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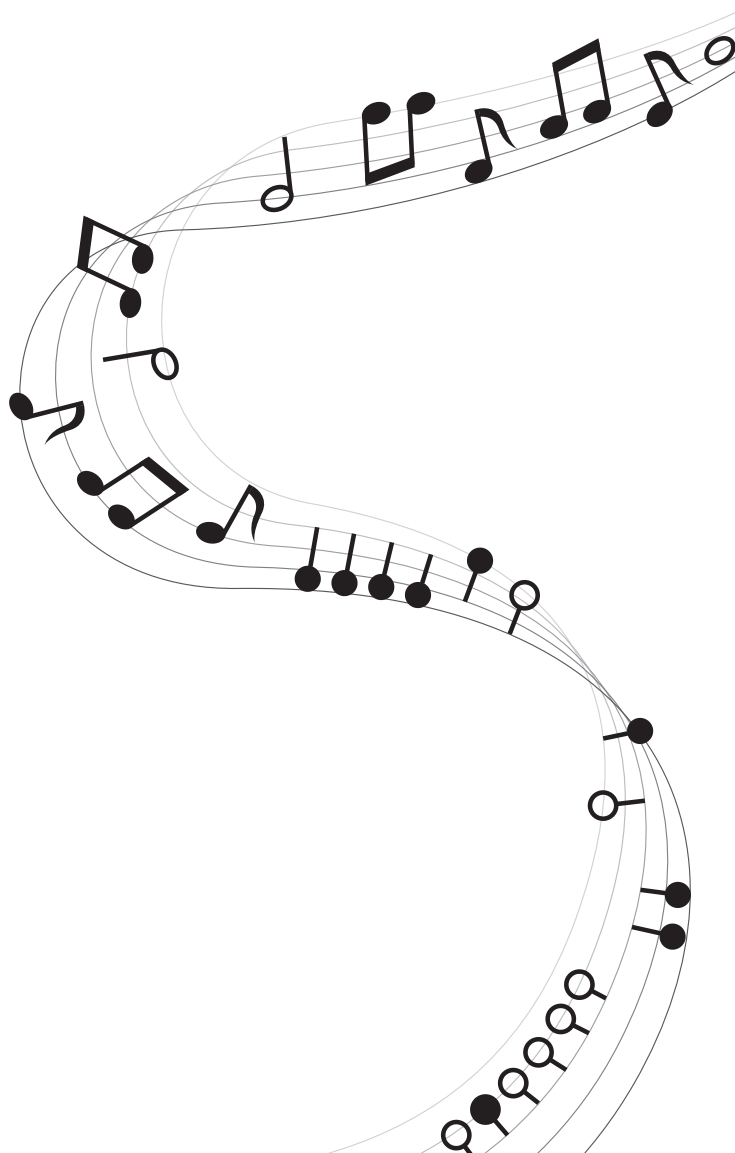
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Chapter 8

General discussion and future perspectives



METHYLATION MARKERS FOR THE MANAGEMENT OF HRHPV-POSITIVE WOMEN

In this thesis, we focused on the use of DNA methylation marker analysis of several host cell genes (*CADM1*, *MAL*, *mir124-2* and *FAM19A4*) in both physician-taken cervical scrapes and self-collected (cervico-)vaginal specimens aiming to improve cervical cancer prevention and the clinical management of hrHPV-positive women. In this chapter, the clinical implications of our findings and possible avenues of future research are discussed.

The progression from cervical precursor lesions to invasive cervical cancer is a long-lasting process that lasts over 15 to 30 years¹. This process is believed to depend on the accumulation of various crucial genetic and epigenetic alterations next to a persistent, transforming hrHPV infection²⁻⁵. Indeed, a longer duration of a preceding hrHPV infection, which can be used as a proxy for duration of lesion existence, is associated with increased levels of chromosomal aberrations in high-grade CIN lesions⁶. In this context, it has been proposed that these (epi)genetic alterations can be used for a molecular classification of cervical precursor lesions, on top of the current morphological (histological) classification (i.e., CIN1, CIN2 and CIN3)². In this concept, CIN1 and CIN2 lesions associated with viral reproduction (productive CIN), known to have a high regression rate, are distinguished by molecular means from CIN2 and CIN3 representing viral transformation (transforming CIN). Transforming CIN could, in turn, be subdivided by the level of (epi)genetic alterations into early (i.e., low levels of molecular aberrations) and advanced transforming CIN (i.e., high level of molecular aberrations), with low and high short-term progression risks for cancer, respectively². Consequently, increased levels of host-cell DNA alterations in cervical scrapes or self-samples of hrHPV-positive women may be seen as a proxy of the severity of the underlying disease. Indeed, methylation levels of host cell genes *CADM1* and *MAL*, which are functionally involved in cervical carcinogenesis, increase proportionally to the degree and duration of existence of underlying cervical disease⁷ and reach very high levels in cervical carcinomas. Accordingly, detection of increased DNA methylation levels might be used for clinical management of hrHPV-positive women.

In this thesis, we have evaluated the clinical performance of DNA methylation marker analysis against classical histological endpoints (i.e., CIN2+, CIN3+ and cancer) in both cervical scrapes and self-samples of hrHPV-positive women. Additionally, whenever longitudinal hrHPV data were available, CIN2/3 lesions were sub-classified according to their known duration of preceding hrHPV infection (PHI) into early transforming CIN (i.e., with <5 years PHI) and advanced transforming CIN (i.e., with ≥5 year PHI), to get an impression about the clinical performance of DNA methylation marker analysis according to cancer progression risk.

Physician-taken cervical scrapes (women of screening age)

From mid-2016, Dutch screening participants (≥ 30 years old) with a hrHPV-positive physician-taken cervical scrape will be triaged by cytology⁸. However, cytology triage has several drawbacks including the need for repeat cytology testing^{9,10}, a subjective test result^{11,12} and a limited sensitivity for AdCAs¹³. Therefore, alternative triage tests for hrHPV-positive women are warranted. In Chapter 4 and Chapter 5, we show that on cervical scrapes of hrHPV-positive women, methylation marker analysis by the *CADM1/MAL/mir124-2* multiplex assay and the *FAM19A4* assay, respectively, have a 100% detection rate of cervical carcinomas, including all AdCAs. The overall clinical performance of both assays in terms of CIN3+ sensitivity and specificity equals that of cytology triage. In line with above concept, advanced transforming CIN2/3 lesions are more likely detected (100%) than early transforming CIN2/3 lesions (42.1%) by the *FAM19A4* assay (Chapter 5).

Since both methylation marker assays show a high reassurance of not missing cervical cancer and advanced disease, methylation marker analysis may in the future be considered as management tool for women with a hrHPV-positive cervical scrape. In line with a strategy of cytology triage of hrHPV-positive women, hrHPV-positive, methylation-positive women should be referred for colposcopy because of a high probability of advanced transforming CIN2/3 or cervical cancer. HrHPV-positive, methylation-negative women could be offered a repeat HPV test after 12-18 months and referred in case of a positive test at that occasion. Given that triage of hrHPV-positive women by methylation analysis has overall the same sensitivity for CIN3+ as cytology, but detects more advanced CIN2/3 lesions than cytology, it can be reasoned that the (pre)cancer risk of hrHPV-positive, methylation-negative women is not higher and probably lower than for hrHPV-positive, cytology negative women. As long as the risk of hrHPV-positive, methylation-negative women for the development of (pre)cancer is not fully elucidated, this strategy is useful for women who prefer full molecular screening.

In addition, we evaluated a combination of DNA methylation marker analysis and cytology as baseline triage strategy for hrHPV-positive women and found a substantially higher CIN2/3+ sensitivity compared to sole baseline cytology triage at a slight decrease in specificity. Both assays appeared partially complementary to each other, with cytology having a moderate sensitivity for all disease categories, i.e. CIN2, CIN3 and cervical cancer, whereas methylation marker analysis displays a steep increase in sensitivity proportional to disease severity^{2,7}. Consequently, combined triage by cytology and methylation marker analysis at baseline would result in immediate detection of the more advanced transforming CIN2/3 lesions and carcinomas, and still reassure the detection of the majority of CIN2 lesions, although at a the cost of a higher colposcopy referral rate.

Thus, besides the above mentioned fully molecular strategy, a combined triage strategy by cytology and methylation marker analysis at baseline may also be advised in settings with adequate cytology. Alternatively, a combination of methylation marker analysis and HPV16/18 genotyping can be a promising molecular alternative to increase the sensitivity for (early-onset) CIN2/3 lesions.

Self-collected (cervico-)vaginal samples (women of screening age)

From mid-2016, non-attendees of the Dutch cervical screening programme will be offered the possibility to participate in cervical screening by self-sampling. Women with a hrHPV-positive self-sample will be advised to obtain a subsequent physician-taken cervical scrape to allow triage by cytology. However, this can lead to substantial loss to follow-up and delay in diagnosis. Previously, it has been shown that direct DNA methylation marker analysis by *MAL/mir124-2* on a lavage self-sample is non-inferior to cytology triage on an additional cervical scrape for detecting CIN3+ lesions. This indicates that methylation marker analysis on self-samples can be a promising molecular triage alternative to cytology. However, clinical performance of methylation-based triage markers can be dependent on the type of sample utilized. Therefore, it is important to gain more data on the clinical performance of methylation marker analysis in different self-sample types. In Chapter 7, we evaluated both hrHPV-positive lavage and brush self-samples by the *FAM19A4/mir124-2* methylation assay. On both self-sample types, *FAM19A4/mir124-2* methylation marker analysis yielded similar sensitivity ($\pm 70\%$) and specificity ($\pm 70\%$) for the detection of CIN3+. These figures equal those obtained for CIN3+ detection by methylation marker analysis of hrHPV-positive cervical scrapes. Therefore, for algorithms involving self-sampling among non-attendees, direct methylation marker analysis is appealing to triage hrHPV-positive women, with a repeat HPV-test after 12-18 months for baseline methylation-negative women.

However, as long as a low (pre)cancer risk of women with a hrHPV-positive, methylation-negative self-sample is not fully established, an additional triage tool could be considered to reassure that no clinical meaningful lesions are missed at baseline. The addition of cytology to algorithms involving self-sampling is, however, unsatisfactory since this requires an extra physician-taken cervical scrape. Alternatively, a combined molecular triage strategy by methylation marker analysis and HPV16/18 genotyping can be considered. Recently, it has been shown that combined molecular triage by *MAL/mir124-2* and HPV16/18 genotyping on lavage self-samples leads to significantly higher sensitivities for CIN2/3+, yet at the cost of a lower specificity¹⁴. In the validation sets of lavage and brush self-samples described in chapter 7, *FAM19A4/mir124-2* in combination with HPV16/18 genotyping revealed CIN3+ sensitivities of 88.5% and 84.7% at specificities of 46.2% and 54.9% for hrHPV-positive lavage and brush self-samples, respectively. These data support that combined methylation marker analysis and HPV16/18 genotyping on self-samples at baseline might be considered to triage screening non-attendees with hrHPV-positive self-samples, when high CIN2/3+ sensitivity is required.

Management of young hrHPV-positive women

In young women (<30 years) who visit the gynaecology outpatient clinic for gynaecological complaints, hrHPV infections are very common and often associated with abnormal cytology results^{15,16}. However, the occurrence of cervical cancer in this age-group is rare¹⁷ and CIN lesions frequently regress spontaneously¹⁸. In chapter 6, we showed that methylation marker analysis by *FAM19A4* in cervical scrapes of hrHPV-positive women <30 years of age, revealed a CIN3+ sensitivity of 50.0% at 81.7% specificity. For cytology, these figures were 86.7% and 56.4%, respectively. The higher sensitivity of cytology for CIN2/3+ probably reflects the better detection of early CIN2 lesions with a regressive nature at this young age, while methylation marker analysis particularly detects cervical cancer and advanced transforming CIN2/3 lesions (Chapter 5). The use of methylation marker analysis in this age category might therefore reduce overtreatment and related treatment morbidity such as cervical insufficiency and preterm delivery.

FUTURE RESEARCH

Clinical validation of methylation assays

To gain further insight in the nature of methylation-negative CIN2/3 lesions, it is valuable to subdivide these lesions and assess their cancer-progression risk. The use of additional molecular markers for further CIN grading such as DNA copy number alterations, immunohistochemical staining for HPV-E4 (a viral protein specific for the productive stage of the viral life cycle), squamous columnar junction markers (keratin 7, AGR2, MMP2 and GDA) and p16^{INK4A}/Ki-67 (biomarker of HPV-E7 over activity in dividing cells) may allow a better sub-classification of these CIN lesions according to cancer-progression risk. With such improved risk stratification, better tailored clinical decisions based on methylation marker analysis can be foreseen.

Furthermore, the effect of methylation marker-based follow-up strategies on patient outcome needs attention. In current clinical practice, most women with CIN2 are treated. However, particularly in young women, most CIN2 lesions are likely to regress. We have shown that more than half of CIN2 lesions are methylation marker negative. A prospective study with close surveillance of hrHPV-positive (young) women with a well overseeable transition zone and harbouring methylation-negative CIN2 or small CIN3 lesions, will answer the question whether these lesions require direct treatment or not. Insight into the clinical behaviour (i.e. progressive or regressive nature) of methylation negative CIN2 or small CIN3 on the basis of the methylation pattern will help to reduce overreferral and overtreatment of these women.

Further determination of the conformity of clinical performance of methylation testing in other cohorts of screening populations from different (European) countries, and on a larger number of women with cervical cancers, including AdCAs and small-cell cancer, will be relevant to provide further proof of effectiveness for the management of hrHPV-positive women. Moreover, given their high sensitivity for advanced high-grade precursor lesions and cervical carcinomas, methylation marker assays may even be considered as a future primary screening tool in an era with reduced HPV-prevalence due to HPV-vaccination.

Advancing technology

At the moment, multiplex qMSP, as described in this thesis, seems most suited for automation and high-throughput clinical application. However, novel technologies, such as next generation, ultra-deep sequencing techniques¹⁹ are upcoming and would allow multi-sample and multi-marker evaluation (at individual CpG level) in parallel in a single reaction. Compared to qMSP, this technique currently requires more laborious and more complicated preparation and analysis steps. Therefore, as long as the number of informative markers is within an acceptable range for multiplex qMSP, there is no urgent need for conversion to a next generation sequencing platform.

Therapeutic possibilities

Re-expression of *CADM1*, *MAL* and *mir124-2* have previously shown to inhibit the growth, viability, motility and/or anchorage independent growth of hrHPV-containing cancer cells²⁰⁻²². Thus, next to their usefulness as biomarkers for early detection, the hypermethylated genes described in this thesis might also serve as attractive therapeutic targets for the treatment of cervical (pre)cancer. Future studies involving the use of DNA methylation inhibitors (i.e. 5'-aza-2'deoxyctidine), HDAC inhibitors or other compounds that trigger epigenetic engineering²³ may shed light on the potential use of these genes as target for therapeutic intervention.

In summary, methylation marker analysis, as described in this thesis, will have clinical implications for the management of hrHPV-positive women in screening and gynaecology outpatient populations.

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