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6. THESIS OUTLINE
1. THE EPIDEMIOLOGY OF CERVICAL CANCER

Cervical cancer is the fourth most common cancer in women worldwide after breast, colorectal and lung cancer\(^1\) (Figure 1). With an estimated occurrence of 528,000 new cases and 266,000 deaths in 2012, it represents a major public health problem. Large differences in incidence and mortality rates have been observed between developed and low-resource countries. Approximately 82% of cervical cancer cases and 85% of deaths have been found in less-developed regions (Figure 1), with the highest incidence and mortality rates observed in Africa, Melanesia and Central America\(^1\). With these figures, cervical cancer is the most common female cancer in Eastern Africa. The high disease burden in developing regions is at least partially due to the absence of cervical screening programmes that have shown to decrease cervical cancer incidence and mortality rates in developed regions\(^2,3\).

In the Netherlands, cervical cancer comprises approximately 1.7% of all newly diagnosed female malignancies. This corresponds to 735 women diagnosed, (age standardized incidence rate 2012: 8.0/100,000)\(^4,5\) and 215 deaths in 2012 (age standardized mortality rate 2012: 2.1/100,000)\(^4,5\). The overall 5-year survival rate of cervical cancer in the Netherlands is 66% and the highest cervical cancer incidence is observed in women aged 40 to 44 years\(^4\).

![Figure 1. Estimated number (per 1000) of the incidence (new cancer cases) and mortality (deaths) in women in developed and developing regions worldwide in 2012. The developed regions comprise all regions of Europe and Northern America, Australia/New Zealand and Japan. The less developed regions comprise all regions of Africa, Asia (excluding Japan), Latin America and the Caribbean, Melanesia, Micronesia and Polynesia. Adapted from GLOBOCAN 2012\(^1\).](image-url)
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2. HPV-MEDIATED CARCINOGENESIS

2.1 Cervical cancer

Infection with a high-risk type of human papillomavirus (hrHPV) is acknowledged as a necessary cause of cervical cancer. Cervical cancer arises in the uterine cervix. The cervix consists of the endocervix (the inner part of the cervical canal), covered with a single layer of glandular columnar cells and the ectocervix (the outer part of the cervix) covered with a multi-layered non-keratinizing squamous epithelium. The border between these two types of epithelium is called the squamo-columnar junction (SCJ) (Figure 3). During puberty, hormonal changes will induce acidification of the vagina resulting in the replacement of a number of columnar cells from the endocervix by metaplastic squamous epithelium. This results in a shift of the SCJ from the ectocervix to the endocervix. The area with metaplastic squamous cells between the original and new SCJ is called the transformation zone (TZ) (Figure 2). Originally, cervical cancers were considered to arise from this zone. Recently, cells from a small, single layered embryonic cell population that localizes at the SCJ of the cervix have been proposed as the more likely precursor of cervical (pre-)cancer. These cells have a unique cuboidal morphology and a typical expression pattern of so-called SCJ-cell specific genes. The SCJ-specific expression profile is also present in the majority of cervical carcinomas. Therefore, it is now suggested that the majority of cervical (pre-)cancers arise from an infection with hrHPV in the SCJ cells, instead of cells from the transformation zone.

Histologically, cervical carcinomas can be classified in different subtypes. Squamous cell carcinomas (SCC) constitutes most cases (80%), followed by adenocarcinomas (AdCA) comprising approximately 15% of cases. The remaining 5% accounts for rare histotypes including neuro-endocrine carcinomas and clear-cell carcinomas.

Figure 2. Anatomy of the uterus and the cervix. SCJ= Squamous columnar junction.
2.2 Precursor lesions of cervical cancer

Cervical cancer develops through different precursor lesions (i.e. cervical intraepithelial neoplasia or CIN), that are traditionally categorized in three groups. In case of CIN1 (mild dysplasia), morphological changes occur up to the lower 1/3 of the epithelium (Figure 3). These lesions are also referred to as low-grade precursor lesions. In CIN2 (moderate dysplasia) the lower 2/3 of the epithelium shows dysplasia, and in CIN3 (severe dysplasia and carcinomas in situ) dysplastic cells are present in more than 2/3 up to the entire epithelium. CIN2 and CIN3 lesions are also referred to as high-grade precursor lesions (Figure 3). According to national guidelines, women with CIN2 and CIN3 are treated by excision of the dysplastic lesion to prevent the development of SCC. It has however been reported that 19-50% of the CIN2/3 lesions spontaneously regress in 3 months to 2 years after development, whereas only approximately 30%, when left untreated, may ultimately progress to cancer. Although the precursor stages of SCCs are well defined, there is still relatively little known about the precursor lesions of AdCAs that are suggested to arise from glandular cells in the endocervix. Currently, the best defined precursor is called adenocarcinomas in situ (ACIS). Women with ACIS are also advised to be treated.

![Figure 3](image)

2.3 Human papillomavirus

DNA from high-risk types of HPV can be detected in virtually all SCCs and in the majority of AdCAs. HPV is a sexually transmitted virus that is commonly found among young, sexually active women. Upon estimation, more than 80% of women will acquire at least one genital HPV infection during their life-time. The HPV prevalence depends on age and the HPV virus is most frequently found in women between the age of 20-25 years (approximately 20%) and gradually decreases with increasing age to as low as 3% in women older than 45 years. Additional factors correlated with an increased risk of HPV infection are a high number of sexual partners, smoking and a young age of first sexual intercourse.
HPVs are small double-stranded DNA viruses that belong to the family of *Papillomaviridae*. The viruses are strictly epitheliotrophic and can be categorized into mucosal (alpha genus, infecting the mucosa of the anogenital region, the oropharynx, and the respiratory tract) or cutaneous (beta and gamma genera, infecting the skin epithelium) depending on their preferential side of infection. The genome of HPV consists of circular DNA of approximately 7900 base pairs. It contains a non-coding long control region (LCR), early (E1, E2, E4, E6 and E7) open reading frames (ORFs) and late (L1 and L2) ORFs (Figure 4).

A new HPV type is defined when more than 10% of the viral DNA sequence of E6, E7 and L1 is different from that of the already known HPV types. To date, more than 170 different types have been characterized of which approximately 40 are known to infect the genital mucosa. Depending on their oncogenic potential, HPV types are classified into low-risk (lrHPV) or high-risk (hrHPV). LrHPV types, such as HPV6 and HPV11, are linked to benign warts or low-grade lesions that do not undergo malignant transformation. HrHPV types on the other hand, may eventually cause malignant transformation of the infected epithelium.

According to phylogenetic criteria presented by the International Agency for Research on Cancer (IARC), twelve mucosal HPV types have been defined as Group 1 human carcinogens and are classified as hrHPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59). Another eight HPV types are rarely but consistently detected as single infections in cervical cancer, but lack evidence of biological activity in tumour tissues (IARC group 2). One of these HPV types (HPV68) has been identified as probably high-risk based on limited evidence of carcinogenicity in humans and sufficient evidence of carcinogenicity in animal experiments (IARC Group 2A). The other seven HPVs (HPV26, 53, 66, 67, 70, 73, and 82) are classified as possibly high-risk (IARC Group 2B) based on limited evidence of carcinogenicity in humans, yet less than sufficient evidence in animal models.

A large difference is observed between the oncogenic potential of the different hrHPV types. HPV16 is known to cause more than 50% of all cervical cancers worldwide and is together with HPV18 (16%) and HPV33 (4%) responsible for 70% of all cervical carcinomas and about 50% of its high-grade precursor lesions. A difference in viral types present in different cervical cancer histotypes is observed, with the exception of HPV16 being prominent in all histotypes. HPV16 (59.3%), 18 (13.2%) and 58 (5.1%) are the most common HPV-types encountered in SCCs, whereas HPV16 (36.3%), HPV18 (36.8%) and HPV45 (5.2%) are most frequently found in AdCAs.
2.3.1 The viral life cycle and productive infections

The HPV infectious life cycle is tightly coupled to the differentiation programme of the infected host epithelium. The complex life cycle begins when micro-lacerations in the mucosa permit the virus to gain access to the epithelial basement membrane\(^\text{32}\) (Figure 5)\(^\text{33}\). The HPV particles are then suggested to enter the basal cells by binding to cell surface receptors, most likely heparin sulphate proteoglycans\(^\text{34,35}\). This results in conformational changes in the capsid proteins and the transport of viral DNA into the nucleus, though the exact manner of viral transport has not yet been fully elucidated.

After infection, the viral DNA is maintained at low copy number episomes in dividing basal epithelial cells\(^\text{36}\). In this stage, expression of the viral genes is very low, which contributes to immune escape. Upon division and differentiation of the basal cells to the supra-basal cells, viral gene expression increases which results in a high-copy number of episomes. Finally, encapsidation takes place in non-dividing, terminally differentiated epithelial cells to yield progeny virions. The viral gene products are functionally involved in this complicated process, and the use of the host replication machinery is essential. This type of infection, in which new viral particles are formed and released, is referred to as a productive infection. These infections may coincide with mild to moderate cellular abnormalities that are histomorphologically comparable with CIN1/2, but do not reflect a true precancerous stage.

The expression of the viral proteins during a productive infection is tightly associated with the different layers of the epithelium (Figure 5)\(^\text{37}\). The viral protein E1 is a DNA helicase that recruits the cellular polymerases and proteins required for viral replication and is mainly expressed in the basal and supra-basal layers\(^\text{38}\). The viral protein E2 regulates transcriptional activation and repression by binding to E2 binding sites (E2BS) within the LCR. It functions as a mediator of expression of the viral proteins E6 and E7. In addition, it aids E1 in binding to the origin of replication and tethers viral DNA to the cells after cell division\(^\text{38,39}\). This protein is mainly expressed in the supra-basal layers.
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The E4 protein is expressed in the mid-layers to upper layers of the epithelium and aids DNA amplification by inducing a G2 cell-cycle arrest\textsuperscript{40,41}. In addition, it is able to destabilize the cytokeratin network and is responsible for viral assembly in the upper layers\textsuperscript{42}. E5 has a role in mediating signals from growth factors and has the capacity to interact with E6 and E7\textsuperscript{43,44}.

Viral E6 and E7 proteins are necessary to create conditions that allow viral replication in differentiated non-dividing epithelial host cells, in which the DNA replication machinery is normally not activated. The expression of the latter viral proteins is tightly regulated to the mid-layer of the epithelium. E6 binds to the human tumour-suppressor protein p53 (TP53) encoded by the $TP53$ gene via an E6-associated protein (E6AP) ubiquitin ligase, thereby targeting TP53 for ubiquitination and degradation through the proteasome\textsuperscript{45}. Since TP53 plays an important role in cell cycle control and apoptosis, targeting TP53 for degradation results in loss of apoptotic processes\textsuperscript{46,47}. E7 binds to the retinoblastoma tumour suppressor protein (pRB) encoded by the $RB1$ gene and disrupts the binding of pRB to E2F transcription factor, thereby releasing E2F which leads to S-phase entry\textsuperscript{33}. Altogether, E6 and E7 expression in mid-differentiated cell layers stimulates a condition of DNA synthesis in the infected host cell, thereby supporting viral replication. Finally, the late genes L1 and L2 are responsible for forming the protein capsid and are expressed in the upper layers of the epithelium.

Figure 5. Schematic representation of a productive infection and the expression of the viral genes. Adapted from Doorbar et al\textsuperscript{22}.

2.3.2 Transforming infections

When deregulated expression of the viral E6 and E7 proteins in the proliferating basal cells occurs, viral transformation may be stimulated\textsuperscript{37}. Such so-called transforming infections are found in a subset of CIN2 lesions and most CIN3 lesions, as well as cervical cancers (Figure 6). In the context of dividing cells, the E6- and E7-encoded proteins function as oncoproteins and the respective genes are referred to as viral oncogenes. A direct result from E6 and E7 deregulation is the altered expression of cell cycle and DNA repair regulators through complex formation with various cellular proteins. The combined disruption of cell cycle
control and the prevention of apoptosis provides a mechanism of malignant transformation by inducing genomic instability\textsuperscript{7,48}. The resulting accumulation of (epi)genetic alterations in the host cell genome affecting both oncogenes and tumour suppressor genes is considered a driving force for malignant progression\textsuperscript{49,50}.

**Figure 6.** Schematic representation of a transforming infection. The deregulation of viral gene expression increases with increasing lesion grade. Proliferating cells are represented by red nuclei. Cells expressing E4 are shown in green and cells expressing L1 are shown in yellow. Persistent deregulated gene expression, as occurs in CIN3 and following viral genome integration, can lead to the accumulation of secondary genetic changes in the infected host cell and development of cancer. Adapted from Doorbar et al\textsuperscript{22}.

Apart from targeting TP53 (as described above), E6 interacts with pro-apoptotic proteins BCL2-Associated X Protein (BAX), BCL2-Antagonist/Killer (BAK), Fas-associated via death domain (FADD) and Apoptosis-Related Cysteine Peptidase 8 (procaspase-8) and targets them for degradation by the proteasome\textsuperscript{51–54}. In addition, E6 activates human Telomerase Reverse Transcriptase (hTERT), the catalytic subunit of the telomerase enzyme.

This enzyme allows telomere lengthening and has to be activated for cells to become immortal. Apart from targeting pRB (as described above), E7 binds other pocket protein family members Retinoblastoma-like 1 (p107) and Retinoblastoma-like 2 (p130). The resulting increased E2F activity becomes evident through the upregulation of E2F-responsive gene products, such as proliferating cell nuclear antigen (PCNA), marker of proliferation Ki-67 (Ki-67), minichromosome maintenance proteins (MCMs), cyclin E (CCNE1) and cyclin-dependent kinase inhibitor 1 (p21)\textsuperscript{55}. Also the cyclin dependent kinase inhibitor 2A (CDKN2A) gene, which resides at the INK4A-ARF tumour suppressor locus and encodes the p16\textsuperscript{INK4A} protein, will become overexpressed as a result of E7 expression in proliferating cells. HPV16-E7 has been shown to upregulate p16\textsuperscript{INK4A} directly through induction of the histone 3 lysine 27-specific demethylase KDM6B, thereby interfering with polycomb repressive complex (PRC)-mediated silencing of p16\textsuperscript{INK4A}\textsuperscript{56}. KMD6B-mediated p16\textsuperscript{INK4A} upregulation, resulting in an inhibition of CDK4/CDK6 activity, has recently been suggested to be essential for the survival of cells from cervical cancer cell lines\textsuperscript{57}. 
Earlier it has been suggested that $p16^{INK4A}$ upregulation mainly results from the absence of a pRB-mediated negative feed-back loop in the presence of HPV16-E7\textsuperscript{58}. Whatever exact mechanism, pRB degradation by E7 prevents a $p16^{INK4A}$-induced cell cycle arrest. Hence diffuse expression of $p16^{INK4A}$ in proliferating cells is therefore considered as a marker for lesions harbouring a transforming hrHPV infection\textsuperscript{59,60}.

Additionally, both E6 and E7 can modulate the DNA methylation machinery, thereby influencing cellular and viral gene expression. E6 can induce the upregulation of the DNA (cytosine-5)-methyltransferase 1 protein (DNMT1) via suppression of p53\textsuperscript{61} whereas E7 associates with DNMT1 directly and stimulates its enzymatic activity\textsuperscript{62}. The activity of DNMT1 enhances methylation-mediated silencing of tumour suppressor genes\textsuperscript{63}. E7 also interacts with histone deacetylases (HDACs) which induces chromatin remodelling and thus changes the accessibility of different gene promoters (e.g. E2F)\textsuperscript{64}.

To date, the exact mechanisms responsible for the deregulation of E6 and E7 expression are still debatable. Viral DNA integration, as found in many cervical cancers and subsets of CIN2/3 lesions, has been suggested as one mode to trigger E6/E7 deregulation\textsuperscript{65}. Upon viral DNA integration, the HPV genome is often disrupted in or near the E2 ORF, thereby affecting the expression of E2, which in turn may stimulate E6/E7 expression\textsuperscript{65,66}. However, not all cervical carcinomas contain integrated viral DNA\textsuperscript{67,68}, and the question remains whether integration results from increasing genomic instability due to E6/E7 deregulation\textsuperscript{67}, or whether integration is the actual cause of genomic instability. Recently, more evidence has emerged that viral integration mostly coincides with transcriptionally active regions in the host cell genome. These findings suggest that integration, as it occurs in stabilized DNA regions, probably takes place before genomic instability. As integration often takes place in or close to genes, this might result in modulation of gene expression that eventually might contribute to carcinogenesis\textsuperscript{69}. Since previous studies have also observed absence of E6 and E7 repression despite an intact E2 ORF\textsuperscript{70,71}, there should exist alternative mechanisms of E6/E7 deregulation. One of them involves altered intraviral control of E6 and E7 expression resulting from epigenetic alterations of the viral genome (for example, methylation of viral promoter regions\textsuperscript{72}). As such, methylation of the CpGs in the E2BS have shown to inhibit the binding of E2, resulting in loss of E2 repression of E6/E7 expression\textsuperscript{71}.

### 2.3.3 Concept of hrHPV induced cervical carcinogenesis

The concept of cervical carcinogenesis is illustrated in Figure 7. Cervical cancer develops through different phases: hrHPV infection, hrHPV persistence, hrHPV transformation, development of precursor lesions and finally invasion to cervical cancer. Backward steps also occur, with hrHPV clearance and regression of cervical precursor lesions to normality. hrHPV infections can either take place in the transformation zone of the cervix (traditional concept)\textsuperscript{46} or in the SCJ cells (new concept)\textsuperscript{10}. 


Despite hrHPV infections being relatively common, most infections (80%) are cleared by the immune system within 1-2 years after exposure (transient infections) and do not result in cervical precursor lesions\(^6,^{74}\).

In the traditional concept, the other 20% will result in a CIN lesion usually reflecting productive hrHPV infection (productive CIN). Only a minority of these infections will persist and gain transforming capacities (after 2-3 years), reflecting transforming CIN lesions. In the new concept, it is suggested that transforming CIN lesions arise from an infection with hrHPV in the SCJ cells. Conversely to infections in the transformation zone\(^10\), the latter infections do not typically lead to in-between conditions facilitating production of viral progeny but are supposed to directly lead to transforming CIN lesions. Regarding both concepts, there is evidence that HPV-tolerance by the immune system, HPV-induced genetic instability and additional host cell (epi)genetic changes are required for progression of transforming CIN towards cancer\(^46,^{75}\). Ultimately, only a fraction of hrHPV-positive women (1-3%) will develop invasive cervical cancer\(^76\). Therefore, cervical cancer can be considered as a rare complication of a persistent hrHPV infection. The progression of transforming CIN to cervical cancer lasts 20-30 years\(^77\).

**Figure 7.** The concept of cervical carcinogenesis. Adapted from Steenbergen et al.\(^75\) The outcomes of an HPV infection of cells in the transformation zone (TZ; traditional concept) and squamocolumnar junction (SCJ) cells (new concept) are represented. The infection can be cleared (no pathological changes in the cervix) or result in a productive infection (productive CIN; CIN1 and a subset of CIN2), or a transforming infection (transforming CIN; remaining subset of CIN2 and CIN3), and finally cervical cancer. Apart from a persistent HPV infection, additional host cell (epi)genetic changes are required for progression towards cancer. Based on the duration of lesion existence, the transforming CIN lesions can be distinguished in early transforming CIN2/3, suggested to have a low short-term progression risk to cancer, and advanced transforming CIN2/3 with a high short-term progression risk.
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From the long latency period between a transforming hrHPV infection and cervical cancer, it can be deduced that CIN2/3 represent a heterogeneous disease group in terms of duration of lesion existence\textsuperscript{75}. Early CIN2/3 lesions are considered to have a low short-term progression risk to cancer, whereas longstanding, more advanced CIN2/3 lesions display a high short-term progression risk for cancer. As such, markers that can determine the duration of existence of a CIN2/3 lesion would be valuable for risk stratification. When known, the duration of preceding HPV infection (PHI) may serve as a proxy of lesion duration, and may be used to discern early from longstanding CIN2/3 lesions, i.e. <5 years and >5 year of PHI, respectively\textsuperscript{75,78}. Like cervical cancers, longstanding, more advanced CIN2/3 lesions are characterized by the presence of multiple molecular (epi)genetic alterations, whereas such alterations are absent or rare in early CIN2/3 lesions\textsuperscript{78,79}. This observation is in line with the concept that accumulation of molecular (epi)genetic host cell alterations is necessary for progression of CIN2/3 to cervical cancer.

3. MOLECULAR PATHOGENESIS OF CERVICAL CANCER

Following a transforming infection with hrHPV, the accumulation of genetic and epigenetic changes in the host cell genome is necessary for malignant progression to cancer\textsuperscript{46}. Genetic changes are considered adaptations in the DNA sequence itself (e.g. DNA mutations and chromosomal copy number alterations). Epigenetic changes are caused by mechanisms that do not affect the genotype. It involves dynamic changes that can switch genes on or off and that can determine which proteins are transcribed (e.g. DNA methylation)\textsuperscript{80}. In this section, the role of host cell DNA methylation in cervical carcinogenesis is discussed in further detail.

3.1 DNA methylation

In somatic cells, DNA methylation typically occurs at cytosines preceding a guanine residue (CpG) in the human genome. The covalent binding of a methylgroup (-CH\textsubscript{3}) to cytosines in CpG dinucleotides is catalysed by DNA methyltransferases (DNMTs) using S-adenosyl methionine (SAM) as a methyl donor\textsuperscript{81}. Where DNMT1 is involved in copying and maintaining DNA methylation distribution amongst daughter strands after replication, DNMT3a and DNMT3b have de novo methylation properties\textsuperscript{82}. The density of CpGs is depending on the genomic location. A low density of CpGs is present in most gene bodies, DNA repeat elements and intergenic regions. In these locations, CpGs are mostly methylated in normal cells (Figure 8). By contrast, a high density of CpGs are mostly found in CpG islands that are present in approximately 60% of the human promoter regions\textsuperscript{83}, replication origins and cis-regulatory transcriptional elements\textsuperscript{84}. The CpGs in CpG islands are mostly unmethylated in normal cells, yet can become methylated during malignant transformation. Unmethylated promoter regions recruit proteins to induce, amongst others, H3K4 trimethylation preventing DNMT activation hence explaining the unmethylated status of most gene promoters\textsuperscript{85}. The unmethylated promoter regions are correlated with the
presence of histone acetyltransferases (HAT) that will induce an open chromatin state (euchromatin) allowing active gene transcription. Conversely, methylation of those CpG islands results in the attraction of high-affinity methyl binding proteins such as methyl CpG binding protein 2 (MECP2). These proteins will recruit histone deacetylases (HDACs) leading to heterochromatin formation and gene silencing. DNA methylation patterns are strongly influenced by epigenetic reprogramming events during embryonic development and are also found to be dramatically changed during (cervical) carcinogenesis.

During carcinogenesis, global hypomethylation in the gene bodies is observed. This is in contrast with the local hypermethylation occurring in the CpG islands located in gene promoter regions (Figure 8). The former can either result from prevention of DNA methylation by DNMT1 after DNA replication or by oxidation of the methylgroup by ten-eleven translocation (TET) enzymes, providing a 5-hydroxyl-methyl CpG (5hmC), a modified CpG that is removed after base-excision repair upon which an unmodified cytosine is re-entered\(^86\). Hypomethylation may contribute to the activation of oncogenes and an increased chromosomal instability\(^87\). In contrast, promoter hypermethylation may result in silencing of genes that might act as tumour suppressor genes (Figure 8)\(^88\).

![Figure 8. DNA methylation of the promoter region induces conformational changes of the chromatin resulting in the abrogation of gene transcription. Unmethylated CpGs are represented by white lollipops; methylated CpGs by black lollipops, blue lines represent acetyl groups on the histones.](image-url)
3.2 DNA promoter methylation of host cell genes in cervical (pre)cancer

Over the last years, many studies have reported on the methylation status of (candidate) tumour suppressor genes in cervical (pre)cancer\textsuperscript{89,90}. Currently, three genes, \textit{Cell Adhesion Molecule 1 (CADM1)}, \textit{Death Associated Protein Kinase 1 (DAPK1)} and \textit{Retinoic Acid Receptor Beta (RARB)} have shown consistent methylation frequencies in more than 5 studies. For other genes described in more than 5 studies, large discrepancies were observed in methylation frequencies. These inconsistencies can be partially attributed to the sensitivity of the methylation detection method used, differences in CpGs analysed per gene and different assay thresholds used to score a sample methylation-positive. With the introduction of genome-wide methylation profiling studies, the knowledge on methylation of genes in high-grade CIN and cancer has increased substantially\textsuperscript{91–93}. A limited number of these methylated genes including \textit{CADM1}, \textit{T-lymphocyte maturation associated protein 1 (MAL)}, \textit{microRNA124-2 (mir124-2)}, \textit{secreted frizzled-related protein 2 (SFRP2)}, \textit{Dickkopf WNT Signalling Pathway Inhibitor 3 (DKK3)} and \textit{Chromosome 13 Open Reading Frame 18 (C13orf18)} have shown to play a functional role in cervical carcinogenesis. Their re-expression in cancer cells led to an inhibition of cell proliferation, migration, tumourogenicity and/or anchorage-independent growth\textsuperscript{94–99}. Methylation-mediated silencing of these genes thus contributes to malignant progression. The biological function of the other genes that are frequently methylated in cervical cancers remains elusive.

In the current thesis, we will focus on promoter methylation of the tumour suppressor genes \textit{CADM1}, \textit{MAL}, \textit{mir124-2} and \textit{Family with sequence similarity 19 (chemokine (C-C motif)-like) memberA4}; (\textit{FAM19A4})). \textit{CADM1} was identified by a candidate gene approach and encodes a cell-surface protein involved in homophilic and heterophilic cell-cell interactions\textsuperscript{100}. \textit{MAL} was identified by genome-wide expression arrays as the most significantly downregulated gene in cervical cancer\textsuperscript{101}. Its gene product is involved in cell polarity and apical transport of membrane and secretory proteins. Methylation of the tumour suppressor genes \textit{CADM1} and \textit{MAL} has previously been shown to induce increased cell proliferation, anchorage independent growth and an increased migratory capacity and tumourogenicity in \textit{in vitro} transformed cells\textsuperscript{94,95}. \textit{Mir124-2} was identified by a candidate gene approach since methylation of this gene was encountered in numerous other cancer types\textsuperscript{102,103}. Methylation of the \textit{mir124-2} host cell gene increased cell proliferation and migration \textit{in vitro}\textsuperscript{96}. \textit{FAM19A4} was identified through methylation-specific digital karyotyping of HPV16 E6/E7 transformed keratinocytes\textsuperscript{97}. Methylation of \textit{FAM19A4} was associated with the acquisition of an immortal phenotype \textit{in vitro}\textsuperscript{93}. 
3.3 Host cell alterations in relation to lesion severity

In a recent study, methylation levels of CADM1 and MAL have shown to be increased proportional to severity of the underlying lesion. A 5.3-fold increase of CADM1 and a 6.2-fold increase of MAL methylation levels was found in CIN2/3 compared to the reference (<CIN1)\textsuperscript{79}. Likewise, a 143.5-fold and 454.9-fold increase, for CADM1 and MAL respectively was found in cervical carcinomas\textsuperscript{79}. These data underscore the concept that an increase in specific epigenetic host cell alterations underlies cervical carcinogenesis. By considering the duration of the HPV infection as a proxy for the duration of lesion existence, it was found that CADM1 and MAL methylation levels are more elevated in CIN2/3 with a longer duration of existence (Previous HPV infection, PHI ≥5 years) (i.e. 11.5- and 13.6-fold, respectively) than in those with a short duration of existence (PHI <5 years)\textsuperscript{75} (i.e. 3- and 3.6-fold, respectively). Recently, it has also been shown that the number of chromosomal alterations increases with lesion severity and duration, with the highest number of alterations detected in cervical cancer and CIN2/3 lesions with a PHI ≥5 years\textsuperscript{78}. CIN2/3 lesions adjacent to cervical SCC (considered to represent the most advanced precancerous lesions), had similar genomic profiles as the SCC\textsuperscript{78}. Thus, markers based on DNA methylation events and chromosomal alterations are promising disease markers that can be considered to reflect the duration and the severity of the CIN lesion.
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4. THE PREVENTION OF CERVICAL CANCER

Two ways of cervical cancer prevention can be recognized: primary prevention and secondary prevention. The main intent of primary prevention is to avert the onset of cervical carcinogenesis by taking the risk factor of cervical cancer, i.e. a hrHPV infection, away in healthy women. Accordingly, prophylactic vaccination is an example of primary prevention. The main intent of secondary prevention is to identify the women with cervical pre-cancer by screening and accordingly treat them to prevent cervical cancer.

4.1 Primary prevention: prophylactic vaccines

Two prophylactic HPV vaccines (Cervarix and Gardasil) have market approval in many countries as of 2015. These vaccines consist of L1-based virus-like particles (VLPs) which resemble the normal HPV particles in morphology, but do not contain viral DNA. Cervarix (GSK) is a bivalent HPV16/18 L1 VLP vaccine that protects against the most prevalent oncogenic HPV types (HPV16 and HPV18). This vaccine was introduced in the Netherlands in 2009, for all 12-year-old girls, with a catch-up vaccination for girls between 13 to 16 years of age.

Gardasil (Merck) is a quadrivalent vaccine that contains L1 VLPs of HPV16/18 as well as of the non-oncogenic low-risk HPV types HPV6/11, which are found in 75-90% of genital warts. Although these vaccines are type-specific, cross-reactivity against other HPV types such as HPV45 or HPV31 does occur. Accordingly, these HPV vaccines may reduce HPV types responsible for approximately 77% of cervical cancers (70% caused by HPV16/18 and 7% by cross-protection).

Currently, also the efficacy of a nonavalent vaccine (protecting against HPV6/11/16/18/31/33/45/52/58) is being evaluated in randomized clinical trials. In a recent trial, it has proven to induce an antibody response to HPV6, 11, 16, and 18 that was non-inferior to the antibody response generated by the quadrivalent vaccine and to prevent infection and disease related to HPV31, 33, 45, 52, and 58 as well. As cervical cancer develops over 15 or more years, the effect of vaccine introduction in reducing cervical cancer incidence will not become evident for some time.

Given that a considerable number of women are not vaccinated (i.e. the coverage of HPV vaccination in the Netherlands was only 58.1% in 2013 and women above 21 years of age have not received vaccination), cervical screening will remain necessary in the foreseeable future for full prevention of cervical cancer. Moreover, since the HPV vaccines presently available do not (yet) protect against all hrHPV types, screening will still be needed for lesions associated with infections of non-vaccine-targeted HPV types.
4.2 Secondary prevention: cervical screening

The success of cervical cancer screening is mostly related to the large time window between a hrHPV infection and the development of cervical cancer. This time window allows for the detection of lesions in the long-lasting premalignant phase. Additionally, the effective treatment procedures available for cervical cancer precursors contribute to efficient cervical cancer prevention by early detection. It is believed that nearly all cervical cancer deaths could be prevented if women and their healthcare providers would fully adhere to screening recommendations and follow-up treatment. Indeed, population-based screening programmes with a call and recall system such as present in the western world, have resulted in a decrease in cervical cancer incidence and mortality. The benefit of screening on the reduction of cervical cancer cases depends on the screening test used, the screening attendance, and the availability of adequate treatment and follow-up algorithms for women with abnormal test results. However, screening also has negative side effects, such as distress about a (false-)positive screening result and the possible side-effects of over-treatment.

The clinical performance of a test is determined by its clinical sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV). The clinical sensitivity is the probability that a test correctly classifies people with clinically meaningful disease i.e. CIN2+ at a preclinical stage as positive (e.g. the percentage of people with disease who are correctly identified as having the condition). Clinical specificity is the probability that a test classifies people without disease as negative (e.g. the percentage of healthy people who are correctly identified as not having the condition).

Predictive values of test results depend on the prevalence of disease in the population. The positive predictive value (PPV) is the proportion of people with a positive test result who have the disease. The negative predictive value (NPV) is the proportion of people with a negative test result who do not have the disease. Ideally, a screening test should have a high clinical performance in terms of above measures to allow effective detection of women with clinically relevant disease in need of treatment, and minimize follow-up procedures of test-positive women without disease. In practice, there is usually a trade-off between the measures. The higher the clinical sensitivity, the lower the number of women with clinically relevant disease that will have a negative test result (false-negatives) and the safer the test will be. This mostly coincides with a lower clinical specificity, resulting in a higher number of women without clinically relevant disease that will have a positive test result (false-positives). This will result in a larger burden (over-referral and unnecessary follow-up) on the population with higher costs and possible side-effects as consequence.
4.3 Cervical screening in the Netherlands

The Pap-smear, which implies the cytomorphological examination of exfoliated cells from the transformation zone of the cervix\textsuperscript{117}, was introduced in the beginning of the 1960s. In the early seventies, regional organized cervical screening programmes were introduced\textsuperscript{118}, that were replaced by a nationwide population-based screening programme in 1988. This screening programme targeted women aged 35-54 years with a physician-taken Pap-smear every three years. This resulted in a decline in cervical cancer incidence from 9.2 per 100,000 women in 1988 to 5.9 per 100,000 women in 2000\textsuperscript{3}. In 1996, the screening programme was restructured to increase screening coverage and efficiency by extending the screening interval and the screening period. This resulted in a screening programme with a physician taken Pap-smear every 5 years for women aged 30-60 years\textsuperscript{118}.

In the current Dutch system, Pap-smears are classified according to the CISOE-A classification system. The letters C (composition), I (inflammation), S (squamous), O (other and endometrium), and E (endocervical cylindrical epithelium) indicate the composition and morphology of the smears. The letter A (adequacy) indicates the adequacy of the smear and, except for inadequate smears, does not affect the follow-up advice. This system can easily be converted into the international Bethesda classification system (Table 1)\textsuperscript{119,120}. In the Netherlands, the majority of screened women (96.5%) has normal cytology (Pap1) and is invited for the next screening round (5 years). Since 5-15% of women with borderline or mild dyskaryosis (i.e. BMD: Pap2, Pap3a1) has or will develop high-grade lesions, these women are followed-up by repeat cytology testing at 6 and 18 months. If ≥BMD is present at either of these repeat tests, women are referred for colposcopic examination of the cervix by a gynaecologist. Women with moderate dyskaryosis or worse (≥Pap3a2) are immediately referred for colposcopy given their high risk of high-grade disease.

### Table 1. Comparison of different classification systems

<table>
<thead>
<tr>
<th>Description</th>
<th>Pap</th>
<th>S</th>
<th>O</th>
<th>E</th>
<th>Bethesda 2001</th>
<th>Management</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inadequate scrape</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Unsatisfactory for evaluation</td>
<td>Repeat cervical scrape</td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1-2</td>
<td>Negative for intraepithelial lesion or malignancy</td>
<td>next invitation screening round</td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1-2</td>
<td>atrophy, negative for intraepithelial lesion of malignancy</td>
<td>next invitation screening round</td>
</tr>
<tr>
<td>Borderline dyskaryosis</td>
<td>2</td>
<td>2-3</td>
<td>3</td>
<td>3</td>
<td>ASC-US/ASC-H</td>
<td>AGC follow-up cytology</td>
</tr>
<tr>
<td>Mild dyskaryosis</td>
<td>3a1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>ASC-H/LSIL</td>
<td>AGC, favour neoplastic</td>
</tr>
<tr>
<td>Moderate dyskaryosis</td>
<td>3a2</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>HSIL</td>
<td>AGC, favour neoplastic</td>
</tr>
<tr>
<td>Severe dyskaryosis</td>
<td>3b</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>HSIL</td>
<td>AGC, favour neoplastic</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>HSIL</td>
<td>AIS</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>5</td>
<td>8-9</td>
<td>7-8</td>
<td>9</td>
<td>Squamous cell carcinoma</td>
<td>Adenocarcinoma</td>
</tr>
</tbody>
</table>

*Adapted from Bulk et al. abbreviations: CISOE-A, Composition, Inflammation, Squamous epithelium, Other abnormalities and Endometrial and Endocervical columnar epithelium- Adequacy of the smear; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade intraepithelial lesion; ASC-US, atypical cells of undetermined significance; ASC-H, atypical squamous cells cannot exclude HSIL; AGC, atypical glandular cells; AIS, adenocarcinoma in situ\textsuperscript{119}.
Although the introduction of cytology-based screening has led to a large decrease in the incidence of cervical cancer in the Netherlands, currently the effects of the screening-programme have leveled-off\footnote{21}. In 2012, still 735 women were diagnosed with cervical cancer, while 215 women died from this disease. The main reasons for missing cervical cancer cases are related to both the performance of the screening test and suboptimal screening compliance. In addition, the follow-up of screen-positive women is not optimal.

First, the cytology test has several drawbacks. It has a relatively low sensitivity for high-grade lesions (50-70\%) which leads to a high number of women with a false-negative test result\footnote{22}. This rather low sensitivity can be explained by incorrect sampling (e.g. vaginal cells instead of cervical indicator cells) or the failure to detect a small number of abnormal cells in a large background of normal cells. The low sensitivity of a single cytology test is therefore compensated by screening women regularly, i.e. every 5 years in the Netherlands. Moreover, cytology does not accurately detect AdCAs and its precursor lesions. This might be due to their higher location in the endocervix, which explains the smaller chance of finding abnormal cells in a standard Pap-smear\footnote{23} and the poor definition of the early precursor lesions for AdCA (atypical glandular cells of undetermined significance (AGUS1 and 2). In addition, cytology is a subjective and labour-intensive test\footnote{24}, with a relatively poor reproducibility resulting in variable accuracy\footnote{25}. It must be noted that in the Netherlands, the sensitivity of cytology is relatively high compared to other countries\footnote{26}. This emphasizes that in order to obtain good cytology performance, expertise, training of cytotechnicians and cytopathologists, and a good quality control (QA) system are required. Without these facilities, quality of cytological screening is not ensured and can be rather poor.

Secondly, a large number of women does not attend the population-based screening programme\footnote{27} due to several reasons (e.g. time constraints, pregnancy, embarrassment, religious and cultural differences)\footnote{28}. Although annually around 700,000 women are invited for a Pap-smear, approximately 280,000 women (35\%) do not attend screening. Since more than 50\% of cervical carcinomas are eventually diagnosed in women that do not attend the screening programme, there is the urgent need to attract these women into the programme\footnote{2,27}.

Given the above, recent efforts to improve the cervical screening programme have focused on increasing screening compliance and on developing alternative screening assays to replace cytology tests.
4.4 Change of the screening programme: HPV testing and triage by cytology

Since hrHPV is causally related to the development of cervical cancer, testing for the presence of hrHPV DNA in cervical scrapes (hrHPV testing) has been proposed as primary screening tool. In the past decade, several randomized controlled trials have evaluated the performance of primary hrHPV testing compared to primary cytology testing\textsuperscript{129-135}. These studies have shown that hrHPV testing is more sensitive in detecting CIN2+ than cytology (i.e. 30% more CIN2 detection and 20% more CIN3 or worse detection (CIN3+). Additionally, several European randomized trials have investigated results over different screening rounds\textsuperscript{129,131,132,135-139} and have shown that the improved detection of CIN2+ by HPV screening in the baseline round resulted in better protection against cervical (pre)cancer in the subsequent round. These studies showed a 50% reduction in CIN3+ lesions detected in the second screening round of women who tested hrHPV-negative at baseline compared to women with normal cytology at baseline. A pooled analysis of different European trials confirmed that a negative hrHPV test at baseline implies a much lower 5-year risk of high-grade CIN and cervical cancer than normal cytology at baseline\textsuperscript{140}. Altogether, trial data support initiation of HPV-based screening from age 30 years and extension of screening intervals to at least 5 years\textsuperscript{140}. Although hrHPV testing increases the protection against cervical (pre-)cancer compared to cytology, it has a 3-4% lower specificity than cytology\textsuperscript{141}. This is due to the fact that HPV tests do not only detect clinically relevant infections associated with CIN2+, but also a substantial proportion of transient HPV infections. To control the number of colposcopy referrals, treatments, and costs compared to primary cytology screening, hrHPV-positive women require triage testing to refer only those women with clinically relevant lesions\textsuperscript{139}. Recently, various studies have compared different triage strategies for hrHPV-positive women in the Netherlands\textsuperscript{142,143}. A triage strategy was considered acceptable for screening if the NPV is at least 98% and PPV is at least 20%. An NPV of 98% denotes that the CIN3+ risk after a negative triage test within 4 years of follow-up is less than 2%. The NPV was derived from the currently accepted risk for CIN3+ after baseline BMD cytology and normal cytology at 6 and 18 months (1.2%)\textsuperscript{144}. The PPV of 20% reflects that at least 1 out of 5 women referred will have CIN3+\textsuperscript{143}. At this time, the best available triage strategies for hrHPV-positive women in the Netherlands are repeat cytology (at baseline and after 6 months to 1 year), and cytology combined with HPV16/18 genotyping at baseline\textsuperscript{142-144}. The Dutch minister of Health has decided that upon implementation of primary hrHPV testing in 2016, women with a hrHPV-positive scrape should be triaged by repeat cytology\textsuperscript{145}. Whereas hrHPV-positive women with normal cytology will receive a repeat test and will be kept under close surveillance, women with abnormal cytology will be referred to the gynaecologist for further follow-up. The new programme will imply 5 invitations for HPV screening at the ages of 30, 35, 40, 50 and 60 years\textsuperscript{146}. Additionally, HPV-positive women with a negative triage test (i.e. normal baseline and repeat cytology at 6 months) at the age of 40, 50 and 60 years will be invited after 5 years because their 5-year CIN3+ risk exceeds the 2% norm in the Netherlands.
4.4.1 Validation of HPV tests

When considering HPV tests for screening, it is important to recognize the difference between analytical sensitivity and specificity versus clinical sensitivity and specificity. Analytical sensitivity and specificity refer to the detection of all hrHPV infections, including transient infections and those associated with high-grade lesions. Instead, clinical sensitivity and specificity point to the detection of only those hrHPV infections that are associated with clinically meaningful lesions. Ideally, the hrHPV test used in clinical settings should detect only women at risk for cervical cancer but not those with transient hrHPV infections.

Currently, many HPV tests are commercially available. HPV DNA can be detected by target or signal amplification assays. Target amplification assays mainly comprise polymerase chain reaction (PCR)-based methods, such as GP5+/6+ PCR, or isothermal amplification methods. The GP5+/6+ PCR is a consensus PCR, amplifying L1 DNA of both lrHPVs and hrHPVs. The presence of hrHPV DNA is subsequently visualized by subjecting the PCR product to an enzyme-immunoassay (EIA) staining procedure with a cocktail of oligoprobes representing 14 hrHPV types (16/18/31/33/35/39/45/51/52/56/58/59/66/68). Alternatively, lrHPV probes can be used to detect lrHPV DNA, or type specific probes can be used for genotyping (in reverse line blot (RLB) or bead based (Luminex) format). An example of a signal amplification assays is the Hybrid Capture 2 (HC2) test (Qiagen). The HC2 is a nucleic acid hybridization assay in which the denatured target DNA will hybridize with a mixture of HPV RNA probes capable of detecting 13 HPV types (16/18/31/33/35/39/45/51/52/56/58/59/68). The formed DNA/RNA hybrids are recognized by an antibody and visualized by electro-chemiluminescence.

The hrHPV tests GP5+/6+ PCR and HC2 are used in the randomized controlled trials discussed above, and have proven to perform well on cervical scrapes in cervical screening programmes. They can be considered as validated reference assays for cervical screening. However, many new hrHPV tests were meanwhile developed and the clinical performance of these tests may differ significantly. Therefore, standards for hrHPV test performance and characteristics in clinical practice have been formulated by an international consortium. These guidelines indicate that the clinical sensitivity for CIN2+ should not be less than 90% and the clinical specificity for CIN2+ not less than 98% of that of a validated reference assay. In addition, the assays should have a sufficiently high intra- and inter laboratory reproducibility. Currently, many hrHPV DNA tests have proven to fulfil the criteria described in the guidelines and are considered clinically validated for primary cervical cancer screening.

4.5 Improving the attendance rate: HPV self-sampling

Since in the Netherlands about half of the cervical carcinomas are diagnosed in about 35% of women who have not been screened regularly, it is of utmost importance to make efforts to get these so-called non-responders into the screening programme. One of these efforts involves lowering the threshold for screening by allowing collection of cervico-vaginal
material by women themselves at home (i.e. self-sampling) instead of visiting a physician for a smear taking. After self-collection, the samples can be sent to the laboratory for further evaluation. Whereas cytology is not reliably applicable to self-collected cervicovaginal specimens because of a lower sample quality in terms of intact (cervical) cells than a cervical scrape (i.e. deficiency of cervical indicator cells), hrHPV testing can be applied rather effectively on this material\textsuperscript{158,159}. Offering self-sampling for HPV testing (HPV self-sampling) as cervical screening tool can thereby lead to an improved protection as it may re-attract non-responders for cervical screening.

Several studies have been performed with a large variety of collection devices for (cervico-)vaginal material including brushes, swabs, lavages, and tampons\textsuperscript{159,160}. Interview surveys have indicated that women prefer self-collection over physician-collection for reasons as the ease-of-use, privacy and time and place of sampling\textsuperscript{161–166}. Consequently, in countries with organized screening programmes, the participation rate of non-responders that were offered self-sampling was higher than when offered a recall invitation for a physician-taken scrape\textsuperscript{164,167–171}. In general, up to 39% of the invited non-responders, returned self-sample material to the laboratory. In addition to an increase in screening compliance, an increase in screening effectiveness is found in pooled data from two large Dutch studies. These data have shown that in the group of non-responders participating in screening through self-sampling, the detection of CIN2/3+ lesions was significantly higher than in the regular screening population\textsuperscript{169}. Studies comparing HPV prevalence using self-sample devices and physician-taken cervical scrapes seem inconsistent and strongly influenced by the kind of device and the HPV test used. However, studies over the last 5-10 years that have used specially developed devices in combination with proper, clinically validated HPV tests have shown a high concordance of hrHPV test results between (cervico-)vaginal self-samples and physician-taken cervical scrapes. Even more importantly, with proper sampler/test combinations, hrHPV testing in self-samples appears to be non-inferior in detecting CIN2+ to hrHPV testing on cervical scrapes\textsuperscript{158,159,172,173}. Based on current data, hrHPV self-sampling will be introduced in the new Dutch screening programme in 2016, as opt-in for non-responders. Herein, a validated combination of self-sampling device and HPV test will be used to reach clinical accuracy comparable to HPV testing on a physician-taken scrape in terms of detection of CIN2+.

Since self-sampling is a cheap method which is preferred by most women over a physician-taken scrape\textsuperscript{167,174}, one might consider using primary self-sampling as an alternative cervical cancer screening tool in the regular screening population. Further evidence from prospective trials will need to be gathered to assess whether clinical non-inferiority of hrHPV self-sampling versus hrHPV testing on cervical scrapes allows implementation as primary screening tool to any screen-women. In the IMPROVE trial which started in April 2015 in the Netherlands, this will be studied in screening invitees. Also in low-resource countries without standard physician-based screening facilities, self-collection can make screening accessible to a large number of unscreened women\textsuperscript{175}. 

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5. ALTERNATIVE TRIAGE METHODS OF HPV-POSITIVE WOMEN

As discussed above, in the new Dutch screening programme HPV-positive women will be triaged by twice (at baseline and after 6 months) cytology testing. In countries with less adequate cytology reading, this triage strategy might not be ideal and alternatives should be considered. Due to its low sensitivity, cytology does not detect all cervical carcinomas\(^\text{176}\). In addition, triage by cytology remains a subjective test. Since only HPV-positive women will be triaged by cytology, cytotecnicians will be aware of the HPV-status, resulting in a possible bias towards the scoring of abnormal cytology\(^\text{177}\). At last, the deficiency of cervical indicator cells in self-samples, does not allow cytology triage. Therefore, there is the need for more objective biomarkers that can be applied to both cervical smears and self-samples. These biomarkers should ideally reassure absence of cervical carcinomas and CIN2/3 lesions in test-negative women. Given the insight in the molecular pathogenesis of cervical cancer, several molecular and morphological markers are currently under investigation for their possible application as alternative triage tool(s) for hrHPV-positive women.

5.1 Viral-related molecular markers

5.1.1 HPV genotyping

The risk for the development of cervical cancer and its precursor lesions is related to the HPV-genotype. Infections with HPV16 and HPV18 are associated with the highest CIN3+ risks, most likely reflecting increased oncogenic potential combined with increased persistence properties of these two types\(^\text{178,179}\). Moreover, women with HPV16/18 related cancers are diagnosed at younger age than women with other HPV type-related cancers\(^\text{17}\). Therefore, HPV16/18-genotyping is useful for the risk-stratification of hrHPV-positive women. However, for screening purposes it is important to consider the long-term CIN3+ risk of HPV16/18-negative women. Since HPV16 and 18 are present in just 50% of CIN3 lesions and 70% of cervical carcinomas, the residual CIN3+ risk in HPV16/18-negative women is too high to be implemented as triage tool. However, if HPV16/18 genotyping is combined with cytology, the 5 year-risk HPV16/18-negative women with negative cytology is around 2% and can be used as triage tool for HPV-positive women when considering cervical scrapes\(^\text{180}\).

5.1.2 HPV E6/E7 mRNA expression

A deregulated expression of HPV E6 and HPV E7 is the driving force of malignant transformation. Since the level of E6/E7 mRNA transcripts has shown to be correlated with the severity of underlying CIN lesion, the detection of E6/E7 mRNA levels has been proposed as triage test for hrHPV-positive women\(^\text{155,181,182}\). Currently, several studies have evaluated E6/E7 transcript analysis of five hrHPV-types (HPV16/18/31/33/45) by a nucleic acid sequence-based amplification (NASBA method). Data from these studies have shown that although this test was able to stratify hrHPV-positive women for colposcopy with a good specificity, the sensitivity was limited compared to cytology, requiring close follow-up of E6/
E7 mRNA-negative women\textsuperscript{155,181,182}. Therefore, this test is currently not considered efficient for triage purposes. Even when 7 HPV-genotypes were used in the assay, the sensitivity of the test was too low for use in clinical practice\textsuperscript{183}. Another, apparently more sensitive mRNA detection assay targeting 14 hrHPV types (Aptima) on the other hand, is highly sensitive but lacks sufficient specificity to become suitable as triage test.

### 5.1.3 HPV DNA methylation

The human cell is prone to infections by viruses and transposons. In order to protect itself from complete take-over by invaders, it has to recognize the invaders and suppress their activity\textsuperscript{184}. One response mechanism is the suppression of the viral genes and transposons by means of DNA methylation of viral and transposon sequences\textsuperscript{185}. During cervical carcinogenesis, the HPV genome has shown to be targeted by DNA methylation resulting in an increase in HPV-methylation with disease severity. Therefore, HPV-methylation analysis has also been proposed as possible triage tool for hrHPV-positive women\textsuperscript{186}.

Until now, a number of studies have been published, describing assays for the detection of HPV16, HPV18, HPV31 and HPV45 methylation. In general, findings on the association of the methylation of specific viral CpG sites and cervical disease are inconsistent. Inconsistencies might be explained by small sample sizes, differences in DNA methylation detection method, different sample types and/or the specific CpGs analysed. To date, only a specific combination of CpGs within the L1 and L2 genes of HPV16 and HPV18 has shown consistent hypermethylation with increasing disease severity between different studies. For other HPV types, the available information is scarce (reviewed in Clarke et al.\textsuperscript{186}). Accordingly, methylation analysis of viral DNA has not yet matured to an efficient triage assay. One aspect in this is that for each hrHPV-type with unique DNA sequence, a unique assay has to be developed and clinically validated, making implementation challenging.

### 5.2 Host cell markers

#### 5.2.1 P16\textsuperscript{INK4A}/Ki-67 dual staining

The overexpression of p16\textsuperscript{INK4A} in proliferating cells serves as a marker for the presence of a transforming hrHPV infection\textsuperscript{59,60}. In addition, Ki-67 is a proliferation marker that has shown to be an independent marker for lesion progression\textsuperscript{187}. In combination, dual staining for p16\textsuperscript{INK4A}/Ki-67 is considered to be a surrogate marker for cell cycle deregulation reflecting transforming HPV infections. Consequently, dual staining of cells for p16\textsuperscript{INK4A}/Ki-67 has been studied as triage marker for hrHPV-positive women in several studies\textsuperscript{188–190}. Data from these studies have shown that p16\textsuperscript{INK4A}/Ki-67 dual staining for triage of HPV-positive women, results in higher sensitivities for the detection of CIN2+ compared to cytology triage. However, p16\textsuperscript{INK4A}/Ki-67 dual staining is still microscopy-dependent and requires a high-quality cytology specimen and an experienced cytopathologist. Cytomorphological analysis is not reliably applicable to self-samples, which forms a drawback in the use of this triage test in screening programmes with HPV self-sampling.
5.2.2 Host cell DNA methylation analysis

Methylation of host cell DNA is frequently encountered in cervical (pre)cancer\textsuperscript{90}. The sensitive techniques available for DNA methylation detection, makes methylation analysis an attractive biomarker for the triage of hrHPV-positive women. Currently, several techniques are available for the detection of DNA methylation, with quantitative methylation-specific PCR (qMSP) as one of the most promising. The key to successful methylation analysis at gene-specific level is based on bisulphite treatment of DNA. During this treatment, unmethylated cytosines are deaminated and converted into uracil whereas methylated cytosines remain unaffected due to steric hindrance by the methyl-group\textsuperscript{191}. Conversion of the unmethylated cytosines is realized in three steps. A sulphonation reaction converts cytosine into cytosine sulphonate which is then deaminated and converted into uracil sulphonate. The last step involves the elimination of sulphate leaving uracil. The conversion of cytosine to uracil leads to sequence changes that can be detected by PCR-amplification\textsuperscript{191}. Methylation-specific PCR (MSP) uses primers specific for the bisulphite-converted methylated target DNA, and often a reference gene not affected by methylation. Using a hydrolysis probe, quantitative measurement of the target is allowed, with analytical sensitivities up to 0.1-1.0% of methylated DNA in unmethylated background DNA.

Although numerous studies have described DNA methylation of several genes known to be to associated with cervical carcinogenesis\textsuperscript{90}, only a limited number of methylation markers have been studied extensively as triage tests for HPV-positive cervical scrapes and self-samples. Most studies performed so far have used panels of methylation markers to reach sufficiently high sensitivities for the detection high-grade CIN and cervical cancer in cervical scrapes\textsuperscript{192-195}. One such panel, CADM1/MAL has shown to be as good as cytology or cytology combined with HPV16/18 genotyping for CIN3+ detection\textsuperscript{192}. Also, methylation analysis of JAM3/EPB4/TERT/C13orf18 and bi-marker combinations of SOX1, PAX1, LMX1A and NKKX6-1 have shown promising results\textsuperscript{193,196}. To date, only two studies have reported single methylation markers, i.e. PAX1 and ZNF582 as promising methylation markers for cervical screening and women with LSIL, respectively\textsuperscript{197,198}. Yet, these markers have not been validated in population-based screening studies and require further investigation.

More recently, methylation marker analysis has proven to be valuable for the direct triage of hrHPV-positive self-samples. Results from a large prospective randomised control trial have shown that methylation analysis by the bi-marker panel MAL/mir124-2 directly on DNA of HPV-positive lavage self-samples, is non-inferior to cytology triage on a physician-taken cervical scrape for the detection of CIN2/3+\textsuperscript{199}. Other methylation markers that have been evaluated in feasibility studies of lavage and/or brush self-samples include JAM3, EPB4, TERT and C13orf18\textsuperscript{200,201}. These markers generally showed good correlation between self-samples and matched physician-taken samples, but have thus far not been evaluated for clinical performance for detection of CIN2/3+ in large self-sample series.
Altogether, these studies have shown that direct molecular triage on hrHPV-positive self-samples is possible and can offer full molecular screening, which eliminates the need for a visit to a physician for a cervical smear. Methylation analysis by qMSP therefore warrants investigation on its clinical value as triage marker for hrHPV-positive women, as is the topic of this thesis.

6. THESIS OUTLINE

This thesis addresses the need for objective disease markers, applicable to both physician-taken and self-collected specimens, for use in triage of HPV-positive women for colposcopy. Molecular markers reflecting DNA methylation events in HPV-mediated carcinogenesis are highly appealing in this respect. Promoter methylation of CADM1, MAL, mir124-2, and FAM19A4 genes has previously shown to be associated with cervical carcinogenesis. This thesis aimed to develop and validate qMSP-based assays specific for these methylation events for triage purposes. Towards this goal, the following studies were performed in this thesis.

To allow efficient triage by DNA methylation analysis, an assay that can determine the methylation status of different markers in one single reaction was developed in the study described in Chapter 2. Thereto, singleplex qMSP assays for CADM1, MAL, mir124-2 and the reference gene β-actin (ACTB) were converted into a multiplex reaction. The multiplex qMSP offered a promising approach for high-throughput diagnostic analysis of the methylation status of multiple genes, performing equally well as its singleplex versions.

In Chapter 3, we evaluated the possible additional value of DNA methylation analysis by CADM1/MAL qMSP to cytology for triaging women with a hrHPV-positive cervical scrape. Combining both tests resulted in substantially higher sensitivities for CIN2/3+, with cytology being more sensitive for CIN2 and DNA methylation analysis for CIN3+. The combination of both triage tests appeared an attractive baseline triage strategy with a high reassurance that no carcinomas or advanced lesions are being missed.

Previously, it was shown that methylation levels increase with the duration of lesion existence. In Chapter 4 we investigated the performance of the CADM1/MAL/mir124-2 methylation assay to detect women with various disease grades, in a series of cervical scrapes enriched for women with cervical and endometrial cancer. Study findings indicated that the assay consistently detects cervical cancer (100%) and the majority of endometrial carcinomas (76%). In addition, the overall methylation positivity and the number of methylated genes increased proportionally to underlying lesion severity.
In Chapter 5, we described the clinical performance of a new methylation assay targeting the FAM19A4 gene to triage hrHPV-positive women by testing their cervical scrapes. The FAM19A4 assay was trained and validated. The assay has a CIN3+ sensitivity of 75.8% at 67.0% specificity, and detects all cervical carcinomas (22/22) and advanced CIN2/3 lesions (29/29). Methylation analysis by FAM19A4 qMSP appeared an attractive triage marker for hrHPV-positive women, detecting at least the advanced lesions and cervical carcinomas in need of treatment.

In Chapter 6, we compared the clinical performance of the validated FAM19A4 methylation assay to cytology and/or HPV16/18 genotyping for the detection of CIN3+ on cervical scrapes of hrHPV-positive women. For this purpose, a prospective observational multi-center cohort study among hrHPV-positive women aged 18-66 years, visiting a gynaecologic outpatient clinic was performed. In hrHPV-positive women of an outpatient population, aged ≥30 years (cervical screening target), methylation analysis by FAM19A4 appeared as a valuable alternative to cytology and HPV16/18 genotyping to identify women with CIN3+.

With a world-wide increasing interest in the introduction of self-collection for non-responders in HPV-based cervical screening programmes, it is of importance to also gain clinical performance data on methylation marker analysis in different self-sample types, especially those collected by the most commonly used self-collection devices, i.e. a lavage-based device and a brush-based device.

In Chapter 7, we therefore assessed the performance of methylation marker analysis by the bi-marker FAM19A4/mir124-2 assay in hrHPV-positive lavage- and brush-collected self-samples of screening non-responder women (PROHTECT studies). DNA methylation analysis of FAM19A4/mir124-2 showed a high and similar CIN3+ detection rate (i.e. 70%) for both self-samples types at 70% specificity. These figures equal those obtained on hrHPV-positive cervical scrapes, supporting direct molecular triage as a reasonable alternative for screening non-responders compared to triage on a physician-taken cervical scrape. In combination with HPV16/18 genotyping, significantly higher sensitivities are obtained, at the cost of a drop in specificity.

In Chapter 8, we present the clinical implications of our findings and possible avenues of future research. In Chapter 9, an English and Dutch summary of the findings of this thesis are presented.
REFERENCES


Chapter 1


Chapter 1


Chapter 1


