+ risk of 5-10%, which is too high to delay follow-up to the next screening round in 3-5 years[7,8], but too low for an immediate colposcopy referral.[9,10] Therefore, additional triage testing of HPV-positive women with normal cytology is still necessary to prevent over-referral for colposcopy. As described before (paragraph 8.1), repeat cytology testing after 6-12 months with or without baseline HPV 16/18 genotyping seems suitable.[7,8,11] Alternative algorithms for triaging HPV-positive women are based on morphological and molecular biomarkers .[12,17]

Since promising cross-sectional and longitudinal results have been described for the use of p16/Ki-67 dual-staining, we evaluated the performance of dual-staining in HPV-positive women with normal cytology and compared the results with those obtained with HPV 16/18 genotyping (Chapter 4). Both strategies may be considered for triage

of HPV-positive women with normal cytology although they performed differently. p16/Ki-67 dual-stained cytology had a higher 3-year sensitivity of 73.3% for CIN3+ compared to HPV 16/18 genotyping (46.7%) and a lower specificity (70.0% versus 78.3%). Referral rates based on p16/Ki-67 dual-stained cytology are slightly higher compared to HPV16/18 genotyping (35.8% versus 28.0%).

In the Netherlands, a 5-year screening interval for HPV positive, triage test negative women is maintained. In addition to the cross-sectional results described before, it is therefore important to assess the 5-year CIN3+ risk of women who were HPV-screen positive, but were triage-test negative. As was shown in chapter 3, HPV-positive women with normal cytology and normal repeat-cytology after 12 months had a 5-year CIN3+ risk of 4.1%. This result is comparable to the 5-year CIN3+ risk of HPV-positive women with normal cytology and a negative baseline HPV 16/18 genotyping test (3.5%) and also comparable to the 5-year CIN3+ risk in case of a negative p16/Ki-67 dual staining (3.3%). This indicates that the three triage strategies in this study rendered a non-negligible 5-year CIN3+ risk and support follow-up within 5 years.

This is in line with other studies. Dijkstra et al[10] evaluated the long-term CIN3+ risks among HPV-positive, triage-test negative women for four different triage strategies and compared them to the CIN3+ risk of HPV-negative women with normal cytology. Their (fourteen years follow-up) data showed that regardless of the triage strategy for HPV-positive women, the long-term CIN3+ risk was as least ten times higher compared to the risk of HPV-screen negative women. Katki et al.[13] also showed that HPV-positive women with normal cytology had a considerable 5-year CIN3+ risk and thus require follow-up.

In summary, to overcome the limitations of the subjective nature of cytology reading and the need for repeat testing, p16/Ki-67 dual-stained cytology as well as HPV16/18 genotyping could be beneficial for the triage of HPV-positive women. Both strategies allow the detection of women with underlying high-grade CIN at baseline. However, repeat testing within 3-5 years remains necessary.

Future perspectives

The results of p16/Ki-67 dual-staining are promising. However, this test is still sensitive to sampling errors. Moreover, although more reproducible than cytology, interpretation is still based on morphology. The threshold for a positive test result is the presence of one p16/Ki-67 dual-stained cell. This emphasizes the need for sufficient cellularity of cytological specimens with sufficient intact abnormal cells to guarantee the clinical applicability of this triage strategy and consequently makes the interpretation of p16/Ki-67 dual-staining susceptible to subjective judgement.

Recently, several non-morphological molecular markers have been suggested to triage HPV-positive women. DNA methylation analysis of tumor suppressor genes have gained

most attention since methylation in cervical cancer has been well established and is easy to detect in histological and cytological specimens.[14, 15] Hesselink et al has validated a CADM1/MAL-methylation test as an objective triage assay for HPV-positive women in cervical screening.[14]

The feasibility of methylation analysis in real-life screening was examined in a large randomized controlled trial in which cytology triage was compared to triage by the bi-marker panel MAL/miR124 methylation test on a self-collected sample. Results showed similar sensitivities for CIN2+[15] and a slightly lower specificity for methylation testing. This study was followed by, Luttmer et al., who demonstrated that promoter methylation analysis of FAM19A4 performs at least non-inferior to cytology for the detection of CIN3+ in HPV-positive women aged ≥30 years from a gynecological outpatient population.[16]

Further, recently published studies show that the extent of DNA promoter methylation of three genes (i.e. CADM1, MAL and FAM19A4) increases with the severity and duration of cervical disease. These epigenetic changes are thought to reflect the presence of a more advanced high-grade CIN lesion with a longer duration of existence.[17] Methylation levels were found to be exceptionally high in cervical cancer[18,19], resulting in a very high sensitivity (>99%) for cervical carcinomas of methylation assays targeting these genes. It can therefore be concluded that such markers are particularly effective in detecting cervical cancer and high grade CIN with a high short term risk of cancer[17,20] (advanced CIN3) and should therefore be treated.

Post-treatment considerations

Post-treatment disease develops in 4-17%[21,22,23] of women treated for high-grade cervical disease. Although most post-treatment disease is detected within 2 years,[24,25,26] the risk is increased for a longer period.[22,27,28,29] Post-treatment disease represents a heterogeneous group, comprising persistent lesions resulting from residual (i.e. incompletely treated) disease with persistence of the same HPV genotype and incident (i.e. early onset) lesions. Post-treatment surveillance should ideally differentiate residual from incident disease and also identify women with a persistent HPV infection. In most European countries, post-treatment women are now monitored with cervical cytology at 6, 12 and 24 months post-treatment. After 3 consecutive negative smears, women return to the screening programme.[30] Limitations are the limited sensitivity of cytology and loss to follow-up.[31] To overcome these drawbacks, the implementation of new techniques is considered.

In line with the implementation of HPV testing in population-based screening, the use of HPV testing in post-treatment monitoring has been studied extensively.[25,32,33,34,35,36,37,38,39,40] Our meta-analysis (Chapter 5) confirmed that HPV testing post-treatment is 15% more sensitive than cytology at the cost of a non-significant 5% decrease in specificity. This result supports a strategy of using HPV testing in post-treatment follow-up. Recently, as

we suggested in our proposed protocol, the Dutch Society of Obstetrics and Gynaecology has incorporated HPV testing in post-treatment surveillance.[41]

In Chapter 7 we evaluated the performance of the bi-marker CADM1/MAL methylation test as a potential tool for monitoring women with post-treatment disease with the aim to discriminate between residual and incident disease. It showed that all post-treatment CIN3 were caused by a persistent (and consequently longer existing) HPV infection and 64% of these lesions were methylation positive (including all 3 carcinomas detected after re-treatment). Additionally, all incidental post-treatment CIN2 that showed a type switch compared to the primary lesion were methylation negative. Combining baseline and longitudinal follow-up results further underlines the concept; a positive methylation status at follow-up is strongly predictive for underlying CIN3+. Nonetheless, a few recurrent CIN2 and CIN3 with a persistent HPV infection remain undetected by the CADM1/MAL methylation test, whereas cytology detected virtually all CIN3. Possible reasons for non-detection of lesions by methylation testing were : i) inadequate sampling of the cervical scrapes as cytology and methylation testing were usually conducted on different cervical samples, and ii) some recurrent CIN2/3 were in fact incident early lesions caused by a reinfection of the cervical epithelium with the same HPV genotype that was present in the primary lesion.

There is increasing evidence that cytology has a better sensitivity than methylation testing for CIN2 whereas the reverse holds for advanced CIN3 and cancer.[17,42] Therefore, DNA methylation analysis is promising supplementary triage test for detection of lesions with a high short-term risk of progressing to cancer that are missed by cytology In settings where cytology performance and infrastructure is less adequate than in The Netherlands, methylation triage testing may be considered not in conjunction but in place of cytology.

Besides being an objective biomarker, an additional, potential advantage of methylation testing is that it can be conducted on self-collected cervico-vaginal specimens. This can simplify the follow-up procedure[15,17,43] and may reduce loss to follow-up which is currently 15-40% of treated women. These options need to be further analyzed in longitudinal implementation studies and cost-effectiveness analyses.

Overall, prevention of cervical cancer is of great importance. Despite prophylactic vaccination and better screening techniques, there is no flawless instrument to prevent cervical cancer. In the past two decades, a better insight into cervical cancer development has offered opportunities to identify novel, morphological and molecular biomarkers. These new biomarkers are useful in screening, diagnosis and post-treatment surveillance and should be further evaluated in the near future.

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