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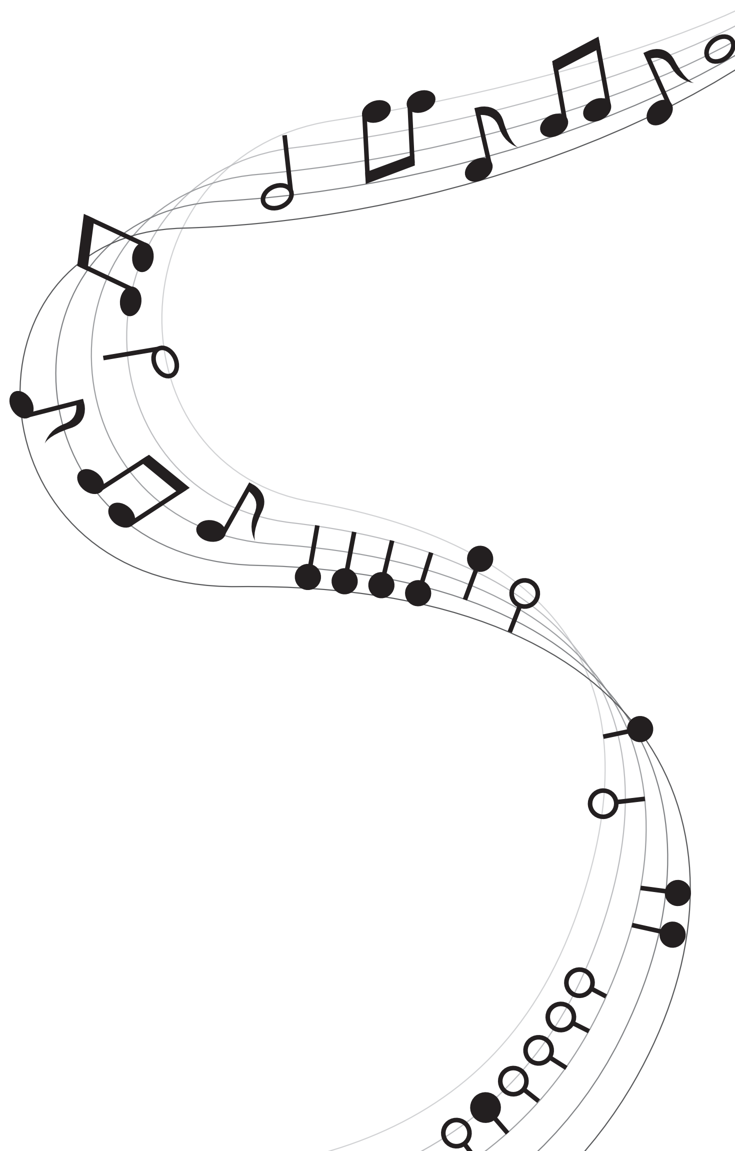
Chapter 9

Summary



SUMMARY

Cervical cancer is the fourth most common female malignancy worldwide. However, the development of cervical cancer can be prevented by effective screening and prophylactic HPV vaccination. In the Netherlands, a nation-wide cytology-based cervical screening programme for women aged 30-60 years, and prophylactic HPV vaccination for girls aged 12 years (since 2009) are in place. The current Dutch screening programme will be renewed in 2016 by implementation of primary HPV testing and offering self-sampling to non-responders. The efficacy of primary HPV screening has been compared with primary cytology screening in several randomized controlled trials, showing that primary HPV screening has a higher sensitivity for detecting high-grade cervical precursor lesions (CIN2/3) and cervical cancer. Also, HPV testing allows offering self-sampling to increase the screening attendance. It is anticipated that the higher sensitivity of the new screening test together with the attraction of more women into the screening programme will yield additional health gains. However, as most high-risk HPV (hrHPV) infections are transient and will clear spontaneously, triage of hrHPV-positive women is required to detect those women with clinically meaningful lesions thereby preventing over-referral and overtreatment and keeping burden and costs within acceptable limits.

In the new Dutch screening programme, repeat cytology triage (i.e., at baseline and at 6 months in case of normal cytology at baseline) will be implemented. Cytology triage requires repeat testing to gain sufficient protection against high-grade disease and cervical cancer, which has shown to be associated with loss to follow-up. Furthermore, the quality of cytology is, given its subjective nature, rather variable and the fear exists that prior knowledge of hrHPV-positivity in an HPV-based screening setting may lead to more false-positive cytology results and consequently more referrals than expected on forehand. In addition, this triage assay is not reliably applicable to self-samples, requiring these women whenever having an hrHPV-positive self-sample to visit the physician for an additional scrape.

This thesis presents recent work evaluating DNA methylation markers as candidate triage tests for hrHPV-positive women. It elaborates on methylation of the promoter regions of the tumour suppressor genes *CADM1, MAL, mir124-2,* and *FAM19A4*, which provide highly appealing disease markers for hrHPV-positive women. The studies described herein could contribute to a more objective manner of cervical cancer screening, both applicable to physician-taken scrapes and self-collected samples.

Chapter 1 provides a general introduction on the epidemiology, molecular pathogenesis and prevention strategies of cervical cancer.

In **Chapter 2,** we described the step-by-step development of a multiplex quantitative methylation-specific PCR (qMSP) that allows the simultaneous detection of the methylation status of several genes. Most studies investigating DNA methylation analysis as triage test, have suggested that combinations of methylation markers are required to reach an optimal CIN2/3+ sensitivity, and multiplex tools therefore would save time and input material. A multiplex qMSP assay for *CADM1, MAL, mir124-2* and the reference gene ß-actin *(ACTB)* was designed, taken into account primer and probe design and PCR reagents, to allow optimal target amplification. The resulting multiplex qMSP showed similar analytical performance as the singleplex qMSPs on serial dilutions of methylated DNA spiked with unmethylated DNA as well as in cervical scrapes. To conclude, this study indicated that multiplex qMSP offers a promising approach for high-throughput diagnostic analysis of the methylation status of multiple genes, which after proper design and validation can be equally specific, sensitive and reproducible as its singleplex reactions.

In **Chapter 3**, we evaluated the performance of bi-marker *CADM1/MAL* methylation analysis of hrHPV-positive cervical scrapes to that of baseline triage by cytology, and also assessed the combination of both tests for the triage of hrHPV-positive women. Whereas *CADM1/MAL* methylation analysis had equal sensitivity for CIN3+ in hrHPV-positive cervical scrapes as cytology at the same specificity, this study importantly showed that both assays in part detect different lesions. Cytology detected with a similar, moderate sensitivity, cellular abnormalities present in CIN2, CIN3 and cancer, whereas the sensitivity of DNA methylation marker analysis increased with the severity of the disease, resulting in a high sensitivity for CIN3+ lesions, ultimately detecting all carcinomas. Accordingly, a combination of both tests resulted in substantially higher CIN2/3+ sensitivities compared to sole baseline cytology triage at a slight decrease in specificity. The combination of both triage tests could therefore serve as an attractive baseline triage strategy for hrHPV-positive women that without a follow-up scrape reduces the risk of missing cervical cancers and advanced high-grade precursor lesions.

In **Chapter 4**, we further evaluated the clinical performance of multiplex *CADM1/MAL/mir124-2* methylation analysis on large series of cervical scrapes from women with cervical and endometrial cancer. DNA methylation analysis of *CADM1/MAL/mir124-2* detected all scrapes of women with cervical cancer (100%). As such, it was further substantiated that methylation analysis had a high reassurance for the detection of cervical cancer in hrHPV-positive women. In addition, methylation analysis showed the capacity to broaden its use on cervical scrapes through the detection of a substantial subset of endometrial carcinomas (76%). Furthermore, this chapter evaluated *CADM1/MAL/mir124-2* methylation in cervical scrapes of women with CIN3, CIN2 or without evidence of CIN2+, and showed that not only the overall methylation positivity, yet also the number of methylated genes increased proportionally to lesion severity.

In **Chapter 5**, we compared the clinical performance of *FAM19A4* methylation analysis and cytology for triage of hrHPV-positive women on cervical scrapes. Using a training-validation set approach, a *FAM19A4* qMSP was generated that performed with a CIN3+ sensitivity of 75.8% (95% CI: 61.1-90.4) at 67.0% (95% CI: 60.3-73.8) specificity in hrHPV-positive cervical scrapes, equalling cytology triage. Next, the validated assay was evaluated in an independent series of hrHPV-positive cervical scrapes in relation to severity and duration of the underlying lesion. Therefore, scrapes from women with cervical cancer, and women with CIN2/3 with a previous hrHPV infection (PHI) of <5 years or ≥5 years, were used. PHI was used as proxy of lesion duration, and accordingly, CIN2/3 lesions were assigned as early and advanced CIN2/3 lesions, respectively. The validated *FAM19A4* qMSP detected all cervical carcinomas (22/22), while cytology detected 86.4% (19/22). Although *FAM19A4* methylation analysis performed equally well for the overall detection of CIN2/3 lesions as cytology (77% (37/48) versus 75% (36/48)), advanced CIN2/3 lesions were more likely detected by *FAM19A4* methylation (100% (29/29) versus 83% (24/29)), and early CIN2/3 lesions by cytology (42% (8/19) versus 63% (12/19)). This study substantiates our earlier findings that cytology and methylation markers in part detect different lesions. It can be concluded that *FAM19A4* is a very attractive triage marker for hrHPV-positive women with an overall equal performance for CIN3+ detection than cytology, yet with a higher reassurance that no carcinomas or advanced cervical disease are missed among hrHPV-positive women.

In **Chapter 6**, we further compared the clinical performance of the *FAM19A4* qMSP to cytology either or not combined with HPV16/18 genotyping on cervical scrapes for the detection of CIN3+ in hrHPV-positive women. For this purpose, a prospective observational multi-center cohort study among hrHPV-positive women aged 18-66 years, visiting a gynaecologic outpatient clinic was performed. It was found that the performance of *FAM19A4* methylation analysis was significantly influenced by age of the women. In women aged ≥30 years (cervical screening target), methylation analysis by *FAM19A4* showed to be non-inferior to cytology for the identification of CIN3+ lesions, yet revealed higher specificities. Therefore, triage by *FAM19A4* methylation analysis was suggested as attractive alternative for women aged ≥30 years visiting an outpatient clinic. In women aged <30 years, *FAM19A4* methylation analysis resulted in a lower sensitivity and higher specificity for detection of high-grade CIN than cytology. HrHPV prevalence in young women is high, most infections are transient and most lesions regress spontaneously, contributing to a very low cancer incidence in this age group. Our findings may contemplate that *FAM19A4* methylation analysis in young hrHPV-positive women might be more efficient than cytology testing, since only the more advanced lesions are detected. These insights would provide an interesting management strategy for young hrHPV-positive women visiting a gynaecological outpatient clinic, given possible treatment morbidity such as cervical insufficiency, and associated risk for pre-term delivery.

In **Chapter 7**, we assessed the performance of *FAM19A4/mir124-2* methylation analysis for hrHPV-positive women on lavage- and brush-collected self-samples. For this purpose, self-collected samples from hrHPV-positive women participating in the PROHTECT studies (non-responders of cervical screening) were used. Following a training-validation set approach, DNA methylation analysis of *FAM19A4/mir124-2* showed a high and similar detection rate for CIN3+ in both self-samples types. More specifically, a CIN3+ sensitivity of 70.5% (95% CI: 60.4-80.6) at a specificity of 67.8 (95% CI: 62.7-73.0) was obtained in hrHPV-positive lavage self-samples. In the brush self-samples, a sensitivity of 69.4% (95% CI: 58.8-80.1) at a specificity of 76.4% (95% CI: 70.2-82.6) was reached. Accordingly, *FAM19A4/mir124-2* methylation analysis may serve as a universal triage assay for hrHPV-positive self-samples. Nonetheless, part of CIN2 and few of CIN3 lesions is likely to remain undetected when using a methylation marker-based triage strategy. Based on our previous work, these lesions are probably early-onset lesions with a low cancer progression-risk. However, as long as the progression risk of methylation-negative CIN2/3 lesions is unknown, combined molecular triage by methylation marker analysis and HPV16/18 genotyping may be considered for triage of women with an HPV-positive self-sample to increase the sensitivity for early-onset CIN2/3. Indeed, combining *FAM19A4/mir124-2* methylation analysis with HPV16/18 genotyping showed significantly higher sensitivity at lower specificity. Direct molecular triage testing of hrHPV-positive self-samples, independent of the collection device used, can optimize the screening programme, by obviating the need for a cytology triage visit to a physician. This strategy is especially attractive for non-responders of the screening programme.

Finally, in **Chapter 8**, we provided the clinical indications of our findings and possible avenues of future research.