GENERAL INTRODUCTION
INTRODUCTION TO CERVICAL CANCER

Cervical cancer arises from the uterine cervix, which is the lowermost portion of the uterus (Fig. 1). The outer part of the cervix (ectocervix) is covered by multi-layered squamous epithelial cells while the inner part of the cervix (endocervix) contains a single layer of glandular columnar cells. The border between these two types of cells is called the squamo-columnar junction (SCJ) and shifts from the ectocervix to the endocervix due to hormonal changes during puberty. The transformation zone (TZ) is the region between the original and the new SCJ. Cervical cancer arises at this zone following a persistent infection with high-risk types of the human papillomavirus (hrHPV). Squamous cell carcinoma (SCC) is the most common histological subtype of cervical cancer and accounts for approximately 80% of the cases, followed by adenocarcinoma (AdCa) which comprises approximately 10-20% of the cervical cancers. The remaining cases consist of rare histotypes such as neuro-endocrine and clear-cell carcinomas. While the introduction of cervical screening programmes has resulted in a large decline in the incidence of SCC, for AdCas an increase in incidence rates has been observed, in particular among young women.

With an estimated 570,000 new cases and 311,000 deaths in 2018, cervical cancer is the fourth most common cancer type among women worldwide and represents ~7% of all female cancers. The vast majority of cervical cancers occur in developing countries where it accounts for nearly 12% of all female cancers. The introduction of organised cervical screening programmes in developed countries has resulted in a major decrease in the incidence of and mortality from cervical cancer. Despite this, in The Netherlands around 800 women are diagnosed with cervical cancer and approximately 200-250 deaths from this disease are reported every year.

Figure 1. The uterus, vagina and cervix including the location of the endocervix, ectocervix and transformation zone.
HPV-INDUCED CERVICAL CARCINOGENESIS

Human papillomavirus

It is well-acknowledged that persistent infection with hrHPV is a necessary cause of nearly all SCCs and the large majority of AdCas\textsuperscript{1,2}. Unlike hrHPV, low-risk HPV types (lrHPV) such as HPV6 and HPV11 are associated with benign wart like lesions. HPV is a common sexually transmitted family of viruses and the majority of both women and men (80%) get infected with HPV shortly after starting sexual intercourse\textsuperscript{8,9}. HPVs are small, non-enveloped DNA viruses belonging to the Papillomaviridae family of which more than 180 different types have been identified\textsuperscript{10,11}. From these 180 types, around 50 are known to infect human mucosal epithelium. Presently, 12 hrHPV types are defined as group I cervical carcinogens (able to cause cervical cancer) by the WHO International Agency for Research on Cancer (IARC)\textsuperscript{12}. These include HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, and HPV59. Out of these 12 hrHPV types, HPV16 is by far the most dominant type causing cervical cancer (61%), followed by HPV18 (10%)\textsuperscript{13}. Together, they are associated with the majority of cervical cancers (>70%) while other hrHPV types are less frequently found. Furthermore, several HPV types are rarely detected in cervical cancer, but show a close phylogenetic relationship and are therefore classified as probable (IARC class 2A; HPV68) or possible (IARC class 2B; HPV26, HPV53, HPV66, HPV67, HPV70, HPV73 and HPV82) high-risk\textsuperscript{14}.

The HPV virus contains double-stranded circular DNA of approximately 7900 base pairs and consists of several genomic regions. The “early” region encodes proteins necessary for viral replication (a.o. E1, E2, E4, E5, E6, and E7), while the “late” region encodes the major and minor viral capsid proteins (L1 and L2, respectively)\textsuperscript{15}. The third region is the so-called long control region (LCR) and contains regulatory sequences and the origin of DNA replication.

An infection with HPV starts when viral particles enter the basal cells of squamous epithelium and may result in either a productive or transforming infection\textsuperscript{16}. The lifecycle of a productive HPV infection is tightly linked to the host differentiation programmes. The viral genome in the undifferentiated infected cells is replicated in conjunction with host DNA and maintained as stable episomes. In these cells, only low levels of the viral proteins are detected. The production of new viral progeny occurs in non-dividing terminally differentiated epithelial cells located in the surface layer of the epithelium. Here, the expression of viral proteins, such as E6 and E7, is increased and facilitates the activation of the DNA replication machinery to allow viral genome amplification in differentiated cells\textsuperscript{15}. Productive HPV infections might give rise to mild or moderate cellular abnormalities.
A transforming HPV infection has been associated with more severe cellular abnormalities and may ultimately lead to cancer. Transforming infections are typified by deregulated expression of the viral early genes E6 and E7 in proliferating basal cells. In these cells, the normal viral life cycle is aborted and E6 and E7 function as oncogenes. As a direct consequence of E6 and E7 deregulation, the expression of critical cell cycle and DNA repair regulators is altered. This results in both genetic and epigenetic host cell changes, ultimately leading to genomic instabilities and the activation of oncogenes as well as inactivation of tumour suppressor genes.

**Concept of hrHPV-induced cervical carcinogenesis**

Despite the fact that most sexually active women become infected with HPV at some point in their life, the vast majority of HPV infections (80%) are transient and will be cleared by the host immune system within 1-2 years without notice. The remaining infections may give rise to cervical precursor lesions (i.e. cervical intraepithelial neoplasia or CIN). These precursor lesions are graded from 1 to 3 based on the severity of dysplasia of the epithelium. CIN1 (mild dysplasia) and a subset of CIN2 (moderate dysplasia) are referred to as low-grade precursor lesions and are considered as the result of a productive infection, while CIN3 (severe dysplasia and carcinomas in situ) and the remaining subset of CIN2 lesions are known as high-grade precursor lesions, and are the consequence of a transforming infection.

The original concept of cervical carcinogenesis states that a minority of the hrHPV-infections in the TZ (20%) will result in a low-grade CIN1/2 lesion. These lesions are characterised by the production of new viral progeny and are therefore referred to as productive CIN lesions. The majority of these productive CIN lesions will regress to normal cervical epithelium upon clearance of the infection by the host immune system. Only a small set of the productive CIN lesions will persist and acquire transforming capacities leading to a transforming CIN lesion. Morphologically, transforming CIN lesions represent high-grade CIN2/3 lesions. While transforming CIN2/3 lesions can develop within 3-5 years following an hrHPV infection, progression to invasive cervical cancer may take 15-30 years. This slow progression provides ample time for early detection and subsequent treatment. Thus transforming CIN2/3 lesions represent a heterogeneous disease of which only a subset is thought to have a high short-term progression risk for cervical cancer.

Herfs and colleagues have proposed a new concept of cervical carcinogenesis. Here, it is suggested that cervical cancer arises from an hrHPV infection in a discrete population of SCJ cells which are considered as potential cervical cancer stem cells. SCJ cells grow in monolayer, and are not permissive for the entire HPV life cycle in contrast to other epithelial stem cells such as cervical reserve cells or basal keratinocytes. Infection of SCJ cells is thought to directly result in a transforming CIN lesion, while an HPV infection of the other cervical epithelial stem cells results in productive CIN lesions that may progress to CIN2/3.
Figure 2. Original and new concept of hrHPV-mediated cervical carcinogenesis. Infection with hrHPV in the transformation zone (TZ) is in most cases cleared by the host immune system. When HPV clearance does not occur a persistent infection results. A small subset of hrHPV infections may then result in productive infections leading to productive CIN lesions (CIN1 and subset of CIN2) of which the majority will regress within 1-2 years. A small part of the productive infections may progress to a transforming infection resulting in transforming CIN lesions (CIN3 and remaining CIN2). According to the new concept, hrHPV infections in squamocolumnar junction (SCJ) cells may directly lead to transforming CIN lesions. Progression from a transforming CIN lesion to invasive cervical cancer can take up to 30 years and is associated with an accumulation of (epi)genetic alterations. Adapted from Steenbergen et al. 16.
MOLECULAR HOST CELL ALTERATIONS

While a persistent infection with hrHPV is required for the initiation and maintenance of a transformed phenotype, additional genetic and epigenetic alterations are needed in order to progress to invasive cervical cancer. Deregulated E6 and E7 expression in dividing cells is known to cause chromosomal instabilities and this likely contributes to an accumulation of alterations in host cell genes over time. Both genetic and epigenetic alterations can result in the functional disruption of tumour suppressor genes as well as the activation of oncogenes. Genetic alterations affect the DNA sequence itself and both copy number aberrations (CNA) and DNA mutations have been detected in high-grade CIN2/3 lesions and cervical cancer. Epigenetic events do not alter the DNA sequence itself, but affect the accessibility of the DNA for transcription factors, thereby influencing gene expression. This can be achieved by modifications of either the histone proteins or bases in the DNA sequence. Such modifications include acetylation, methylation, phosphorylation and ubiquitination. In this thesis, we will focus on epigenetic alterations during cervical carcinogenesis, in particular miRNA expression and DNA methylation.

microRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules with an average of 22 nucleotides in length. By interacting with the 3' UTR of target mRNAs, they are key players in the posttranscriptional regulation of gene expression. In cancer, miRNAs are often found deregulated and they can either function as tumour suppressors or oncogenes (oncomiRs) depending on the genes and pathways they target.

The biogenesis of miRNAs starts with the transcription of miRNA genes which are primarily located in introns of protein coding genes. Following transcription, the primary miRNA (pri-miRNA) of ~1000 nucleotides is cleaved by the RNAse III enzyme Drosha and its cofactor the double-stranded RNA (dsRNA)-binding protein (dsRBP) DiGeorge critical region 8 (DGCR8) resulting in a precursor miRNA (pre-miRNA). The pre-miRNA is subsequently exported from the nucleus to the cytoplasm via Exportin 5 (XPO5) which creates a transport complex with Ran-GTP (Fig. 3). In the cytoplasm, the pre-miRNA is further cleaved by the RNAse III enzyme Dicer in cooperation with trans-activation-responsive RNA-binding protein (TRBP) which generates a ~22 nucleotide mature miRNA duplex. One strand of this duplex is incorporated in the RNA induced silencing complex (RISC) and guides the complex to target mRNAs resulting in mRNA degradation or translational repression. The tightly regulated biogenesis of miRNAs is accomplished by several miRNA processing proteins of which some have been described above. A more detailed description of this process including the proteins that are involved is reviewed in and described in Chapter 2. This leads to a miRNA duplex of ~22 nucleotides from which one strand is incorporated in the RNA induced silencing complex (RISC) together with an Argonaute (AGO) protein. The miRNA then guides the complex to target mRNAs resulting in their degradation or translational repression.
Figure 3. The pathway of microRNA processing. The first step in the tightly regulated process of microRNA biogenesis is the transcription of miRNA genes resulting in a primary miRNA (pri-miRNA). The pri-miRNA is subsequently cleaved by the RNase III enzyme Drosha and its cofactor the double-stranded RNA (dsRNA)-binding protein (dsRBP) DiGeorge critical region 8 (DGCR8) resulting in a precursor miRNA (pre-miRNA). The pre-miRNA is transported from the nucleus to the cytoplasm by Exportin 5 (XPOS) and its cofactor Ran-GTP followed by further cleavage by the RNase III enzyme Dicer in cooperation with trans-activation-responsive RNA-binding protein (TRBP).
In cervical cancer, many miRNAs have been found deregulated in cervical cancer tissues and cell lines including miR-9, miR-21, miR-203 and miR-375 \(^{25-27}\). Interestingly, both E6 and E7 are known to alter the expression of miRNAs. For example, E6 was shown to downregulate miR-218, miR-23b and miR-34 \(^{28-30}\), while E7 was reported to increase the expression of the miR-15a/16-1 cluster \(^{31}\). Furthermore, Wilting et al. showed that the altered expression of a number of miRNAs is associated with chromosomal alterations or changes DNA methylation of several genes that are frequently found during cervical carcinogenesis \(^{26,32}\). The above mentioned studies have all focused on intracellular miRNAs, while recent studies have reported on altered miRNA expression patterns in exosomes from cervical cancer cells \(^{33}\). Exosomes are small extracellular vesicles that are secreted by cells, including tumour cells \(^{34}\). Honegger and colleagues have reported on a miRNA signature that they found to be highly expressed in exosomes secreted by cervical cancer cells. Interestingly, upon E6/E7 silencing the expression of this miRNA signature was significantly altered in exosomes which indicates the involvement of HPV on exosomal miRNA composition \(^{33}\).

**IsomiRs**

Recent advances in small RNA sequencing technology have revealed that miRNAs exist as multiple length and sequence variants, termed isomiRs \(^{35}\). IsomiRs can comprise elongations, trimmings, editing and non-templated additions (NTA) that can occur at the 5’ or 3’ ends or both. These sequence and length differences are generated by miRNA processing proteins that include ribonucleotidyl transferases (rNTAses), exonucleases and RNA editing enzymes. In addition, heterogeneity in length can also arise from imprecise cleavage by Drosha and Dicer. To date, little data exist on the role of miRNA processing proteins in cervical carcinogenesis.

**DNA methylation**

DNA methylation is a process in which a methyl group \((\text{CH}_3)\) is covalently bound to the DNA and specifically occurs at cytosines preceding a guanine residue \((\text{CpG})\) in the human genome. The transfer of a methyl group is catalysed by the DNA methyltransferases (DNMTs) DNMT1, DNMT3A and DNMT3B. While DNMT1 is the key maintenance methyltransferase, DNMT3A and DNMT3B are de novo DNMTs that are involved in the methylation of unmethylated DNA \(^{36}\). Genomic regions that contain a high density of CpG sites are called CpG islands, and are generally found in promoter regions of protein-coding genes \(^{37}\). In normal cells, these CpG islands are mostly unmethylated while during malignant transformation local hypermethylation of CpG islands in promoter regions is commonly observed. Hypermethylation of promoters typically results in transcriptional repression and often occurs in tumour suppressor genes.

In cervical carcinogenesis, DNA methylation has gained much attention. Interestingly, the viral oncoproteins E6 and E7 have been shown to affect the expression and activity of DNMTs \(^{38}\). Following p53 degradation induced by E6, the transcription activator specificity protein 1 (Sp1)
binds the promoter of DNMT1 and enhances its expression. In addition, E7 has been shown to both directly and indirectly interact with DNMT1. The indirect mechanism involves E7 binding to pRb which results in release of E2F and subsequent binding of E2F to DNMT1. E2F binding to the promoter of DNMT1 results in overexpression of DNMT1. The potential role of E6 and/or E7 on the expression and activity of DNMT3A and DNMT3B remains to be elucidated.

Aberrant DNA methylation of a number of candidate tumour suppressor genes has been found in CIN lesions and cervical cancer. These methylated genes have been identified by either genome-wide or targeted gene approaches and include CADM1, EPB41L3, FAM19A4, GHSR, MAL, miR-124-2, PAX1, SST, and ZIC116,39-41.
CERVICAL CANCER PREVENTION

In general prevention of cancer is defined on different levels. The prevention of disease in healthy people is called primary prevention. The use of prophylactic HPV vaccines in young HPV naïve women is a good example. Secondary prevention is the early detection and subsequent treatment of (pre)cancer in asymptomatic women. This is the case in cervical cancer screening. Tertiary prevention is the treatment of patients with clinical cancer in order to prevent the progression of the disease. In this section, we will focus on primary and secondary prevention in the context of cervical cancer.

Primary prevention: Prophylactic HPV vaccines
Prophylactic vaccines consist of L1-based virus-like particles (VLPs) that mimic the morphology of native viral particles but lack viral DNA and are therefore non-infectious. Presently, three prophylactic HPV vaccines are available. These include 1) the bivalent Cervarix® vaccine (GlaxoSmithKline) which provides protection against HPV16 and HPV18, 2) the quadrivalent Gardasil® vaccine (Merck) which protects against HPV16 and HPV18 and low-risk types HPV6 and HPV11, and 3) the nonavalent Gardasil9® vaccine (Merck) which provides protection against HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52, and HPV58. For both the bivalent and the quadrivalent HPV vaccine cross-reactivity against other oncogenic HPV types such as HPV31 and HPV45 has been observed. Importantly, HPV vaccines have shown to be highly effective and safe.

In 2009, the bivalent HPV vaccine was introduced in The Netherlands for all 12 year old girls. The vaccination uptake rate was initially 58% (2010) and considerably declined over recent years to 46% in 2017.

Secondary prevention: hrHPV-based cervical screening
In the early seventies cervical cancer screening by cytology was introduced in three regions in the Netherlands. Up to 1988 this intervention gradually spread over the country and in 1996 a nation-wide screening programme was formally introduced. The slow progression of cervical precancerous lesions to invasive cancer provides ample time for early detection and timely treatment, explaining the success of moderately sensitive cytology screening tool.

The introduction of population-based cervical screening programmes using cytology has resulted in a substantial decline in the incidence of cervical cancer from 9.2 per 100,000 women in 1988 to 6.9 per 100,000 women in 2014. However, disadvantages of cytology include its subjective nature and its limited sensitivity for cervical (pre)cancer (~60%). Importantly, recent studies have demonstrated that primary hrHPV testing is more sensitive in detecting CIN2/3 and cervical cancer compared to primary cytology testing. As a result in The Netherlands in...
2017 primary hrHPV testing has replaced cytology-based screening. A schematic overview of the Dutch cervical screening programme is depicted in Figure 4.

In order to increase participation, the new screening programme offers self-collection of cervico-vaginal specimens (self-sampling) for hrHPV testing. Women who do not attend the Dutch screening programmes, i.e. 30-40% of the invited women, so-called non-attendees, are at increased risk of developing cervical cancer. More than half of cervical carcinomas are detected in these women. Offering self-sampling for hrHPV testing (HPV self-sampling) to non-attendees has been shown to increase the efficacy of the screening program. Recent research has shown that HPV self-sampling is equally effective in detecting CIN3 and cervical cancer when compared to hrHPV testing on physician-taken specimens. Another novel development is the use of urine for HPV testing. The detection of hrHPV in urine for cervical cancer detection is feasible and is currently under intense investigation.

HPV tests detect both transient HPV infections and HPV infections associated with disease (CIN lesions). Although primary hrHPV testing with a clinically validated test has a higher sensitivity for CIN3 and cervical cancer detection than cytology, its specificity is 3-5% lower compared to cytology-based testing. This is related to the fact that clinically validated hrHPV tests to a certain extent still detect transient hrHPV infections that do not give rise to cellular abnormalities. In order to avoid over-referral and overtreatment of women with a positive HPV test, an additional test, so-called triage test, is used to identify women with clinically meaningful cervical disease (CIN lesions) that need referral to the gynaecologist. In The Netherlands triage testing of hrHPV-positive women is done by cytology at baseline and 6 months (repeat cytology).

Repeat cytology was implemented to overcome the relatively low sensitivity of cytology testing. Since cytological examination of self-samples is not reliable, women testing positive for hrHPV on their self-sample need to have a physician-taken smear for triage testing by cytology. The extra physician taken smear results in a prolonged diagnostic track and is expected to result in a loss to follow-up of ~20%. Given these restraints, alternative more objective triage methods based on molecular techniques applicable to both cervical scrapes and self-samples are presently developed.

Alternative triage methods
Several morphological and molecular markers have been investigated as potential triage strategy for the management of hrHPV-positive women. At present, two methods have been clinically validated and are now commercially available. These include the p16/Ki-67 co-expression assay (CINtec® PLUS; Roche) and the FAM19A4/miR124-2 DNA methylation assay (QIASure®
Methylation Test; Qiagen). Concomitant expression of the biomarkers p16^{INK4A} and Ki-67 rarely occurs in the same cell, but when it does it is considered to indicate HPV-mediated transformation of the cell\(^{66}\). Furthermore, the DNA methylation assay from Qiagen is a multiplex quantitative methylation specific PCR (qMSP)-based assay that detects promoter hypermethylation of the genes FAM19A4 and miR124-2 in both cervical scrapes and self-samples.

**Figure 4. Schematic overview of the Dutch cervical screening programme.** Women aged 30-60 years are invited to undergo screening once every five years. Screening consists of a physician-taken sample (cervical smear) which is tested for the presence of hrHPV. When tested hrHPV positive, the same cervical smear is also assessed cytologically. If abnormal cells are present, the woman is referred to a gynaecologist. If there are no abnormal cells, the woman is advised to have a second smear test made in 6 months. As an alternative, Dutch women may participate using a self-sampling device. Using this device, the woman can collect a sample from the vagina herself. This material is tested for hrHPV in the laboratory. No cytological assessment can be performed using this material. Therefore, if hrHPV is found, the woman will still need to have a primary care provider perform a smear test. Adapted from \(^{54}\).
Molecular markers based on host cell alterations driving carcinogenesis and that can be applied on both cervical scrapes and self-samples are highly promising. At present, several DNA methylation markers have been tested for triage of hrHPV-positive women with encouraging results for their ability to detect CIN2/3 and cervical cancer\textsuperscript{16,67,68}. In addition, Tian and colleagues have reported on a number of altered miRNAs as promising triage tool for hrHPV-positive women\textsuperscript{69}. They showed that a 3-miRNA panel exhibits superior performance over cytology-based triage testing for high-grade CIN detection in hrHPV-positive cervical scrapes.

Altogether these studies have shown that molecular triage of hrHPV-positive women based on miRNA or DNA methylation markers is feasible. Despite this, limited data is available on the use of molecular markers for triage of hrHPV-positive women in non-invasive material such as self-samples and urine and warrants further investigation. Ultimately, improved knowledge of epigenetic alterations during cervical carcinogenesis may enable better risk stratification of hrHPV-positive women for the development of cervical cancer, which is the focus of this thesis.
THESIS OUTLINE

As described above, persistent infection with hrHPV is necessary but not sufficient for the development of cervical cancer. Additional genetic and epigenetic alterations are required for malignant transformation and have shown to accumulate with progression of disease. Despite our increasing knowledge on HPV-mediated carcinogenesis, the process of malignant transformation initiated by HPV is still not completely understood. A better understanding of epigenetic alterations contributing to cervical carcinogenesis may aid in the identification of clinically relevant lesions in need of immediate treatment. To establish this purpose, we applied both targeted and genome-wide approaches to evaluate alterations in miRNA expression and DNA methylation. A more detailed description of the studies performed in this thesis is listed below.

In Chapter 2, we provide an overview of currently available findings on alterations of miRNA processing proteins as well as enzymes involved in isomiR biogenesis during HPV-induced carcinogenesis. Processing of miRNAs and isomiRs is tightly regulated by a number of proteins and alterations in any of these proteins may disturb efficient processing and could drastically alter miRNA and isomiR function. Several miRNA processing proteins were found altered in HPV-induced cancers, including Drosha, DGCR8, Dicer, and AGO. Interestingly, in some cases viral oncoproteins E6 and E7 were implicated in their deregulation. For enzymes involved in isomiR biogenesis such as ADAR, TENT4B, AND TUT1 only limited data is available in HPV-induced cancers. Despite this, TCGA and in-house data suggest their potential involvement in early stages of cancer development and indicate the strong need for further investigation.

The most commonly employed method for accurate miRNA expression analysis is reverse transcription-quantitative PCR (RT-qPCR). In order to reliably analyse miRNA expression using RT-qPCR, adequate data normalisation is essential to remove nonbiological, technical variations. In Chapter 3, we evaluated 11 candidate reference genes in different cervical specimens (i.e. tissues, scrapes, and self-samples). By using three commonly employed algorithms (GeNorm, NormFinder, and BestKeeper) we identified the biologically most stable reference genes for each sample type. Our results demonstrate that the most optimal reference genes may differ between specimen types and that adequate normalisation could improve data interpretation.

Although numerous studies have focused on altered miRNAs in cervical tissues, data on the diagnostic use of altered miRNAs in cervical scrapes is limited. Therefore, in Chapter 4 we set out to evaluate the clinical value of eight previously identified miRNAs in cervical scrapes. The expression of these miRNAs was previously shown to be altered in cervical tissues from women with CIN3 or cervical cancer due to either methylation-mediated silencing or chromosomal alterations. In this study, we found similar miRNA expression patterns in cervical scrapes.
compared to cervical tissues. A miRNA classifier consisting of miR-15b and miR-375 showed good clinical performance and detected the majority of CIN3 and all of the cervical cancers. Combining HPV16/18 genotyping with the 2-miRNA classifier further improved the detection rate of CIN3. Interestingly, both miRNAs were shown to be functionally involved in cervical carcinogenesis by affecting cervical cancer cell viability.

In 2017, The Netherlands took the lead in offering women that do not participate in cervical screening, so-called non-attendees, self-sampling for hrHPV-testing. Women that test hrHPV-positive on their self-sample require triage testing. Since cytological examination of self-samples is not reliable, molecular triage markers that can be directly applied on self-samples are needed. In recent years, deregulated miRNAs have emerged as promising triage markers for risk stratification of hrHPV-positive women. Due to the large overrepresentation of non-disease related cells in self-samples, molecular triage markers such as altered miRNAs identified in cervical tissues are not necessarily of good clinical value in self-sampled material. Therefore, in Chapter 5 we performed a genome-wide miRNA discovery screen directly on hrHPV-positive self-samples. A panel of 9 miRNAs was identified with strong discriminative power for CIN3 detection. Validation by RT-qPCR in an independent large series of hrHPV-positive self-samples and subsequent logistic regression analysis resulted in a 5-miRNA classifier consisting of let-7b, miR-15b, miR-20a, miR-93, and miR-222). This 5-miRNA classifier showed good clinical performance for both CIN3 (67% detected) and cervical cancer (93% detected).

Recent studies have demonstrated that altered DNA methylation markers exhibit good clinical performance for CIN3 and cervical cancer detection. However, as mentioned above, these markers do not necessarily perform well in self-samples. In order to identify the most discriminative DNA methylation markers for CIN3 and cancer detection in self-samples, in Chapter 6 we performed a genome-wide DNA methylation marker discovery screen directly on hrHPV-positive self-samples.

This resulted in the identification of 12 DNA methylation markers for CIN3 detection. Analysis by quantitative multiplex PCR (qMSP) in independent series of hrHPV-positive lavage and brush self-samples followed by logistic regression analysis revealed a 3-gene classifier consisting of ASCL1, LHX8, and ST6GALNAC5 with high discriminatory power for CIN3 detection applicable to self-samples of both devices. Validation of this 3-gene classifier by qMSP in large independent sets of hrHPV-positive self-samples showed a very good and reproducible clinical performance in both lavage and brush self-samples for CIN3 detection. Importantly, the 3-gene classifier was able to detect all cervical cancers.
Urine sampling for hrHPV-testing is emerging as an alternative to cervical sampling. Recent studies have established that this manner of non-invasive sampling is preferred over cervical sample collection either by physician or by self-sampling. Previous studies by us and others have focused on DNA methylation analysis in cervical tissues, scrapes and self-samples for cervical cancer detection but limited data exist on DNA methylation analysis in urine. Therefore, in Chapter 7, we tested six previously identified DNA methylation markers in urine samples from cervical cancer patients and compared the results with cervical scrapes. HrHPV testing as well as DNA methylation analysis was compared between different urine components and revealed highly similar results. A strong agreement was found between hrHPV testing on urine and scrapes and 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 show a good discriminatory power for cervical cancer detection.

In Chapter 8, we present a summary of our findings and discuss the clinical implications and future applications.
REFERENCES


