Chapter 9
Summary and future perspectives
Summary

Cervical cancer represents an important health issue. It is the third most common female malignancy and the fourth leading cause of cancer death in women worldwide. National population-based cervical cancer screening programs have reduced the incidence of cervical cancer significantly in the western world. However, there is room for improvement in the technical aspects and performance of the different screening tests, participation rates of the target screening populations, compliance to follow-up and the efficacy of treatment of cervical neoplasia.

Until now, the most widely used cervical cancer screening test is cytology-based. However, methodology for primary screening for cervical cancer is currently changing in many countries, including the Netherlands. HR-HPV testing is becoming the preferred primary screening test over cytology. By HR-HPV testing the sensitivity for detecting high-grade (pre)malignant cervical lesions (CIN2+) is much higher. Because of this increased sensitivity, more CIN2+ lesions will be detected and less cervical cancers will be missed. A disadvantage of the HR-HPV test for primary screening however is the lower specificity, due to the unavoidable identification of women with transient HPV infections that will not develop into CIN2+ lesions. To prevent unnecessary referral to gynecologists and associated high costs, there is an urgent need for risk stratification by triage testing of HR-HPV positive women after population-based primary screening.

A screening test based on detection of DNA methylation may further increase the efficacy of current population-based cervical cancer screening programs. DNA methylation of the promoter region of genes is an early event in cervical carcinogenesis. Quantitative methylation specific PCR (QMSP) is a specific and sensitive method that allows high throughput analysis, which makes it suitable as a potential screening tool for (premalignant) cervical neoplasia. Different studies explored DNA methylation markers as a screening test for the detection of (pre)malignant cervical neoplasia. In chapter 2, a systematic literature search was performed to summarize studies analyzing methylation markers in cervical scrapings by QMSP in different patient groups. In this review, 37 studies describing 61 genes were selected for analysis and data were stratified for studies using methylation markers as a primary screening test versus studies using methylation markers as a triage test in HR-HPV positive women. Methylation analysis as primary screening test for detection of CIN2+ revealed 6 genes (EPB41L3, HS3ST2, JAM3, NKX6, SOX9, and ZNF582) with relatively high sensitivity (49%-93%) and specificity (67%-100%). Methylation analysis as a primary screening test for detecting cervical cancer revealed 4 genes (PAX1, CCNA1, EPB41L3, and JAM3) with consistently high sensitivity (72%-100%) and specificity (82%-100%). Methylation analysis used as a triage test in HR-HPV positive women revealed 6
genes (DKK3, SFRP2, MAL, CADM1, JAM3 and EPB41L3) that showed a combined sensitivity and specificity for the detection of CIN2+/CIN3+ lesions comparable or higher than for other triage strategies known in literature such as p16INK4a immunohistochemistry, HPV16/18 genotyping and cytology testing. There is a need for standardization of the current approaches to validate the diagnostic potential of new methylation markers. Furthermore, well-performing, reproducible methylation markers from literature should be selected and prospectively evaluated in population-based cohorts.

Since cervical cancer screening in many countries, including the Netherlands is going to change to primary hrHPV screening, the performance of the hrHPV test is of great importance. Multiple hrHPV tests are now available and in order to have a reliable hrHPV test, it is important that the test meets certain requirements. In chapter 3, we demonstrate that the widely-used Cervista HPV HR test fulfills the cross-sectional clinical performance and reproducibility criteria of international guidelines for HPV test requirements for primary cervical cancer screening in women 30 years and older. Both the relative clinical sensitivity and specificity were non-inferior to that of the well known Hybrid Capture 2 (HC2) test (non-inferiority score tests, $P=0.043$ and $P<0.0001$, respectively). Intra- and inter-laboratory agreements were 92% (kappa=0.83; $P<0.001$) and 90.4% (kappa=0.80; $P<0.001$), respectively. Although, the Cervista HPV HR test fulfilled the clinical performance and reproducibility criteria and was non-inferior to the HC2 test, Kinney et al. signaled that the Cervista HPV HR test was 2-4-fold more likely to give positive HPV test results in women >30 years with normal cytology compared to the HC2 test, suggesting that the Cervista HPV HR assay is significantly less specific than the HC2 assay. Other studies did not reflect this opinion. In chapter 4, the diagnostic performance of the Cervista HPV HR test is compared to the HC2 test in a Dutch population-based cervical cancer screening program. We included 900 scrapings of women with normal cytomorphology; specificity was 90% (95%CI: 87.84-91.87) for the Cervista HPV HR test and 96% (95%CI: 94.76-97.37) for the HC2 test with 93% agreement between both tests (kappa=0.5, $p<0.001$). The detailed analysis of the discordant cases revealed that most (57/60) tested true HPV negative with PCR-based HPV assays; of these cases 56% were defined as Cervista triple-positive, because the FOZ (fold-over-zero) value of the 3 mixes exceeded the second cut-off of 1.93 (as set by manufacturer). We show that when the second cut-off was set at 5.0, specificity improved significantly without affecting sensitivity. External validation of this new cut-off at 5.0 in triple-positive scrapings of women selected from a multisite population-based cross-sectional study (SHENCCASTII) revealed that 22/24 histological normal cases tested HPV-negative in the Cervista HPV HR test, while CIN2+ lesions remained HPV-positive. Both the inter- and intra-laboratory reproducibility of the Cervista HPV HR test improved when using this new cut-off set at 5.0. Therefore, we concluded that the specificity of the Cervista HPV HR test could significantly be improved by increasing the
second cut-off from 1.93 (default setting) to 5.0, without affecting the sensitivity of the test in a population-based screening setting.

As we show in our systematic review, described in chapter 2, the search for methylation markers with both high sensitivity and specificity for detection of CIN2+ lesions needs to be continued. Therefore in chapter 5 innovative genome-wide methylation analysis is used to identify new methylation markers for high-grade cervical intraepithelial neoplasia (CIN2/3). Enrichment and capturing of methylated DNA from normal cervices and CIN2/3 lesions followed by next-generation sequencing (MBD2-seq) was performed to identify differential methylation regions (DMRs). The top 15 highest ranking differentially methylated genes were selected and validated by MSP in two steps: on the same DNA used for MBD2-seq and on DNA samples from an independent patient cohort with (pre)malignant cervical neoplasia. For further diagnostic evaluation, the best differentiating methylation markers were tested with quantitative MSP (QMSP) in cervical scrapings from 2 cohorts: 1) cervical cancer patients vs. healthy controls and 2) patients referred from population-based screening with an abnormal Pap smear. With genome-wide MBD2-seq, 176 DMRs comprising 163 genes were identified. After verification and validation of the top 15 genes with MSP, 9 genes showed significant differential methylation in normal cervices versus CIN2/3 lesions (p<0.05). Subsequently, methylation levels of 8/9 genes were significantly higher in carcinoma compared to normal scrapings. In scrapings from patients with abnormal cytology for all 8 genes methylation levels increased with the severity of the underlying histological lesion. In addition to the 8 new genes, also our previous four-gene panel (C13orf18, JAM3, EPB41L3 and TERT) was analyzed. The combination AL590705.4/EPB41L3/JAM3 and AL590705.4/C13orf18/JAM3 revealed equally high sensitivity for CIN2+ (74-76%) compared to hrHPV testing (79%), while specificity was significantly higher (71-76%) compared to hrHPV testing (42%) (p≤0.05). With this genome-wide DNA methylation analysis we identified new CIN2/3 specific methylation markers. The diagnostic performance of our new methylation panel shows comparable sensitivity to hrHPV testing for CIN2+, but with higher specificity to prevent referral for unnecessary colposcopy. The next step before implementation in primary screening programs will be validation in population-based cohorts. In addition to improvement of the efficacy of current cervical cancer screening methodology, the low participation rate is another aspect of population-based screening programs for cervical neoplasia that could be improved 13. In the Netherlands around 35% of women are so-called non-responders and do not show up after the screening invitation. Reaching out to these non-responders is important, as half of all cervical cancers are found in the non-responder group 14,15. Introduction of self-sampling methods for hrHPV testing increased participation up to 39% for these non-responders 16. Studies have shown that detection of hrHPV in self-sampled specimens is feasible and shows similar test accuracy in detecting CIN2+ lesions compared to physician-taken cervical scrapings 16-18. However,
due to the relatively low specificity of the hrHPV test, an independent triage test is necessary. In chapter 6, our study is described in which non-responding women from population-based screening were invited to self-collect a cervico-vaginal specimen for hrHPV testing and hrHPV positive women were referred to a physician for triage liquid-based cytology. In the physician-taken triage material, DNA methylation analysis of C13ORF18, JAM3, EPB41L3 and TERT was compared with cytology in 128 hrHPV positive women. DNA methylation analysis of JAM3 showed the highest combined specificity (88%) and sensitivity (82%) for detection of CIN3+, whereas cytology showed a specificity of only 48% and a sensitivity of 91%. Out of 39 women with abnormal cytology and normal histology (false-positive by cytology), 87% were negative for JAM3 and 90% for C13ORF18 methylation. In addition, feasibility of DNA methylation analysis directly on brush-based self-sampled specimens was assessed. Agreement between DNA methylation analysis performed directly on the matched self-sampled material and physician-taken samples was 88% for JAM3 (κ=0.75, p<0.001) and 90% for C13ORF18 (κ=0.77; p<0.001). From this study we conclude that DNA methylation analysis as a triage test in hrHPV positive women is an attractive alternative to cytology. Furthermore, direct triage on self-sampled specimens could optimize the screening program, as this would eliminate the need for an additional physician-taken scraping.

Apart from optimizing screening strategies for cervical cancer, there is also ample room for improvement of different aspects of current standard of care of cervical cancer patients. In advanced stage cervical cancer locoregional recurrence remains a frequent cause of death after (chemo)radiation. For these patients the value of routinely performing adjuvant post radiation hysterectomy has been debated. Early selection of patients with residual disease who are most likely to benefit from salvage surgery has been suggested. To identify these patients with central residual disease an examination under anesthesia with cervical biopsy 8-10 weeks after completion of (chemo)radiation has routinely been performed at our institution since 1994. The high frequency of locoregional recurrences in the operated patients (46%) suggested that the number of patients who may be salvaged might increase if more extensive surgery was performed. In chapter 7, we show that isolated residual central disease, detected 8-10 weeks after (chemo)radiation is a strong negative prognostic factor (HR 3.59; 95%-CI: 2.18-5.93, p<0.001). More extensive salvage surgery did not improve locoregional disease-free survival nor disease-specific survival, but did significantly increase treatment-associated morbidity and should not be recommended.

Currently, primary (chemo)radiation is considered the standard treatment for cervical cancers FIGO stage 2B and higher. However, response to (chemo)radiation varies in patients. During chemoradiation autophagy has been related to DNA damage response in multiple
tumor models. In chapter 8, we describe in cervical cancer the role of ATG13-mediated autophagy in response to irradiation. In a large series of pre-treatment primary cervical cancer tissue, high punctate staining of ATG13, representative for autophagy, was observed in 51% (136/268) of the cervical cancer patients using immunohistochemistry. ATG13 puncta and LC3 puncta were also detected in HPV-positive HeLa and SiHa cervical cells. Irradiation induced the formation of GFP-LC3 positive autophagosomes in HeLa-GFP-LC3 cells. Bafilomycin-A1 treatment caused an enhanced accumulation of irradiation-induced autophagosomes, demonstrating the presence of an autophagic flux in these cells. Thus we demonstrate that ATG13 plays an essential role in irradiation-induced autophagy. Inhibition of the autophagy machinery sensitized cervical cancer cells to irradiation, which is an interesting strategy to be further explored for the treatment of cervical cancer.
Cervical cancer screening methodology is in transition. The currently most widely used cytology-based test has several limitations, as it is based on subjective interpretation of morphological alterations in exfoliated cervical cells. Moreover, it has a relatively low sensitivity for detecting CIN2+. We are now facing a shift towards primary cervical cancer screening by molecular testing for the presence of hrHPV. Since the sensitivity of the hrHPV test is much higher, more CIN2+ lesions will be detected, offering women better protection in less screening rounds. However, the lower specificity of the hrHPV test compared to cytology-based testing will cause excess false-positive tests especially among young healthy women, which will lead to unnecessary clinical management, associated higher costs and emotional burden for these women. Therefore, a triage test for hrHPV positive women is necessary. Based on sensitivity (~60%) and specificity (~90%) results from literature, the cytology-based test is currently advocated as the most optimal triage test. However, these sensitivity and specificity results for triage testing with cytology are based on test results where the cytotechnician was not informed about the HPV status of the patient. Recent studies have shown that knowledge of HPV status of a sample by the cytotechnician leads to a higher frequency of cytological abnormal (≥ASCUS) scores in HPV positive samples, consequently resulting in a higher referral rate to gynecologists. Therefore, there is a need for objective markers that preferably can be determined in the same sample as used for hrHPV testing.

In this thesis we show that DNA methylation markers might serve as the next step in cervical cancer screening. Both our previous and current studies as well as studies by other groups indicate a variety of markers of which the methylation levels increase with the severity of the underlying CIN lesion. Currently, there are no suitable single methylation markers for primary cervical cancer screening yet. However, combining different genes from literature and testing these in large population based screening cohorts might result in a methylation panel with equally high sensitivity (90-95%) as hrHPV testing, but with higher specificity (≥85%). There might be some concerns about the costs, comparable to debates on costs of implementing hrHPV testing. With QMSP, high-throughput analysis can be performed and by finding a methylation panel with both high sensitivity and high specificity, costs of unnecessary referrals will diminish in the end leading in higher cost-effectiveness.

Even more interesting and probably more feasible to implement in practice in the near future is the role of methylation markers as a triage test in hrHPV positive women. The currently tested methylation markers in literature all show high specificity, but this is merely due to the use of different cut-off values. Furthermore sensitivity for the detection of CIN2+...
lesions is still not high enough. For biomarker validation in cervical screening, Pepe et al. recommended a five-phase framework with preclinical exploratory studies, assessment in non-invasive samples, retrospective longitudinal studies, prospective screening studies and prospective intervention studies. At present most methylation markers have not been tested according to all five phases. Until now, only one randomized controlled non-inferiority trial was performed, but in this trial only two methylation markers were evaluated. Besides test performance also reproducibility is important. In order to ensure high quality of the described methylation markers, intra- and inter reproducibility of selected markers needs to be analyzed in future studies. Another advantage of methylation markers is that, in contrast to cytology, methylation markers are directly applicable to self-collected samples. This will improve population-based screening by reaching the non-responders.

The prevalence of CIN lesions and cervical cancer will decrease, because of primary prevention of cervical cancer by prophylactic HPV vaccinations. In 2009 the vaccine Cervarix® against hrHPV 16 and 18 was introduced in the Netherlands. Although, primary prevention by HPV vaccination will contribute to an important decrease in the prevalence of (premalignant) cervical cancer, secondary cervical cancer screening remains important, as there are some caveats for HPV vaccination. First, it will take 10-15 years before the effect of vaccination on cervical cancer prevalence will be seen. Second, not all 12 year old girls are vaccinated, since only 50-60% of the girls show up for the vaccination program in the Netherlands. Concerns about long term side-effects, parental refusal or religious beliefs are all factors that influence participation rates in the vaccination program. Third, immunization will fail in some women and women will be only protected against HPV 16 and 18, although cross-protective efficacy against four other HPV types has been shown. Finally, available prophylactic vaccines are indeed exclusively prophylactic and have no therapeutic effect in clearing HPV infections that are already present. Therefore, it is safe to conclude that secondary prevention by means of cervical cancer screening remains important for many years from now. In fact, improving the current efficacy of screening becomes even more important as screening for a disease with a lower prevalence by definition will increase the number of false positive results. A screening test with high specificity becomes therefore more important than ever.

A different aspect of cervical cancer screening is the age at which women are invited to be screened for the first time. In the Netherlands women will be first invited to attend screening at the age of 30. This policy was based on the estimation that progression from CIN to cervical cancer generally takes 10-15 years and that the mean age of first sexual intercourse, when the risk of contracting a genital HPV infection was highest, was around 20 years. Currently, the mean age of first sexual intercourse of girls in the Netherlands has decreased to around 17 years. Therefore, the onset of cervical cancer screening at the age of 30 might be relatively late. In a study by De Bie et al. it was reported that one-
third of the women diagnosed with cervical cancer was under the age of 30, of which 80% was between 27 and 30 years of age. Therefore, lowering the age for the first invitation for screening from 30 to 27 years might prevent cervical cancer also in this age group. The problem however in this young population is that many women will have a transient HPV infection resulting in an increase of false-positive hrHPV tests. Again by means of a highly specific triage test this problem can be overcome.

In summary, primary HPV screening for cervical cancer is in the implementation phase in many countries world-wide. A highly sensitive and specific triage test by means of methylation markers is on the brink of being developed. Many possible methylation markers have been discovered and now the time has come for independent and population-based validation of these markers to develop a uniform, reproducible and highly efficient screening test.
Reference list

SUMMARY AND FUTURE PERSPECTIVES


