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Chapter 1

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1. Cervical cancer epidemiology

Cervical cancer is the fourth most common cancer among women worldwide.\(^1\) In 2018, according to the GLOBOCAN estimates, there were 569,847 new cases of cervical cancer and 311,365 deaths from cervical cancer worldwide.\(^1\) Almost 85% of cervical cancers occur in women living in developing countries, with the highest incidence rates in Africa, Latin America and Melanesia (Figure 1). In comparison, the lowest cervical cancer incidence rates are observed in Western Asia, Western Europe, Northern America, Australia and New Zealand.

The dissimilarity in cervical cancer incidence and mortality between less and more developed regions is mainly caused by differences in the availability of preventive strategies. In most developed countries organized cervical screening programmes have led to a significant decrease in cervical cancer incidence due to early diagnosis and effective treatment of cervical cancer and precancer.

In 2017, more than 800 new cervical cancer cases and 206 cervical cancer related deaths were observed in the Netherlands.\(^2\)

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**Figure 1.** World age-standardised incidence rates of cervical cancer in 2018 (rate per 100,000) (adapted from Globocan).
2. HPV and cervical cancer

2.1 Cervical cancer

Cervical cancer develops in the cervix uteri, which is the lower part of the uterus. The outer part of the cervix (ectocervix) has multiple layers of squamous epithelium, while the inner part (the endocervix) has a single layer of glandular columnar epithelium. These two types of epithelium meet at the squamocolumnar junction (SCJ) (Figure 2). Due to hormonal changes in a woman’s adolescent life, metaplastic squamous epithelial cells replace the columnar epithelial cells, causing the SCJ to move more inwards the cervical canal. The region between the old and new SCJ is called the transformation zone (TZ), which is thought to be the most vulnerable to neoplastic changes (Figure 3).

Classified by histological subtype, 80% of cervical carcinomas are squamous cell carcinomas (SCC) and 15% are adenocarcinomas (AC). The remaining 5% are more rare types of cervical cancer such as neuro-endocrine and clear-cell carcinomas.

![Figure 2. Anatomy of the uterus and cervix.](image)
2.2 Cervical precancer

Cervical SCC develops through premalignant lesions called cervical intraepithelial neoplasia (CIN) (Figure 4). In CIN grade 1 dysplasia is seen in less than one third of the width of the epithelium (mild dysplasia), in CIN grade 2 dysplasia is seen in two thirds of the width of the epithelium (moderate dysplasia), and in CIN grade 3 dysplasia is seen in more than two thirds of the width of the epithelium (severe dysplasia). When atypical cells invade the basal membrane, the lesion is graded as SCC.

All CIN lesions can progress, persist or regress. CIN3 has the lowest chance of spontaneous regression and is considered a true premalignant stage, and therefore is always treated. Treatment is performed by a large loop excision of the transformation zone (LLETZ), in which the cervical transformation zone and lesion is excised using a loop-shaped electric wire. In contrast, CIN1 has the lowest progression risk and the highest chance of spontaneous regression. Consequently, a wait-and-see policy with follow-up after one year is warranted. The chance of spontaneous regression of a CIN2 lesion is somewhere in between the regression chances of CIN1 and CIN3, however remains very uncertain. Consequently, there is no uniform consensus regarding treatment of CIN2: women are often advised to undergo treatment in order to prevent possible progression to cancer, however, for women in their fertile life phase with a future child wish it can be decided, depending on the size of the lesion and the colposcopical aspect, to follow a wait-and-see policy.

Unlike for SCC, precursor lesions of AC of the cervix are less well defined. The only well-defined precursor lesion of AC is adenocarcinoma in situ (AIS), which is comparable to CIN3 for SCC, and is an indication for treatment. Treatment of AIS is most often performed via conisation.
2.3 Human Papillomavirus

A persistent infection with high-risk human papillomavirus (HPV) is the necessary cause for the development of cervical cancer.\textsuperscript{6-9} HPVs are small, non-enveloped, double-stranded viruses that belong to the Papillomaviridae family. The viral genome is about 8,000 base pairs long and contains eight genes\textsuperscript{10}, which are grouped into early and late genes. The early genes (E1, E2, E4, E5, E6 and E7) encode proteins that are necessary for viral replication, whereas the late genes (L1 and L2) encode the capsid proteins.\textsuperscript{11}

So far, more than 200 HPV types have been identified based on DNA sequence data showing genomic differences. Approximately 40 HPV types are known to infect the genital mucosa.\textsuperscript{12} HPVs are subdivided into high-risk (hr) and low-risk (lr) types, according to their oncogenic potential. High-risk HPV types are considered as carcinogenic and can cause high-grade CIN and cervical cancer, whereas low-risk HPV types can cause benign, wart-like lesions and CIN1 and CIN2 lesions. At this moment, twelve different HPV types are considered high-risk (i.e. HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 [IARC class 1]\textsuperscript{13}. In addition, one HPV type is considered as ‘probably carcinogenic’ (HPV68 [IARC class 2A) and several other types are considered as ‘possibly carcinogenic’ (HPV26, 53, 66 and 70 [IARC class 2B])\textsuperscript{13}. HPV16 accounts for approximately 50% of cervical cancers worldwide, followed by HPV18 (~20%).\textsuperscript{14} HPV types 31, 33, 45, 52 and 58 account for an additional 19% of...
cervical cancers.\textsuperscript{15} In addition to causing cervical cancer, HPV, particularly HPV type 16, is associated with other types of cancer (e.g. vulvar and vaginal, anal, penile, and oropharyngeal).\textsuperscript{16-20}

The lifetime risk of acquiring at least one genital HPV infection is estimated at 80%.\textsuperscript{21, 22} Transmission of HPV is mainly through sexual contact.\textsuperscript{23} Hence, like all sexually transmitted infections, the prevalence of HPV infections is highest within the first ten years after sexual debut.\textsuperscript{24, 25} In the Netherlands, HPV prevalence is the highest between the age of 18 and 24 years (\textsim{\textpercent}{20}).\textsuperscript{22} With increasing age, the incidence decreases to less than 5\% in women aged 45 and older.\textsuperscript{26, 27}

In the Netherlands, an increasing HPV prevalence among women participating in cervical screening (women aged 30-60 years old) has been observed in recent years. In a study conducted between 1999 and 2002 a mean HPV prevalence of \textsim{\textpercent}{3} was observed.\textsuperscript{28} A subsequent study conducted between 2003 and 2005 showed a mean HPV prevalence of \textsim{\textpercent}{5}, followed by a mean prevalence of \textsim{\textpercent}{9} in a study conducted between 2013 and 2015.\textsuperscript{27} Moreover, studies from the Netherlands, the United Kingdom, Scotland, Finland, Sweden and the United States showed an increase in the incidence of oropharyngeal squamous cell carcinoma (OSCC)\textsuperscript{30-35} and the proportion of HPV-positive OSCC\textsuperscript{36-38}, most likely explained by an increase in the prevalence of oral HPV infections. Based on this, it is suggested that we are currently encountering an HPV epidemic\textsuperscript{39}, most likely explained by a change in sexual behaviour, e.g. increasing numbers of sex partners and increased oral sexual activity.\textsuperscript{40, 41}

\section*{2.4 HPV induced cervical carcinogenesis}

The concept of cervical carcinogenesis consists of different steps, starting with HPV infection, transition of productive to transforming HPV infection, development of cervical precancer, and finally invasive cancer (Figure 5). Approximately 80\% of HPV infections have a transient character as they are cleared by the host's immune system within 1-2 years. These transient HPV infections do not result in visible cervical precancerous lesions.\textsuperscript{7, 42} The remaining 20\% of HPV infections will persist.

Viral particles entering the basal cells of the squamous epithelium via microabrasions may give rise to a \textbf{productive infection}. In a productive infection new viral particles are formed and released from shedding terminally differentiated cells. These infections may give rise to mild to moderate cellular abnormalities, which are histologically recognisable as CIN1 or CIN2 lesions. These so-called productive CIN lesions do not reflect actual cervical precancer and, usually, spontaneously regress within 1-2 years.\textsuperscript{43}
In contrast to productive infections, **transforming infections** may give rise to CIN2, CIN3 and cervical cancer. A transforming infection occurs when there is a deregulation in the expression of the viral E6 and E7 oncoproteins resulting in a deregulated cell cycle.\(^{44}\) The E6 oncoprotein targets the p53 tumor suppressor protein, thereby interfering with p53-apoptosis and cell cycle control mechanisms.\(^{45, 46}\) The E7 oncoprotein targets the retinoblastoma tumor suppressor protein (pRB) and leads to increased E2F activity with as a consequence uncontrolled cell proliferation. In addition, complex formation of E6 and E7 with other cellular proteins does also contribute to the virus-mediated transformation process.\(^{47, 48}\) Both oncoproteins can modulate the DNA methylation machinery, thereby influencing cellular and viral gene expression.\(^{43}\) Only 1-3% of the productive HPV infections will persist and gain transforming capacities. A consequence of the deregulated E6 and E7 expression in case of a transforming infection is chromosomal instability, which contributes to the accumulation of both genetic and epigenetic aberrations in host cell genes, which may ultimately result in malignant transformation.\(^{45, 49, 50}\) The step from HPV infection to invasive cervical cancer takes 15-30 years.\(^{50, 51}\)

The above indicates that CIN lesions represent a heterogeneous group of lesions, consisting of both productive and transforming lesions, and both early and advanced lesions, the latter indicating a high short-term progression risk to cancer (Figure 5). Only a small part of CIN2/3 lesions will progress to invasive cancer. However, it is impossible to predict which CIN lesions will, and which will not, progress to cancer. Consequently, all women with CIN3, and a large proportion of women with CIN2, are advised to undergo treatment by a large loop excision of the transformation zone (LLETZ), in order to prevent possible progression to cancer. Based on these treatment guidelines, in the United States a two-tiered grading system is used, dividing CIN lesions into low-grade squamous intraepithelial lesions (LSIL, i.e. CIN1) that do not require direct treatment, and high-grade squamous intraepithelial lesions (HSIL, i.e. CIN2 and CIN3) that do require direct treatment.\(^{52}\)

It is known that treatment of all high-grade CIN lesions does lead to a considerable amount of overtreatment of potentially regressive CIN lesions. Therefore, current research in the field of cervical cancer prevention aims to identify biomarkers that are able to distinguish between advanced CIN2/3, requiring direct treatment in order to prevent development into cancer, and early CIN2/3 that allow a wait-and-see policy because of a high chance of spontaneous regression.
Figure 5. Schematic presentation of HPV-mediated cervical carcinogenesis (adapted from Steenbergen et al. 2014).
HPV = human papillomavirus; CIN = cervical intraepithelial neoplasia

3. Cervical cancer prevention

Prevention can take place at three different levels: primary, secondary, or tertiary. **Primary prevention** aims to prevent the onset of disease, by reducing risk factors or providing protection in healthy individuals. **Secondary prevention** aims to detect and treat disease in a subclinical and non-symptomatic stage in order to prevent progression (e.g. cancer screening programmes). **Tertiary prevention** aims to decrease the burden of disease among individuals with established clinical stage disease.

Given the long duration from HPV infection to cervical cancer it is unfeasible to study the effect of preventive strategies using cervical cancer as endpoint. Consequently, intermediate endpoints are often used to study the effect of preventive strategies. These surrogate endpoints for cervical cancer are chosen based on their role in the pathway of cervical carcinogenesis. For evaluation of the effectiveness of HPV vaccination preventive strategies in cervical cancer research the following endpoints, in decreasing order of strength, are often used as intermediate endpoints: CIN3+ and CIN2+ yield, and 12 and 6 month (type-specific) HPV persistence.

3.1 Primary cervical cancer prevention: prophylactic HPV vaccination

Primary prevention of cervical cancer aims to prevent the onset of disease by providing protection by prophylactic HPV vaccination. Prophylactic HPV vaccines are designed to prevent initial HPV infection and subsequent HPV-associated lesions, and are based on the discovery that the major structural L1 gene of HPV
automatically folds into non-infectious virus-like particles (VLPs) which mimic the viral capsid.\textsuperscript{53}

Currently, three HPV vaccines are available. Firstly, the bivalent vaccine (Cervarix\textsuperscript{®}, GSK) is directed against HPV16 and HPV18, which are the most carcinogenic HPV types and are estimated to cause approximately 70\% of all cervical cancers worldwide.\textsuperscript{54} Secondly, the quadrivalent vaccine (Gardasil\textsuperscript{®}, Merck) is also directed against HPV 16 and HPV18, but in addition also targets two low-risk HPV-types (HPV6 and HPV11), and therefore also protects against the majority of anogenital warts.\textsuperscript{55, 56} Thirdly, the nonavalent vaccine (Gardasil-9\textsuperscript{®}, Merck) is directed against the same HPV types as the quadrivalent vaccine (6, 11, 16 and 18), as well as high-risk types 31, 33, 45, 52, and 58.\textsuperscript{57} Although protection of HPV vaccines is type specific, a significant degree of cross-reactivity against other HPV types has been demonstrated.\textsuperscript{58, 59}

Large randomized controlled trials have shown HPV vaccination to be effective in the prevention of vaccine-type related cervical precancer, especially among HPV-naive women (93-100\% efficacy).\textsuperscript{54-57} Moreover, protection against cervical precancer associated with non-vaccine HPV types has been shown to different extents.\textsuperscript{60-62}

Several countries have introduced HPV vaccination for young girls. In the Netherlands, three-dose vaccination with the bivalent HPV vaccine was introduced in 2009 for girls aged 12, with catch-up vaccination for girls aged 13-16 years old (i.e. born in 1996 to 1993). In 2014, two-dose vaccination has been implemented \textsuperscript{63}, and recent studies suggest that one-dose vaccination might also be effective.\textsuperscript{64, 65} Up to now, participation in the HPV vaccination programme in the Netherlands has been suboptimal. In 2017, HPV vaccine uptake was 45.5\%.

Since the introduction of HPV vaccination programmes for girls, knowledge on the role of HPV in other cancer types, such as anal, penile, vaginal, vulvar and oropharyngeal cancer, has been obtained, and effectiveness of the HPV vaccines against other anogenital diseases has been shown. Consequently, registration indications of HPV vaccination have been extended to other HPV-related cancers and all three available HPV vaccines have been licensed for use in males.\textsuperscript{66, 67} Implementation of male HPV vaccination could protect males against HPV-associated diseases, and, in addition, could reduce transmission of HPV, thereby increasing herd immunity and cervical cancer prevention. Several countries, for example The United States, Australia, Finland and Canada, have implemented gender-neutral HPV vaccination. However, in many countries it remains a point of discussion whether to implement gender-neutral HPV vaccination. Although vaccination of boys is likely to be less efficient
on a population level than increasing the vaccination uptake among girls, it is expected to increase herd immunity by preventing virus transmission. According to recent estimations of a Dutch study, for each additional female to be vaccinated, two males should be vaccinated to obtain a similar effect. However, gender-neutral HPV vaccination is still expected to be cost-effective under the current uptake and vaccine costs. A decision of the Dutch Health council about implementation of gender neutral vaccination is expected by the end of this year.

3.2 Secondary cervical cancer prevention: cervical screening

Secondary prevention of cervical cancer aims to detect and treat early stage disease in women without symptoms in order to prevent progression. Since the progression of CIN to cervical cancer is slow, and since CIN lesions can be detected relatively easy and can be treated effectively, secondary prevention of cervical cancer via screening is possible. The effectiveness of screening depends on the clinical performance of the screening test, the attendance rate, the adequacy of follow-up algorithms, and the availability and effectiveness of treatment. The disadvantages of screening also depend on the clinical accuracy of the screening test and the adverse effects of (over)treatment.

The clinical performance of a test is determined by its sensitivity -the percentage of women with meaningful disease (i.e. high-grade CIN) who are correctly identified as having the condition- and its specificity -the percentage of women without disease who are correctly identified as not having the condition. Ideally, a screening test has a 100% sensitivity and a 100% specificity, which would mean that the test identifies all patients with disease, allowing treatment of all women with high-grade CIN, and that the test correctly not-identifies all women without disease, limiting the number of unnecessary follow-up tests and treatments. However, in practice, there is a balance between these two parameters: an increase in sensitivity often results in a decrease in specificity, and vice versa (Table 1).

Predictive values are influenced by the prevalence of disease in the population, and are often more meaningful for evaluation of a screening test. The positive predictive value (PPV) is the proportion of women with a positive test result who have underlying disease. The negative predictive value (NPV) is the proportion of women with a negative test result who do not have the disease.
Table 1. Explanation of screening test characteristics and an overview of harms and benefits of screening outcomes (adapted from Naber et al. 2017).

<table>
<thead>
<tr>
<th>(PRECURSOR OF) DISEASE</th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>True positive</td>
<td>Benefits: early detection of progressive disease, enabling early treatment and reducing morbidity and mortality from disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Harms: detection and treatment of non-progressive disease due to overdiagnosis and overtreatment</td>
<td></td>
</tr>
<tr>
<td>False positive</td>
<td>Benefits: -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Harms: unnecessary follow-up testing, leading to anxiety and stress</td>
<td></td>
</tr>
<tr>
<td><strong>Negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>False negative</td>
<td>Benefits: -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Harms: false reassurance, potentially leading to a later diagnosis</td>
<td></td>
</tr>
<tr>
<td>True negative</td>
<td>Benefits: justified reassurance</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Harms: -</td>
<td></td>
</tr>
<tr>
<td><strong>Positive predictive value (PPV):</strong></td>
<td>true positives among test positives</td>
<td></td>
</tr>
<tr>
<td><strong>Negative predictive value (NPV):</strong></td>
<td>true negatives among test negatives</td>
<td></td>
</tr>
</tbody>
</table>

Sensitivity: true positives among those with disease

Specificity: true negatives among those without disease
3.2.1. Cytology-based cervical screening

The cytology test, or the Pap-smear, involves the cytomorphological evaluation of exfoliated cells from the transformation zone of the cervix (Figure 6). Cervical cytology was first described in 1941 as a method to detect the presence of cervical precancer and cancer. This finding resulted in the worldwide use of cervical cytology as a standard test for cervical screening. Initially, the collected cervical cells were smeared directly onto a microscope slide (i.e. conventional cytology). Nowadays, the cervical cells are transferred into a vial with preservative liquid. Subsequently, in the laboratory a layer of cells is applied on a slide by centrifugation (i.e. liquid-based cytology). Liquid-based cytology allows the preparation of several high-quality slides and allows the use of the obtained cellular material for additional testing. Cervical cytology slides are assessed by cytotechnicians and -pathologists and are classified according to standard classification systems. Internationally, the Bethesda classification system for reporting cervical or vaginal cytological diagnoses is most commonly used. Women with an abnormal cervical cytology result are subsequently referred to a gynaecologist for colposcopy.

In the Netherlands, cytology was first introduced in the 1960s, and regionally organized screening programmes were set up in the 1970s. The incidence and mortality of cervical cancer declined ~50% since the 1960s. In 1988 a national
screening programme, in which women aged 35-54 years old were invited every three years for a Pap-smear, started. In 1996 the programme was restructured, and women aged 30-60 years old were invited every five years.\textsuperscript{77}

In the Netherlands cytology slides are assessed according to the CISOE-A classification (KOPAC-B in Dutch). This classification interprets smears using each letter as an item to be scored: C for composition, I for inflammation, S for squamous epithelium, O for other abnormalities and endometrium, E for endocervical columnar epithelium, and A for adequacy of the smear.\textsuperscript{78} Based on the CISOE-A score a Pap-score is determined. The CISOE-A classification can be easily converted into the international Bethesda classification (Table 2). In 2016, the majority (93.5\%) of women participating in cervical screening in the Netherlands had normal cytology (Pap 1) and were therefore referred back to the next screening round five years later.\textsuperscript{79} 3.8\% of women had borderline (Pap 2) or mild (Pap 3a1) dyskaryosis (BMD) cytology and were advised to repeat-cytology after six months.\textsuperscript{79} If BMD or worse (≥BMD) was present at the repeat-cytology test, women were referred to the gynaecologist for colposcopic examination of the cervix. Approximately 0.9\% of women who participated in screening in 2015 had moderate dyskaryosis (Pap 3a2), severe dyskaryosis (Pap 3b), suspicion of carcinoma in situ (Pap 4), or suspicion of invasive disease (Pap 5) and were directly referred for colposcopy.\textsuperscript{79} Finally, in 1.6\% of women the cervical smear was unsatisfactory for evaluation (Pap 0).\textsuperscript{79}

In the Netherlands, and in many other countries, cytology-based cervical screening has led to a significant decrease in cervical cancer incidence and mortality.\textsuperscript{80-84} However, in recent years, this decrease seems to have levelled-off (Figure 7)\textsuperscript{85-88}, most likely as a consequence of limited sensitivity of cytology and the increase in HPV prevalence that has been observed over the last decades (see ‘2.3 Human Papillomavirus’).

Cytology-based screening has a few constrains. The biggest constrains are the limited sensitivity and the subjectivity of the cytology test for detection of high-grade CIN (50-65\%).\textsuperscript{80, 89-91} Moreover, participation in cytology-based cervical screening is limited (60.3\% in 2016).\textsuperscript{79, 92} In order to overcome these constrains and to further reduce cervical cancer incidence and mortality, a new HPV-based screening programme was introduced in the Netherlands in 2017.
Table 2. Classification of cervical cytology (adapted from Bulk et al. 2004 and Bulkmans et al. 2004).

<table>
<thead>
<tr>
<th>Description</th>
<th>Inadequate</th>
<th>Normal</th>
<th>Borderline dyskaryosis</th>
<th>Mild dyskaryosis</th>
<th>Moderate dyskaryosis</th>
<th>Severe dyskaryosis</th>
<th>Carcinoma situ</th>
<th>CISOE-A</th>
<th>Bethesda 2001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pap-score</td>
<td>Pap0</td>
<td>Pap1</td>
<td>Pap2</td>
<td>Pap3a1</td>
<td>Pap3a2</td>
<td>Pap3b</td>
<td>Pap4</td>
<td>Pap5</td>
<td>HSIL</td>
</tr>
<tr>
<td>CISOE-A</td>
<td>C0</td>
<td>S1, O1-2, E1-2</td>
<td>S2-3, O3, E3</td>
<td>S4, E4-5</td>
<td>S5, O4-5</td>
<td>S6, O6, E6</td>
<td>S7, E7</td>
<td>S8-9, O7-8, E9</td>
<td></td>
</tr>
<tr>
<td>Unsatisfactory for evaluation</td>
<td>NILM</td>
<td>ASC-H</td>
<td>ASC-US</td>
<td>LSIL</td>
<td>AGC favour neoplastic</td>
<td>AIS</td>
<td>SCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CISOE-A: C = composition, I = inflammation, S = squamous epithelium, O = other abnormalities and endometrium, E = endocervical columnar epithelium; A = adequacy. NILM = negative for intraepithelial lesion or malignancy; ASC-H = atypical squamous cells cannot exclude HSIL; ASC-US = atypical squamous cells of undetermined significance; AGC = atypical glandular cells; LSIL = low grade squamous intraepithelial lesion; HSIL = high-grade squamous intraepithelial lesion; AIS = endocervical adenocarcinoma in situ; SCC = squamous cell carcinoma; AC = adenocarcinoma.
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Figure 7. World standardised incidence and mortality rates from cervical cancer in the Netherlands (rate per 100,000 women) (adapted from www.cijfersoverkanker.nl).

3.2.2 HPV-based cervical screening

Several clinical studies have evaluated the use of HPV testing for cervical screening. Four European randomised trials (Swedescreeen, POBASCAM, ARTISTIC and NTCC) showed that HPV testing significantly reduced the detection of CIN3+ at the second screen with 47-66% as compared to conventional cytology. Moreover, pooled results of these trials showed a 60-70% better protection against cervical cancer. Based on these results, several countries have implemented, or are planning to implement, HPV-based screening.

In the Netherlands, in 2011 the Health Council advised the Ministry of Health to replace cytology with primary HPV testing in cervical screening. Consequently, a new, HPV-based cervical screening programme has been implemented in the Netherlands in 2017. The rationale behind the switch from cytology to HPV-based screening is highlighted in the discussion of this thesis.

As most HPV infections are cleared spontaneously, only a small proportion of HPV infections persists and eventually lead to the development of cervical precancer. Adjunct testing (i.e. triage testing) of HPV-positive women is required to identify the subset of women with clinically relevant cervical disease. In the new cervical screening programme in the Netherlands, triage of HPV-positive women is performed by (reflex-)cytology at baseline cytology and repeat cytology after 6 months. If cytology, either at baseline or at six months follow-up, is abnormal (≥BMD), women are referred for colposcopy. If HPV-positive women have normal cytology at baseline
and at six months follow-up, women are referred back to the next screening round five years later. Different studies have shown that cytology with repeat-cytology is a feasible triage strategy.\textsuperscript{99, 100}

Primary HPV testing detects high-grade CIN earlier than cytology. Results of different studies showed that long-term high-grade CIN risks after a HPV-negative test result are much lower than risks after a negative cytology result.\textsuperscript{29, 101-103} In a study evaluating data from seven European studies, six-year CIN3+ risk for HPV-negative women was found to be 0.27%, compared to 0.97% for women with a negative cytology result.\textsuperscript{103} In addition, a Dutch follow-up study showed that long-term CIN3+ risks in HPV-negative women aged 40 years and older were 72% lower than in HPV-negative women under 40.\textsuperscript{104} Based on these results, \textit{screening intervals} for HPV-negative women aged 40 years and older are extended to 10 years within the new programme.\textsuperscript{98}

Approximately half of the cervical cancers that occur in the Netherlands is diagnosed in the 35\% of women not attending cervical screening.\textsuperscript{92} Consequently, it is important to decrease the number of non-attendees. Studies have showed that offering HPV \textit{self-sampling} to non-responders (i.e. women who do not participate in routine screening) increases participation rates.\textsuperscript{105-112} In Dutch studies, self-sampling attendance rates up to 34.2\% were found among screening non-responders.\textsuperscript{110-114} Based on these results, HPV self-sampling for non-responders is implemented in the new cervical screening programme in the Netherlands.\textsuperscript{98}

\section*{4 Thesis outline}

\subsection*{4.1 Challenges in HPV-based cervical screening}

The first part of this thesis focuses on strategies to overcome challenges within HPV-based screening, e.g. validation of HPV assays, strategies for triage of HPV-positive women and extension of screening intervals.

\subsubsection*{4.1.1 Validation of HPV assays}

Worldwide, HPV testing is increasingly being incorporated in clinical protocols and screening guidelines. Nowadays, a substantial number of different HPV assays is available\textsuperscript{115}, however, the clinical performance of these assays differ significantly. Therefore, one of the challenges within HPV-based screening is establishing and maintaining criteria for validation of new HPV assays.
In the four large European randomized trials that demonstrated the efficacy of HPV-testing, the GP5+/6+ PCR-enzyme immunoassay (EIA) and the Hybrid Capture-2 (HC2) assay were used. Therefore, these assays can be considered as validated reference assays for cervical screening. Based on this, an international team of experts formulated equivalence criteria for validation of HPV assays (i.e. the Meijer criteria). These criteria state that the clinical sensitivity of a new HPV assay for detection of CIN2+ should not be lower than 90%, and the clinical specificity for CIN2+ not lower than 98%, compared the GP5+/6+ PCR-EIA or the HC2 assay. Before a new HPV assay can be used for screening purposes, non-inferiority compared to the GP5+/6+ PCR-EIA or the HC2 assay should be shown. Currently, many HPV assays have been proven to fulfil these criteria and are therefore considered clinically validated for primary cervical screening.

The international validation guidelines described by Meijer et al. have been useful for translation of HPV testing into clinical practice. However, one of the difficulties for validation of assays is obtaining suitable test material. The VALGENT (Validation of HPV Genotyping Tests) framework facilitates the comparison and validation of HPV tests by providing a set of samples derived from women attending routine screening, enriched with cytological abnormal samples. In order to allow comparison with other HPV assays, each VALGENT panel includes a clinically validated comparator assay.

In chapter 2 we evaluate the clinical performance of the HPV-Risk assay for detection of high-grade CIN, in comparison to the performance of a clinically validated comparator assay (Hybrid Capture 2).

4.1.2 Triage of HPV-positive women
HPV-testing has a 3-4% lower specificity compared to cytology due to the transient nature of many HPV infections. Because of this lower specificity, additional triage testing of HPV-positive women is needed to distinguish women with a transient HPV infection from those with a high-risk of cervical precancer and cancer who should be referred for colposcopy. Consequently, one of the challenges within the new HPV-based cervical screening programme in the Netherlands is finding the most suitable strategy and developing new triage tools.

Within the new cervical screening programme in the Netherlands, triage of HPV-positive women is performed by cytology. Even though different studies showed
that cytology with repeat-cytology is a feasible triage strategy\textsuperscript{99, 100}, cytology testing also has a couple of limitations. The first limitation of cytology is that it is a subjective test. The quality of cytology strongly depends on the assessor’s experience and training, and therefore significantly differs between countries. In addition, international studies indicate that prior knowledge of the HPV-status influences cytological assessment.\textsuperscript{123-125} Secondly, a limitation of cytology triage is that it can only be performed on a cervical smear, since self-sampled cervicovaginal material does not contain sufficient intact cervical indicator cells for a reliable cytological assessment. Consequently, within the new cervical screening programme in the Netherlands, women who test HPV-positive on their self-sample, still have to go to their general practitioner for a cytology triage smear. These reasons underline that there is a need for more objective biomarkers that, ideally, can be performed on self-sampled cervicovaginal material.

A way to make cytology more objective is immunohistochemistry of the cervical cytology slide with \textbf{p16/Ki-67 dual-staining}. P16 is a negative cell-cycle regulatory protein.\textsuperscript{126} Normally, p16 is expressed at very low levels in healthy cells, while it is overexpressed in cervical-cancer cell lines in which pRb is inactivated by the HPV E7 oncoprotein.\textsuperscript{127, 128} Ki-67 is a proliferation marker. Simultaneous expression of the anti-proliferative p16 protein and the proliferation marker Ki-67 should exclude each other in cells under normal physiological conditions.\textsuperscript{129, 130} When a cell shows p16/Ki-67 co-expression, which can be identified by double staining, this is a sign for cell-cycle deregulation and points to HPV-induced high-grade CIN lesions. P16/Ki-67 dual-stained cytology has shown promising results compared to sole cytology triage for triage of HPV-positive women.\textsuperscript{129, 131-133} A limitation of p16/Ki-67 dual-stained cytology is that it, like cytology, cannot be performed on self-sampled cervicovaginal material. In addition, the threshold for p16/Ki-67 positivity is the presence of one single cell staining positive for both p16 and Ki-67, which accentuates the need for qualitatively good samples with sufficient cellularity.\textsuperscript{129, 130, 132-135} Another limitation is that, although the addition of immunohistochemistry makes cytology more objective, the assessment of a p16/Ki-67 dual-stained cytology slide remains partially subjective. Therefore, more objective, non-morphological molecular markers have gained attention in recent years.

In \textit{chapter 3} we evaluate the cross-sectional and longitudinal performance of p16/Ki-67 dual-stained cytology for triage of HPV-positive women with normal cytology in a population-based screening cohort.
Another application of p16/Ki-67 dual-stained cytology is to monitor post-treatment women for recurrent CIN (rCIN).

In chapter 4 we evaluate the performance of p16/Ki-67 dual-stained cytology for post-treatment monitoring of women treated for high-grade CIN.

Another option for triage or follow-up of HPV-positive women is repeat HPV testing. HPV testing is objective and can be performed on self-samples. In addition, it seems practical to re-test HPV-positive women for the presence of HPV since women who have cleared the HPV infection are no longer at risk for high-grade CIN. However, little research has been performed on the absolute residual CIN2+ risk after an HPV-negative repeat test, and therefore it is unsure if HPV-positive, repeat HPV-negative women truly have low CIN2+ risks. Moreover, a strategy consisting of repeat HPV testing is expected to lead to a high number of referrals for colposcopy and has the disadvantage of losing women to follow-up.

In chapter 5 we evaluate the five-year risk of HPV infection and CIN3+ in HPV-positive women with normal cytology and a negative repeat HPV test.

Another known triage strategy is HPV genotyping. The risk for development of cervical precancer and cancer is related to the genotype. HPV genotypes 16 and 18 are associated with 70% of the cervical cancers and 50% of the CIN3 lesions. Previous studies have shown that these two types have a high oncogenic potential and are more likely to persist, which makes HPV16/18-genotyping an interesting strategy for stratification of HPV-positive women. A limitation of HPV16/18-genotyping is that the residual risk for HPV16/18-negative women is still relatively high, since approximately 30% of the cervical cancers and 50% of the CIN3 lesions are caused by other HPV-genotypes. Therefore, HPV16/18-genotyping can only be implemented when combined with another triage tool, for example cytology.

Finally, a relatively new method for triage of HPV-positive women is with the use of DNA methylation markers. These markers are based on the fact that specific (epi)genetic changes of host cell genes are necessary for the development of cervical cancer. Tumor suppressor genes produce proteins that have an inhibitory effect on the cell cycle, thereby preventing unrestricted cell division and associated
tumor growth. In normal conditions, tumor suppressor genes are unmethylated, while methylation of these genes can lead to silencing. Once silenced, the tumor suppressor gene can no longer fulfil its inhibitory function, which may eventually lead to tumor growth. Research has shown that methylation of certain genes is functionally involved in (cervical) carcinogenesis. Consequently, methylation marker analysis of different genes (e.g. CADM1, MAL, mir124-2 and FAM19A4) showed a good performance for detection of cervical cancer and high-grade CIN in HPV-positive women. Currently ongoing studies aim to identify the most suitable methylation markers and to assess the long-term CIN3+ and cancer risk for women with a negative methylation test.

In chapter 6 we aim to identify feasible strategies for triage of HPV-positive women in the second round of HPV-based screening.

4.1.3 Extension of screening intervals

Within the new HPV-based screening programme, screening intervals for HPV-negative women aged 40 years and older will be extended to 10 years within the new programme. For HPV-positive women the five-year screening interval remains as it is uncertain whether risks for HPV-positive, triage-negative women are low enough to justify extension of the screening intervals beyond five years. Therefore, research is needed to evaluate the long-term safety of a negative triage test.

In chapter 7 we evaluate the long-term CIN3+ risk of women with different combinations of HPV and cytology test results in order to evaluate if screening intervals can be safely extended.

4.2 Future perspectives in HPV-based cervical screening

4.2.1 Implementation of HPV self-sampling

Studies have shown that offering HPV self-sampling to non-responders attracts women into cervical screening. Based on these results, HPV self-sampling is offered to non-responders within the new screening programme in the Netherlands. It is believed that a proportion of women who do normally attend clinician-based sampling for cervical screening would also prefer self-sampling. Moreover, implementation of self-sampling as a primary screening option could make
cervical screening more cost-effective as it would greatly reduce the number of GP visits. However, before HPV self-sampling can be considered as a primary screening option for the total screening population instead of only for non-responders, non-inferiority as compared to clinician-collected HPV testing needs to be assessed.\textsuperscript{148-150}

In chapter 8 we aim to assess non-inferiority of HPV testing on self-collected samples as compared to clinician-based samples for detection of CIN2+ and CIN3+ within an organized screening setting.

Questionnaire surveys evaluating women’s experiences with self-sampling and clinician-based sampling in non-responders populations, showed that self-sampling was very well accepted and experienced as more convenient, less embarrassing, less uncomfortable and less painful than clinician-based sampling.\textsuperscript{27, 151-155} Moreover, when asked for their preference for future screening, most women indicated to prefer self-sampling over clinician-based sampling for future screening. Although it is believed that women who normally do attend screening via clinician-based sampling would also prefer self-sampling, little research into their experiences and preferences has been performed.

In chapter 9 we evaluate women their experiences with self-sampling as compared to clinician-based sampling, and assess their preferences for future screening.

Finally, chapter 10 provides a general discussion in which we review the rationale and future perspectives of the new Dutch HPV-based cervical screening programme and discuss how the results presented in this thesis may contribute to further improvement of cervical screening.
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