CHAPTER 8
SUMMARY AND GENERAL DISCUSSION
SUMMARY

Cervical cancer is the fourth most common cancer among women worldwide, with about 570,000 new cases annually. It represents approximately 7% of all female cancers with the majority in low and middle income settings. In order to decrease the incidence of and mortality from cervical cancer, effective prevention strategies have been developed. These include prophylactic human papillomavirus (HPV) vaccination (primary prevention) and cervical screening programmes (secondary prevention). The introduction of cervical screening has substantially declined the incidence of cervical cancer, but in recent years this decrease has levelled off. This suggests that changes regarding the detection methods and attendance (~65% of invited women in The Netherlands) are needed to facilitate further reduction in cervical cancer incidence. In 2017, The Netherlands replaced cytology-based screening by HPV-based screening, because HPV-based screening has been shown to provide better protection against cervical precancerous lesions (termed cervical intraepithelial neoplasia or CIN) and cervical cancer than cytology. In order to increase participation rates, HPV testing on self-collected cervicovaginal material (HPV self-sampling) for women not-attending regular screening (so-called non-attendees) was also introduced. To identify HPV-positive women with clinically meaningful cervical disease in need of immediate intervention, an additional test (triage test) is needed. In the Netherlands, HPV-positive women are triaged with cytology and repeat cytology after 6 months. Cytology testing of self-samples is however not reliable. As a result, women testing hrHPV-positive on their self-sample need to visit their general practitioner for a cervical scrape. As this results in a 20% loss-to follow-up, molecular triage markers that are directly applicable on self-samples are required.

Considering the above, this thesis focused on altered microRNA (miRNA) expression and DNA methylation as potential triage tool for hrHPV-positive women in different types of cervical specimens, including self-samples and cervical scrapes. The studies described herein, and summarised below, improve our knowledge on molecular events during cervical carcinogenesis and provide promising triage markers for (early) detection of cervical (pre)cancer in screening programmes. The findings presented in this thesis are illustrated in Figure 1.

In Chapter 2, current knowledge on altered regulation of the miRNA machinery in HPV-induced cancers is summarised. We provided an overview of known alterations in miRNA processing proteins in HPV-induced cancers, which include besides cervical cancer, other anogenital cancers, such as anus, penile, vagina, and vulvar, and a rising subset of head and neck cancers. MiRNAs are small non-coding RNAs that regulate gene expression post transcriptionally and are often deregulated in cancers, including HPV-induced cancers. Their biogenesis is a tightly regulated multistep process accomplished by several proteins. After miRNA transcription in the nucleus, the primary miRNA (pri-miRNA) is cleaved by the RNase III enzyme Drosha and its cofactor the double-stranded RNA (dsRNA)-binding protein (dRBP) DiGeorge critical region 8.
(DGCR8) resulting in a precursor miRNA (pre-miRNA) of ~60-70 nucleotides. The pre-miRNA is subsequently exported to the cytoplasm by the export receptor exportin 5 (XPO5), followed by cleavage by the RNase III enzyme Dicer in cooperation with trans-activation-responsive RNA-binding protein (TRBP). From the resulting mature miRNA duplex, one strand assembles into the RNA-induced silencing complex (RISC) together with a member from the Argonaute (AGO) protein family and guides the complex to target mRNAs for their degradation or translational repression. In addition to the mature miRNA sequence, miRNA variants exists (so-called isomiRs) that can be generated by changes in Drosha and Dicer cleavage or miRNA processing proteins such as terminal nucleotidyltransferases (NTases). The summarised findings in our review indicate that miRNA processing proteins Drosha, DGCR8, Dicer and AGO2 are deregulated in HPV-induced cancers. This deregulation is contributed by either or both viral oncoproteins and genomic alterations.

Furthermore, NTases involved in isomiR generation such as TENT2, TENT4A and TUT7, are altered at the genomic and/or expression level in cervical cancers, and some already at the precancerous stage. This indicates that NTases are likely to be involved during the early steps of cancer development and that the generated isomiRs could provide promising biomarkers for early cancer diagnosis. Future studies are needed to elucidate the exact role of NTases during carcinogenesis and their modulation might provide a potential therapeutic avenue.

The most commonly employed method for accurate miRNA expression analysis is reverse transcription-quantitative PCR (RT-qPCR). The measured expression can be divided into the biological expression and a technical bias component. The latter is unwanted and can be introduced because of differences in amount and quality of starting material, RT efficiency, and qPCR performance. In order to reliably analyse miRNA expression using RT-qPCR, adequate data normalisation is essential to remove non-biological, technical variations. Inadequate data normalisation can reduce or exaggerate the biological variability of the miRNA, which might lead to data misinterpretation and incorrect conclusions. In Chapter 3, we defined the optimal methods to normalise miRNA expression analysis using RT-qPCR. We selected a panel of 11 candidate reference genes from genome-wide miRNA profiling data of cervical tissues, self-samples, and the literature. The 11 candidate reference genes were analysed in different types of cervical specimens (i.e. tissues, scrapes, and self-samples) with different disease grades. We assessed the expression stability of the 11 candidate reference genes by combining three commonly employed algorithms (GeNorm, NormFinder, and BestKeeper). The calculation of signal-to-noise ratios (SNRs) and P values between control and disease groups were used to evaluate the effect of normalisation of two marker miRNAs. Higher absolute SNR values imply larger differences between the groups that are compared and are therefore wanted. We identified miR-423 as suitable reference gene for all sample types, to be used in combination with RNU24 in cervical tissues, RNU43 in scrapes, and miR-30b in self-samples. In general, data
normalisation using the two selected reference genes increased SNRs, while normalizing to the most commonly used reference genes in the literature, introduced variation and compressed SNR. Taken together, our results demonstrate that the most optimal reference genes may differ between specimen types and that adequate normalisation could improve data interpretation.

![Diagram of epigenetic changes in cervical carcinogenesis](image)

**Figure 1. A schematic representation of the epigenetic changes involved in cervical carcinogenesis and described in this thesis and the triage markers derived from these studies.** Adapted from Steenbergen et al. Normal: normal cervix; Productive CIN: CIN1/2; Transforming CIN: CIN2/3.

In **Chapter 4**, we studied the feasibility of miRNA testing in cervical scrapes. We evaluated the clinical performance of eight previously identified miRNAs on cervical scrapes to triage hrHPV-positive women in cervical screening. The expression of these eight miRNAs (miR-9, miR-15b, miR-28, miR-100, miR-125b, miR-149, miR-203a, and miR-375) was found to be altered in cervical tissues of precancerous lesions and cervical cancer due to either methylation-mediated silencing or chromosomal alterations.

First, by using RT-qPCR we confirmed the expression of 6 out of 8 miRNAs in 58 cervical tissues consisting of normal cervical epithelium, high-grade CIN lesions (CIN2/3), squamous cell carcinomas (SCCs) and adenocarcinomas (ACs). Next, we evaluated whether altered expression of the 6 miRNAs was also observed in cervical scrapes. Two hundred and twenty-five hrHPV-
positive cervical scrapes from women without cervical disease, CIN3, SCC or AC were analysed by RT-qPCR. Most of our findings in cervical scrapes were comparable to those obtained in tissue samples. To identify the most discriminative miRNA marker panel for hrHPV-positive cervical scrapes from women with CIN3, we performed multivariable logistic regression analysis and identified a 2-miRNA classifier consisting of miR-15b and miR-375 with an area under the curve (AUC) of 0.622 for CIN3 detection. Additionally, combining this 2-miRNA classifier with HPV16/18 genotyping further improved classification of CIN3 lesions with a sensitivity of 63% and specificity of 77%. Importantly, all carcinomas were detected using the 2-miRNA classifier either alone or in combination with HPV16/18 genotyping. Furthermore, functional analysis of miR-15b and miR-375 revealed that both miRNAs affect cervical cancer cell viability and this indicates that biologically relevant miRNAs are promising disease markers. Our findings show the potential of miRNA expression analysis in cervical scrapes for triage of hrHPV-positive women in cervical screening.

Given the particular need for molecular markers for triage testing of hrHPV-positive self-samples, and in follow-up of above data we questioned whether triage testing by miRNAs might also be applicable to HPV-positive self-samples. Since marker performance is known to be sample type dependent, we decided to perform a genome-wide miRNA discovery screen directly on self-samples rather than taking a more conventional approach based on tissues. Therefore, in Chapter 5 we performed, for the first time, genome-wide small RNA sequencing (sRNA-Seq) on 74 hrHPV-positive self-samples. Following extensive statistical analysis we identified a panel of 9 miRNAs with an AUC of 0.89 for CIN3 detection. To ensure that the identified miRNAs are related to cancer development, we analysed in-house available genome-wide miRNA profiles of cervical tissues consisting of normal cervical epithelium, CIN2/3 lesions and SCCs. For the majority of miRNAs we observed similar expression patterns as observed in self-samples and all miRNAs except one showed increased expression in SCC compared to controls. Subsequent validation by RT-qPCR in an independent set of hrHPV-positive self-samples (controls, CIN3, and SCC) followed by logistic regression analysis revealed a good clinical performance for a 5-miRNA classifier consisting of let-7b, miR-15b, miR-20a, miR-93, and miR-222 with an AUC of 0.78 for CIN3+ detection. This 5-miRNA classifier was able to detect 67% of CIN3 (32 out of 48) and 93% of SCCs (38 out of 41) at 65% specificity. These findings show that it is feasible to detect altered miRNA expression associated with CIN3 and cervical cancer in self-samples. Hence, miRNA expression analysis offers a novel and promising molecular triage strategy directly applicable to self-samples.

In addition to miRNAs, other epigenetic events also provide interesting molecular triage markers. Previous studies by others and us have focused on DNA methylation analysis in different types of cervical specimens, including self-samples. These findings have shown the potential of DNA methylation testing for triage of hrHPV-positive women, even in impure samples that contain few lesional cells such as self-sampled material. In order to obtain a methylation marker panel with
optimal clinical performance on self-samples, we performed in Chapter 6 a genome-wide DNA methylation screen (Infinium 450K array) directly on self-sampled material. Discovery studies on self-samples have not been performed before. We identified 12 DNA methylation markers with good discriminative power for CIN3 that were subsequently analysed by multiplex qMSP in large series of hrHPV-positive lavage (n = 245) and brush self-samples (n = 246). Logistic regression analysis of the qMSP data resulted in a 3-gene methylation classifier consisting of ASCL1, LHX8 and ST6GALNAC5, with optimal clinical performance for CIN3 detection. The 3-gene methylation classifier was subsequently validated by multiplex qMSP in large independent series of self-samples from a screening cohort of non-attendees (n = 100 lavage and n = 287 brush) and showed an excellent and reproducible clinical performance for CIN3 detection in hrHPV-positive lavage self-samples (AUC = 0.88; sensitivity = 74%; specificity = 79%) and brush self-samples (AUC = 0.90; sensitivity = 88%; specificity = 81%). Importantly, all carcinomas (both SCC and AC) were detected using the 3-gene methylation classifier. This study indicates that the 3-gene methylation classifier provides a powerful triage method for the detection of both CIN3 and cervical cancer in hrHPV-positive self-samples, which is superior to other currently available triage tools.

Finally, we explored to potential of hrHPV DNA and methylation testing in urine as an alternative self-sampling method for cervical cancer detection. Urine sampling recently gained much interest as another non-invasive manner of cervical sampling. It has been established that urine sampling is preferred over cervical sample collection either by physician or by self-sampling. While studies on DNA methylation analysis in cervical tissues, scrapes and self-samples are plentiful, limited data exist on DNA methylation analysis in urine. Therefore, in Chapter 7, we tested hrHPV and six previously identified DNA methylation markers (FAM19A4, GHSR, PHACTR3, PRDM14, SST, and ZIC1) in urine samples and paired cervical scrapes from cervical cancer patients. HrHPV DNA test results were compared between different urine components (native urine and sediment) and cervical scrapes and yielded a strong to near-perfect agreement (sediments versus native: kappa= 0.79; 95% CI 0.58-1.00, sediments versus scrapes: kappa=0.85; 95% CI 0.64-1.00). In addition, DNA methylation levels detected in urine were moderately to strongly correlated to those detected in cervical scrapes. Furthermore, logistic regression analysis revealed that all DNA methylation markers showed a good discriminatory power for cervical cancer detection with an AUC varying from 0.744 (PHACTR3) to 0.887 (SST). Our results indicate the potential of hrHPV DNA and DNA methylation testing in urine for the detection of cervical cancer.

In conclusion, in this thesis, we showed altered miRNA and DNA methylation profiles associated with cervical cancer development in different types of cervical specimens, including cervical scrapes, self-samples and urine. Most alterations were already observed at the precancerous stage and increased with severity of the disease. Genome-wide screens performed on hrHPV-positive self-samples identified novel promising miRNA and DNA methylation markers for CIN3 and cervical cancer detection.
We showed that DNA methylation marker based triage of hrHPV-positive self-samples was able to detect all cervical carcinomas and the majority of CIN3 lesions with an AUC of (close to) 0.9. The new DNA methylation markers described in this thesis perform as good as the well clinically validated bi-marker panel FAM19A4/miR124-2, has competitive performance versus other triage options today, and is compatible with self-collected cervicovaginal material (Chapter 6). With these performance characteristics, this marker panel warrants further clinical validation to confirm its suitability in self-collected cervical specimens and potentially urine.

Although many studies have reported on the involvement of miRNAs in cervical cancer, their potential as triage markers for hrHPV-positive women remains largely unexplored. In fact, our study on miRNAs in self-samples (Chapter 5) is the first to determine genome-wide miRNA profiles and their clinical applicability in hrHPV-positive self-samples. The feasibility of miRNA detection in self-collected cervicovaginal material has opened the way for further evaluation of their suitability and accuracy as triage markers in clinical specimens of HPV-positive women.
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In this thesis, we aimed to identify and validate molecular markers for triage of hrHPV-positive women in both physician-taken samples and self-collected cervicovaginal material such as self-samples and urine. The findings presented in this thesis have increased our understanding of molecular alterations during the development of cervical cancer, and pave the way for a transition towards triage testing by molecular markers for the clinical management of hrHPV-positive women in cervical screening programmes, thus opening the way to full molecular cervical self-screening. Nevertheless, a number of research questions still remain to be elucidated. Although cervical precursor lesions can appear morphologically similar, recent data indicate that especially CIN2 lesions have different clinical behaviour and are (epi)genetically heterogeneous\(^2\)\(^{-4}\). Furthermore, recent immunohistochemical and molecular data suggests that a distinction can be made between early and advanced CIN2/3 lesions, defined by a cancer-like methylation profile, chromosomal aberrations and association with a long standing persistent HPV infection (>5 years)\(^2\)\(^,\)\(^5\)\(^,\)\(^6\). While early CIN2/3 lesions display a low cancer risk, advanced CIN2/3 lesions are suggested to have a high short-term progression risk to cancer. In addition to CIN lesions, cervical cancers represent a heterogeneous group of tumours that displays distinct molecular profiles\(^7\)\(^,\)\(^8\). In this respect, it is most important to further sort out how the molecular and genetic profiles of CIN2/3 lesions and the (epi)genetically different groups of cervical cancer are related in order to find out the best biomarkers for early detection and susceptibility for different cervical cancer treatment modalities. To further improve our understanding of the mechanisms driving cervical cancer development additional functional studies, viral characteristics and the interplay with the microenvironment need to be considered as well.

Molecular host cell alterations: associations and underlying mechanisms/Data integration

We have studied genome-wide changes in miRNA expression and DNA methylation patterns in HPV-positive self-samples of women with CIN3 versus women without cervical disease, resulting in the identification of a number of biomarkers for CIN3 detection. Previous studies have revealed frequent chromosomal aberrations during cervical carcinogenesis\(^9\) and characterised the mutational landscape of cervical cancer\(^7\)\(^,\)\(^10\)\(^{-12}\). In general, the alterations increase with progression from cervical precancerous lesions to cancer, but it is still unclear how these different aspects interact over time resulting in the development of cervical cancer.

Role of copy number aberrations

In 2014, Thomas and colleagues performed a meta-analysis on microarray comparative genomic hybridisation (CGH) studies and found that gain at 3q, loss at 3p and loss at 11q were the most frequent alterations in cervical cancer\(^9\). In CIN2/3 the most frequent aberration was gain at 1p. Interestingly, miRNAs often reside in genomic regions prone to chromosomal abnormalities in cancer cells\(^13\). Accordingly, a study by Wilting et al. revealed that altered expression of several miRNAs (miR-
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9, miR-15b, miR-28, miR-100, and miR-125b) was directly linked to chromosomal aberrations. Experimental analysis of miR-9 (linked to gain of 1q) showed that altered miRNAs linked to chromosomal alterations are of functional significance and contribute to cervical carcinogenesis. Based on the assumption that similar to HPV, molecular aberrations with biological relevance are most promising biomarkers, we evaluated the clinical potential of miR-9, miR-15b, miR-28 and miR-100 for triage of hrHPV-positive cervical scrapes, as described in Chapter 4. Indeed it was found that these miRNAs linked to a chromosomal aberration were of diagnostic value.

However, out of the nine most promising miRNA markers identified by our genome-wide discovery screen described in Chapter 5, it appeared that only one, miR-15b, resides in a genomic region (i.e. at chromosome 3q) that is frequently altered in cervical cancer. This indicates that miRNAs not linked to chromosomal aberrations are also promising disease markers.

Besides miRNA encoding genes, also, enzymes involved in miRNA processing may be affected by chromosomal aberrations. For example, one of the major players in miRNA biogenesis, the RNAse III enzyme Drosha, is often overexpressed in cervical cancer and its overexpression was found to be associated with a frequently occurring gain of 5p (described in Chapter 2). Furthermore, a recent study by Verlaat and colleagues identified several altered DNA methylation markers in CIN2/3 and cervical cancer that are associated with a 3q gain. Similar to these findings, two of the genes in the 3-gene methylation classifier from Chapter 6 are located on a known gained region in cervical cancer, i.e. \textit{LHX8} and \textit{ST6GALNAC5} both located on 1p. These observations are in contrast to the classical Knudson two-hit hypothesis that states that DNA methylation of a tumour suppressor gene promoter on one allele is accompanied by a loss of the second allele, as has previously been shown for the tumour suppressor miR-375. It has been suggested that associations between DNA methylation and CNA are due to redistribution of DNA methyltransferases. Whether this is the case in cervical cancer, and whether this explains hypermethylation of gained regions needs to be elucidated by additional integration of longitudinal measurements of DNA methylation and CNA in cervical carcinogenesis.

\textbf{Epigenetic interactions}

A number of miRNAs become methylated during cervical carcinogenesis which influences their expression. For example, miR-203 and miR-375 were found to be methylated in cervical cancer as well as precancerous lesions, and their expression could be restored upon treatment with a demethylating agent. However, at present only limited data exist on methylated miRNAs in cervical cancer and to the best of our knowledge there are no studies that have compared methylation and expression of miRNAs on a genome-wide scale. Our genome-wide miRNA and DNA methylation profiles of self-samples from women with CIN3 or without cervical disease (Chapter 5 and 6) could be integrated to extract this information. However, ideally we would expand this dataset with genome-wide profiles of cervical cancers to explore this phenomenon in different disease stages.
Molecular classification of cervical lesions: TCGA data, viral status and beyond

Although the aforementioned integration strategies are informative, they focus on the integration of a rather limited number of molecular features and lack a comprehensive approach. Recently, TCGA published the most comprehensive data integration of molecular features in cervical cancers performed to date. This publicly available dataset includes profiles on CNA, miRNA, mRNA and DNA methylation of 228 primary cervical cancers as well as protein profiles of 155 cervical cancers. Integration of these datasets revealed the molecular heterogeneity of cervical cancer. Three molecular clusters were identified that distinguished squamous carcinomas with high keratin expression from those with low keratin expression and adenocarcinomas. Characterisation of miRNA expression profiles identified 6 miRNA clusters of which one cluster almost exclusively overlapped with adenocarcinomas. The latter showed high expression of miR-375 suggesting that altered expression of this miRNA is histotype-associated.

In fact, our previous research and data presented in Chapter 4 indicate that miR-375 expression is downregulated in SCC compared to normal epithelium, which has been associated with both a chromosomal loss at 2q35 and increased methylation of its promoter region. Moreover, we show (Chapter 4) that miR-375 functions as a tumour suppressor gene in squamous epithelial cells. Since TCGA data found high miR-375 expression in adenocarcinomas, it would be of interest to study whether its functional role differs between histotypes. Furthermore, TCGA data showed that the keratin-high cluster expressed significantly higher levels of miR-203a compared to the keratin-low cluster.

Similar to miR-375, reduced expression of miR-203a has been associated with DNA methylation in CIN lesions and SCC and miR-203a has been shown to function as tumour suppressor. Hence, it would of interest to determine how the miRNAs identified in this thesis and miRNAs targeted by DNA methylation relate to the different subgroups of cervical cancer defined by the TCGA analysis.

Furthermore, TCGA data revealed three DNA methylation clusters that correspond to the levels of hypermethylated CpG islands (high, intermediate, and low). Most adenocarcinomas were DNA methylation-high whereas the squamous carcinomas contained a mix of DNA methylation profiles. While previous studies have shown that cervical cancers display high DNA methylation levels and CNA, TCGA data revealed that cervical cancers with CNA-high profiles do not overlap with DNA methylation-high profiles.

This suggests that the development of cervical cancer may vary between individuals. While the methylation clusters are based on all methylated genes combined, the high methylation levels of the markers identified in Chapter 6 in all cancers may well be cluster independent. Because
the TCGA dataset lacks data of normal cervix and CIN lesions it remains to be determined whether similar clusters are also evident in CIN3 lesions. For this purpose, several institutions are currently putting together the Pre-Cancer Genome Atlas, the precancer variant of TCGA\textsuperscript{22}. Since the microenvironment plays a key role in cancer development, which has also been shown for cervical cancer\textsuperscript{23,24}, the Pre-Cancer Genome Atlas aims to analyse the microenvironment, in addition to host cell alterations, of precancerous cells to help identify the lesions that will likely advance to invasive cancer.

Besides the integration of host cell changes and the microenvironment, alterations related to viral protein expression should also be considered and included in integration studies. For example, analysis of p16 overexpression combined with Ki-67 expression, reflecting HPV E7 transforming activity, revealed heterogeneous expression patterns particularly within CIN2 lesions\textsuperscript{25}. A high immunoscore characterised by extensive p16 and Ki-67 expression was associated with a reproducible CIN3 grading. Additionally, analysis of viral E4 expression (marker for the onset of a productive infection) and DNA methylation levels of the host cell genes CADM1, MAL, and miR124-4 (marker for an advanced, transforming infection) in tissue biopsies demonstrated heterogeneous molecular patterns in CIN2/3 lesions, with CIN2/3 lesions being either E4 positive, E4 and methylation positive or methylation positive\textsuperscript{6}. In line with these findings, a recent study by Verlaat et al. on multiple DNA methylation markers showed that HPV-positive scrapes from women with CIN2/3 lesions display a heterogeneous methylation pattern, with only a subset of CIN2/3 lesions being characterised by a cancer-like methylation profile\textsuperscript{2}. Together with previous studies on DNA methylation markers that have demonstrated that within the group of CIN2/3 elevated DNA methylation levels are associated with a longstanding HPV infection (so-called advanced CIN2/3 lesions)\textsuperscript{5,16,26}, a cancer-like methylation profile is expected to imply a higher short-term risk of progression to cancer. These data indicate that a combined analysis of viral activity (e.g. E4, p16, Ki-67 expression) and molecular host cell alterations (e.g. DNA methylation) could improve the clinical management of women with CIN2/3.

Since the studies described above and also the studies described in this thesis are cross sectional studies, these data cannot be used to relate our findings on molecular host cell changes with the clinical course of CIN2/3 CIN lesions. In order to expand our understanding of the clinical behaviour of CIN2/3 lesions, our group is currently conducting a longitudinal study with 24 months follow-up where small CIN2/3 lesions are not treated, but monitored every 6 months (CONCERVE study; NTR6069)\textsuperscript{27}. DNA methylation testing of the host cell genes FAM19A4 and miR124-2 will be performed on self-samples collected at baseline and during follow-up to evaluate the value of DNA methylation for the detection of persisting CIN2/3 lesions.
Advancing technologies

Several technologies exist for the assessment of miRNA expression profiles. For genome-wide profiling, small RNA sequencing (sRNA-Seq) is commonly employed. Besides the assessment of known miRNAs, this technique allows for the identification of novel miRNAs. However, due to ligation bias in sRNA-Seq methods the accurate quantification of miRNAs has been limited\(^\text{28}\).

To minimise this bias, recent efforts have focused on optimizing ligation-based sRNA-Seq methods and resulted in an improved sRNA-Seq protocol, called AQ-seq (accurate quantification by sequencing)\(^\text{28}\). This protocol utilizes adapters with four terminal degenerate nucleotides and a high concentration of polyethylene glycol (PEG) and was shown to minimise adapter ligation bias.

For genome-wide DNA methylation profiling of self-samples we used the Infinium HumanMethylation450 BeadChip array (Infinium 450K array), which is the most common method for genome-wide methylation profiling to date. Such an approach would also be favourable for the identification of methylation markers for cervical cancer detection in urine, the feasibility of which has been shown in Chapter 6. Towards this goal, the improved Methylation EPIC Beadchip Infinium array, as introduced by Illumina in 2016 which measures over 850.000 CpG sites may be the method of choice\(^\text{29}\). Furthermore, so-called third-generation sequencing technologies were recently introduced and allow for DNA methylation analysis without prior bisulphite conversion. Examples of such technologies are single-molecule real-time (SMRT) DNA sequencing and Nanopore sequencing\(^\text{30,31}\).

In addition to sRNA-Seq, we also used a targeted approach for miRNA quantification. We used the well-known Taqman miRNA assay which is a traditional quantitative PCR (qPCR) method that uses target-specific stem-loop reverse transcription (RT) primers. This method is highly sensitive and useful for validation studies. In order to reduce costs and sample material, we included a multiplex RT step that allows for simultaneous transcription of a set of miRNAs including reference genes. Reliable miRNA expression analysis using qPCR relies on adequate data normalisation, which is essential for correct data interpretation. Therefore, in Chapter 3, we evaluated candidate reference genes for their potential to accurately normalise miRNA expression data in different types of cervical specimens (i.e. tissues, scrapes and self-samples). We identified miR-423 as suitable reference gene for all sample types, to be used in combination with RNU24 in cervical tissues, RNU43 in scrapes, and miR-30b in self-samples. Similarly, Nilsen and colleagues most recently identified miR-423 as one of the most optimal reference genes for normalisation of qPCR data in cervical cancer tissues\(^\text{32}\).
Despite the availability of various detection methods for efficient and accurate quantification of miRNAs, only some of these approaches have been tested for their ability to distinguish between highly similar miRNA sequences. Although these methods possess high sensitivity for specific sequences, they are not adequately specific and closely related isomiRs are also detected. Therefore, further advancements in isomiR detection methods are needed in order to accurately quantify individual isomiRs.
FUNCTIONAL RELEVANCE AND UNDERLYING MECHANISMS

Functional relevance
A number of studies have shown that biologically relevant miRNAs might provide promising markers for cervical (pre)cancer detection. Indeed, functional analysis of miR-15b and miR-375 (Chapter 4) revealed an effect of both miRNAs on cervical cancer cell viability suggesting their involvement in cervical carcinogenesis. In addition, preliminary data on miR-93 (Chapter 5) showed that its inhibition reduced cervical cancer cell viability while inducing cell death (unpublished data).

These findings are in line with a recent report from Li et al. in which miR-93 inhibition was shown to inhibit cell proliferation and increase apoptosis of cervical cancer cell lines. Furthermore, they found that miR-93 targets BTG3, a member of the anti-proliferative B-cell translocation gene (BTG)/transducer of ErbB2 gene family. Interestingly, we also identified a BTG family member as direct target of miR-93 in cervical cancer cells using the Ago-HITS-CLIP technology (unpublished data).

The Ago-HITS-CLIP method (Argonaute High Throughput Sequencing after Cross-Linked Immunoprecipitation) stabilizes RNA-protein complexes by UV cross-linking in live cells followed by immunoprecipitation of RISC-associated miRNAs. This method allows for the identification of direct miRNA targets on a genome-wide scale.

In addition to miR-93, we also performed Ago-HITS-CLIP analysis on miR-15b and besides known targets such as CCND1 (Cyclin D1), we identified putative novel miR-15b targets that are currently being validated.

Furthermore, isomiRs have been found differentially expressed in various tissue types and cell lines (see also Chapter 5) and were shown to actively interact with RISC which indicates their functional significance. However, studies that report on isomiR function are scarce. Therefore, more evidence is needed in order to unravel the exact functional role of isomiRs, including their involvement in cervical cancer.

Despite numerous studies on the functional significance of miRNAs and their targets in cervical carcinogenesis, studies on altered DNA methylation markers tend to mainly focus on their role as potential disease marker. One of the few studies on the functional role of a methylated gene in HPV-induced carcinogenesis focuses on PRDM14, a marker that we investigated in Chapter 7. Repression of PRDM14 expression mediated by methylation of its promoter was found to induce apoptosis-resistance of HPV-transformed cells, indicating that PRDM14 exerts a tumour suppressive role in HPV-induced cancers. While for some of the methylated genes described in Chapter 6 and 7 a functional role in cancer has been described, i.e. ASCL1 is essential for
the survival of small cell lung cancer (SCLC) with neuroendocrine features\textsuperscript{43}, and suppresses the tumourigenicity of glioblastoma stem cells by promoting neuronal gene expression\textsuperscript{44}, their functional characterisation in cervical carcinogenesis is still to be awaited.

To more comprehensively determine the biological significance of individual molecular events, functional genomic screens are commonly employed. This powerful tool enables the discovery of gene function, pathways and interactions on a genome-wide scale and is based on measurable phenotypes. Examples include gain-of-function screens that utilise libraries of miRNA mimics and loss-of-function screens that result from using libraries of CRISPR-Cas9 (clustered interspaced short palindromic repeats/CRISPR-associated protein) guide RNAs (sgRNAs). The latter uses the CRISPR-Cas9 technology which is now widely adopted as targeted genome editing tool and offers new unbiased opportunities for global investigation of gene function.

**Mechanisms**

As described above, many molecular alterations characterise the development of cervical cancer. This raises the question which are the underlying mechanisms driving these alterations. The main contributors to HPV-induced molecular host cell changes are the viral oncoproteins E6 and E7. Both E6 and E7 are for example able to induce DNA methyl transferases (DNMTs) and thereby affect DNA methylation. The degradation of p53 initiated by E6 releases the transcription factor specificity protein 1 (Sp1) which then binds to the promoter of DNMT1 and results in DNA hypermethylation\textsuperscript{45}. In addition, E7 can directly bind to and activate DNMT1\textsuperscript{46}. Accordingly, DNMT1 was shown to be upregulated in CIN3 and cervical cancer\textsuperscript{47}. Besides its effect on DNMTs, E7 has been demonstrated to modulate KDM6A and/or KDM6B histone 3 lysine 27-specific demethylases which leads to histone demethylation of genes that were silenced such as p16(INK4A)\textsuperscript{48}.

Both viral oncoproteins have also been shown to modulate the miRNA processing machinery (described in Chapter 2). Both E6 and E7 have been implicated in the regulation of Drosha, DGCR8, and Dicer, which influences miRNA expression and subsequently affect biological processes\textsuperscript{49,50}. Based on the results described in Chapter 2 it may be speculated that also terminal nucleotidyltransferases (NTases) are deregulated in cervical cancer.

Furthermore, because miRNAs control the expression of many protein-coding genes and thereby regulate a diverse array of biological processes, it is has been suggested that mutations affecting miRNA function may be involved in carcinogenesis\textsuperscript{51}. Several miRNA mutations have been found in cancer and were shown to affect miRNA expression and function\textsuperscript{51,52,53}. These include mutations affecting miRNA sequences, mutations in miRNA target sites, and mutations in proteins involved in miRNA processing. While several mutations have been identified in cervical cancers including mutations in PIK3CA, EGFR, FBXW7, and KRAS\textsuperscript{7,11,12}, at present no miRNA mutations have been reported.
However, TCGA data revealed putative driver mutations in Dicer, one of the major players in miRNA biogenesis (Chapter 2). The potential effect of this mutation on the processing of miRNAs and their function remains to be determined.

**Diagnostic Implications**

The development of biomarkers for early cancer detection can be divided in five phases, as proposed by Pepe et al. (Table 1)\(^{54}\). In the last decade, many studies have been conducted on DNA methylation testing for triage of HPV-positive women which has resulted in major advancements in their detection and performance in cervical specimens.

Presently, DNA methylation triage testing is at phase 4 and some of the identified biomarkers have been translated into commercially available CE-IVD tests (e.g. QIAsure® Methyla
tion Test, GynTect®). The relatively unexplored potential of miRNAs as triage markers for cervical screening puts them at the start of biomarker development (phase 1).

The genome-wide discovery screens performed in this thesis have identified novel miRNA (Chapter 5) and DNA methylation (Chapter 6) markers for triage of hrHPV-positive self-samples that show good clinical performance for CIN3 and cervical cancer detection. Furthermore, our results on miRNAs in cervical scrapes (Chapter 4) and DNA methylation markers in urine (Chapter 7) represent one of the still limited number of studies to demonstrate the potential clinical application of these molecular markers in cervical scrapes and urine for cervical (pre)cancer detection. Taken together, our studies have identified and evaluated promising miRNA and DNA methylation markers (phase 1) applicable to different types of (noninvasive) cervical specimens that now await further clinical validation in large independent population-based screening cohorts.

**miRNA markers**

In Chapter 4, we evaluated the clinical performance of miRNA markers in HPV positive cervical scrapes and found a 2-miRNA classifier consisting of miR-15b and miR-375 combined with HPV16/18 genotyping which detected a major subset of CIN3 (63%) and all cervical carcinomas at a specificity of 77%. To date, only one other study reported on the clinical applicability of miRNAs in cervical scrapes for HPV-based cervical screening\(^{55}\). Here, a combination of miR-375 and miR-424 resulted in a sensitivity of 78.1% for CIN3+ detection at specificity of 80%.

Furthermore, the genome-wide discovery screen on self-samples performed in Chapter 5 identified altered miRNA markers with high discriminatory power for CIN3. The identified 5-miRNA classifier showed a good clinical performance and was able to detect the majority of CIN3 (67%) and almost all cervical cancers (93%) at a specificity of 65% in HPV-positive self-samples.
Table 1. Phases of biomarker development for early cancer development. Adjusted from Pepe et al.54.

<table>
<thead>
<tr>
<th>Phases</th>
<th>Type of study</th>
<th>Primary aims</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1</td>
<td>Preclinical exploratory studies</td>
<td>Identification and prioritisation of potentially useful biomarkers</td>
</tr>
<tr>
<td>Phase 2</td>
<td>Clinical assay development</td>
<td>Assess the clinical performance of biomarker assay for cancer detection</td>
</tr>
<tr>
<td>Phase 3</td>
<td>Retrospective longitudinal repository studies</td>
<td>Evaluate the capacity of biomarker to detect preclinical disease (before clinical diagnosis)</td>
</tr>
<tr>
<td>Phase 4</td>
<td>Prospective screening studies</td>
<td>Determine detection rate and false referral rate of biomarker-based screening test in relevant population</td>
</tr>
<tr>
<td>Phase 5</td>
<td>Cancer control studies</td>
<td>Estimate the reductions in cancer mortality afforded by the biomarker-based screening test</td>
</tr>
</tbody>
</table>

Since our study was the first to evaluate miRNA expression profiles in self-samples and evaluate its clinical applicability, we were unable to relate our findings to the performance of other miRNA markers in self-samples. However, compared to previously investigated DNA methylation markers in self-samples, we obtained a nearly comparable clinical performance for CIN3+ detection37,56.

Taken together, our findings on cervical scrapes and self-samples indicate that the analysis of more than one miRNA improves the detection of cervical disease. Moreover, these results suggest the potential of miRNAs as triage markers in HPV-based screening, but clinical validation is needed in large independent screening cohorts.

While previous studies have shown that DNA methylation markers detect advanced CIN2/3 lesions with high short-term progression risk to cancer1,5,16,26, a most recent study from our group performed on cervical scrapes found indications that miRNAs have a similar performance in early and advanced CIN2/3 lesions and did not detect all cervical cancers57. It remains to be determined whether this observation is miRNA and/or sample type specific, and which CIN2/3 lesions are detected by the 5-miRNA classifier from Chapter 5.

DNA methylation markers
A large number of validation studies have shown the strong performance of DNA methylation markers in HPV triage (current test at phase 4; Table 1)26,37,56,58–62. These studies have revealed that DNA methylation testing exhibits competitive performance over other triage options today64, detects relevant advanced CIN2/3 lesions with high short-term risk of progression to cervical cancer1,5,16,26, shows high reproducibility60, detects (nearly) all cervical cancers (Vink et al. submitted, and 26,62,63), and provides long term reassurance against CIN3+ risk (Dick et al. submitted and 64).
By a genome-wide screen on self-samples to define an optimal methylation marker panel, as described in Chapter 6, we identified the 3-gene DNA methylation classifier (ASCL1, LHX8, ST6GALNAC5) that showed an excellent sensitivity for CIN3 (74-88%) and cervical cancer (100%) in both lavage and brush self-samples at a specificity of 80%. This 3-gene classifier performs as good as the well clinically validated FAM19A4/miR124-2 DNA methylation triage test. These findings highlight the need for further validation in prospective clinical trials.

In addition to self-samples, we have shown that DNA methylation testing in urine is feasible and may provide a promising strategy for cervical cancer detection (Chapter 7). Recent studies have demonstrated that urine sampling is preferred over self-sampling\(^{65,66}\), and may potentially attract more women to the cervical screening programme. The potential of methylation testing in urine for the detection of cervical precancerous lesions has been demonstrated by Feng et al.\(^{67}\) and is currently being tested in the SOLUTION study at the Amsterdam UMC.

**Combinatorial analysis of miRNAs and DNA methylation**

Present data indicate that miRNAs and DNA methylation would potentially detect partially different stages of cervical cancer development, suggesting that their combined analysis might benefit (pre)cancer diagnosis. Indeed, the study from Babion et al. demonstrated that a 3-miRNA panel could complement DNA methylation analysis of FAM19A4 for the detection of cervical precancerous lesions\(^{57}\).

Moreover, in a preliminary analysis, where the miRNA and DNA methylation markers identified in Chapter 5 and Chapter 6 respectively were combined, we observed that their combination improved the clinical performance for CIN3 and cervical cancer detection when compared to their individual performance (unpublished data). These findings are in line with a previous report on lung cancer where the combined analysis of miRNAs and DNA methylation markers was shown to synergistically improve the detection rate of lung cancer\(^{68}\).

**Full molecular screening**

In The Netherlands, primary HPV screening is currently complemented with (repeat) cytology as triage strategy. Due to the unreliability of cytology testing on self-samples, women testing hrHPV-positive on their self-sample need to visit their general practitioner for a cervical scrape which results in loss to follow-up.

The miRNA and DNA methylation markers described in this thesis (Chapter 5 and 6) provide promising alternative triage strategies for hrHPV-positive women and pave the way for a transition to full molecular cervical screening of self-samples. In addition to self-samples, urine-based full molecular screening can also be envisioned.
In a full molecular screening setting, only hrHPV-positive women with a positive molecular triage test may need referral to the gynaecologist. DNA methylation markers (and potentially miRNA markers) have shown to detect nearly all cervical cancers and relevant advanced CIN2/3 lesions with high short-term risk of progression to cervical cancer. Indeed, a positive methylation triage test meets the 2-3 year CIN3+ risk threshold for colposcopy referral in the Netherlands (20%) (Dick et al. submitted). Although it has been shown that methylation triage negative women have a low 14-year CIN3+ risk similar to cytology triage negative women, a way to reduce the number of colposcopy referrals is to ask women who are molecular triage negative at baseline for a repeat hrHPV test at 24 months, and refer or triage with methylation in case of a hrHPV-positive test at that occasion. Since ~65% of the HPV positive women at baseline have cleared their HPV infection this procedure would identify women with persistent hrHPV infections > 2 years and most of the remaining clinically relevant cervical precancerous lesions at the costs of low number of colposcopy referrals.

As DNA methylation assays detect nearly all cervical cancers including the majority of rare histotypes and hrHPV negative carcinomas (Vink et al., submitted), some realistic near-term improvements would place methylation marker testing at the forefront of screening. With the increasing uptake of prophylactic HPV vaccines, methylation analysis can serve as a good assay to rule out the low numbers of cervical cancers caused by hrHPV genotypes not covered by the vaccine or characterised by absence of hrHPV.

**Novel therapeutic developments and options**

When women are diagnosed with early stage cervical disease, many are cured with radical surgery. Also, women that present with locally advanced disease often respond favourably to chemoradiation. However, in case of metastatic, recurrent or persistent disease the prognosis is usually poor. Since cervical cancer still leads to an estimated 311,000 deaths in 2018, there is need for novel therapeutics. Several strategies have been or are currently tested for the treatment of cervical cancer that includes cellular-based strategies such as adoptive immunotherapy and non-cellular strategies like checkpoint inhibition. At present, only very few therapeutics based on miRNAs or DNA methylation have entered preclinical trials for the treatment of cancer, and may well be of interest given their role in cervical carcinogenesis.

**miRNA therapeutics**

Since miRNAs are able to regulate important cellular processes, they have been proposed as promising therapeutic tools for cancer management. There are two strategies to manipulate miRNA expression and activity depending on whether they need to be up- or downregulated. The first strategy involves the inhibition of oncomiRs which is accomplished by single stranded oligonucleotides that contain the complementary sequence of the target miRNA (so-called
miRNA inhibitors or antimiRs\textsuperscript{73}. By directly binding the mature target miRNA, antimiRs can block miRNA function. The second strategy aims to increase the expression of tumour suppressive miRNAs using miRNA mimics. MiRNA mimics are synthetic double-stranded small RNA molecules that contain the corresponding miRNA sequence and thereby mimic their function. In 2013, the first miRNA mimic (MRX34; Mirna Therapeutics) entered the clinic in a multicentre phase I trial in various cancer patients\textsuperscript{73,74}. This trial was however terminated because of severe immune-related adverse events. Recently an antimiR directed against miR-155 (MRG-106; mirage Therapeutics) entered a phase I clinical trial for patients with cutaneous T cell lymphoma and preliminary results are encouraging\textsuperscript{75}.

**Figure 2. Concept of full molecular cervical screening.** Primary hrHPV testing performed on self-samples or urine in combination with miRNA and/or DNA methylation triage testing could improve the current screening situation using self-samples, which is shown as a reference below the dotted line.

**Therapeutic implications of DNA methylation and histone deacetylation**

The most successful epigenetic drug to date is 5-azacytidine (5AC), a DNA demethylating agent that is FDA approved for the treatment of myelodysplasticsyndrome\textsuperscript{76} and that is currently in clinical trials for the treatment of various other cancer types. DNA demethylating drugs are modified cytidines that incorporate into replicating DNA and bind the catalytic sites of DNMTs thereby irreversibly inhibiting DNMT activities and mediating their proteasomal degradation\textsuperscript{77}.
Another class of epigenetic drugs that may also hold promise for the treatment of HPV-induced cancers and precancerous lesions are histone deacetylase (HDAC) inhibitors. HDACs are enzymes that induce deacetylation of histones which is associated with a more condensed chromatin state and transcriptional gene silencing\(^7^8\). Recently, the pan-HDAC inhibitor Vorinostat was shown to stabilise the expression of tumour suppressors such as p53 and pRb targeted by viral oncoproteins E6 and E7 and to induce cell death of HPV16-positive cervical cancer cells\(^7^9\). This drug is already in phase 1 clinical trial for the treatment of head and neck cancer (HNC) and acute myeloid leukaemia (AML)\(^8^0,^8^1\).
REFERENCES


Summary and General Discussion


