CHAPTER

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1. CERVICAL CANCER

1.1. Epidemiology

With an estimated 528,000 new cases and 266,000 deaths in 2012, cervical cancer is the fourth most common cancer in women worldwide with an age standardized incidence rate (ASIR) of 14.0 per 100,000 women. The majority of cervical cancers (85%) occurs in developing countries, where it is responsible for almost 12% of all cancers in women.1,2 In Eastern and Middle Africa cervical cancer is the most common cancer in women, with ASIRs of 42.7 and 30.6 per 100,000 women, respectively. The lowest registered incidence rates are found in Western Asia (4.4), Australia and New Zealand (5.5 per 100,000), Northern America (6.6 per 100,000), Northern Africa (6.6 per 100,000) and Western Europe (7.3 per 100,000) (Figure 1).1

Efficient programmed screening is the most important factor that affects the observed trends in incidence of and mortality from cervical cancer, since it leads to an earlier detection of (precursors of) cervical cancer, and better survival owing to earlier treatment.3,4 The introduction of regular cervical screening in the Netherlands coincided with a decrease in incidence of cervical cancer from 9.2 per 100,000 women in 1988 to 5.9 per 100,000 women in 2000.3

In 2012, 6.8 per 100,000 women were diagnosed with cervical cancer and 1.6 per 100,000 women died as a consequence of this disease.1 The 5-year overall survival rate in women with cervical cancer in the Netherlands is 67%.5 The 5-year survival rate depends on the stage of cervical cancer and is 60-99% in women with FIGO stage I and II, and 10-40% in women with FIGO stages III and IV.6

Figure 1. World age-standardised incidence rates (ASIR) of cervical cancer in 2012 (rate per 100,000). [Source: GLOBOCAN].1
1.2. The uterine cervix

Cervical cancer arises from the uterine cervix, which is the lower segment of the uterus and is located between the uterus and vagina (Figure 2). The cervix consists of two parts: the ectocervix (outer part of the cervix on the vaginal side) which is covered by multi-layered squamous epithelial cells and the endocervix (inner part of the cervix on the uterine side) lined by a single layer of glandular columnar epithelial cells. The border between the two types of epithelial cells is called the squamo-columnar junction (SCJ). Due to hormonal changes from puberty onwards, squamous epithelial cells will replace columnar epithelial cells at this junction. This leads to a dynamic shift of the SCJ from the ectocervix into the endocervix. The macroscopically visible region between the old and new position of the SCJ is referred to as the transformation zone (TFZ) of the cervix. The metaplastic cells of this zone are most vulnerable for neoplastic changes. Therefore, according to the classic concept of cervical cancer development, it is thought that most cervical cancers have their origin on cells of the TFZ (Figure 2). However, recent data support the hypothesis that most cervical cancers develop from a discrete population of single layered, cuboidal epithelial cells of embryonic origin in the SCJ, instead of from metaplastic epithelial cells of the TFZ. These presumed highly transformation-susceptible cells are characterized by a specific immunohistochemical staining pattern using SCJ specific antibodies (i.e., keratin7, AGR2, CD63, MMP7, and GDA).

There are different histological subtypes of cervical cancers of which the majority (80%) comprises squamous cell carcinomas (SCCs), which are generally localized in the ectocervix. A smaller fraction of cervical cancers comprise adenocarcinomas (ACs) (15-20%) that are often found in the endocervix. Rarely (5%) other histotypes are diagnosed, such as neuro-endocrine and clear-cell carcinomas.

Figure 2. Anatomy of uterus and cervix. [Adapted from micro2tele.com].
1.3. Precursor lesions of cervical cancer

Cervical SCCs develop via premalignant lesions called cervical intraepithelial neoplasia (CIN). These CIN lesions are divided into CIN1, in which the lower one third of the epithelium shows atypia (mild dysplasia), CIN2 with atypical cells in the lower two thirds of the epithelium (moderate dysplasia), and CIN3, in which more than two thirds of the epithelium shows atypia (severe dysplasia or carcinoma in situ). When the atypical cells invade the basal membrane, lesions are graded as SCC (Figure 3).

All CIN lesions can regress, with the highest regression rate for CIN1 and the lowest regression rate for CIN3. The reverse is true for the progression rate to cervical cancer, with the highest rate seen for CIN3 lesions, of which 30-50% may ultimately progress into cervical cancer within 30 years. Since CIN2 and CIN3 lesions have a higher relative risk for progression to cervical cancer compared with CIN1, they are considered as the true cancer precursor lesions and are referred to as high-grade CIN, whereas CIN1 lesions are regarded as low-grade CIN. Although only a subset of high-grade lesions will progress into cervical cancer, it is not possible to distinguish between non-progressive and progressive high-grade lesions. Therefore, treatment by a loop electrosurgical excision procedure (LEEP) is advised for all women with high-grade CIN to prevent the development of SCC. This approach in practice results in overtreatment.

In contrast to SCCs, little is known about the precursor stages of ACs, which are supposed to arise from glandular cells of the endocervix. A classification system similar to the precursors of SCC has been proposed but reproducibility is low and remains controversial. The best defined precursor is adenocarcinoma in situ (ACIS) in which treatment is needed.

Figure 3. Schematic presentation of histological classification of cervical precursor lesions. [Adapted from Woodman et al 2007]
2. HUMAN PAPILLOMAVIRUS (HPV)

Persistent infection with HPV is the necessary cause for developing cervical cancer. HPV can be detected in almost all SCCs (99.7%) and in 94-100% of ACs of the cervix. HPVs are non-enveloped double-stranded DNA viruses and belong to the Papillomaviridae family. The HPV genome is approximately 8,000 base pairs long and can be divided into early genes encoding proteins necessary for viral replication (E1, E2, E4, E5, E6 and E7), and late genes, which encode the major and minor viral capsid proteins (L1 and L2).

HPVs are explicitly epitheliotropic and can be subdivided into mucosal and cutaneous types, which can infect mucosal and cutaneous epithelial cells, respectively. Presently, more than 120 different HPV types have been identified of which 40 can infect the genital mucosa. These types are subdivided in low-risk (lr) types that are associated with benign, wart-like lesions and low-grade CIN lesions, and high-risk (hr) types that are considered oncogenic and can cause high-grade CIN and cervical cancer. Twelve types are defined by the World Health Organisation (WHO) as being hrHPV, i.e. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 (IARC class 1). Two types are being recognized as ‘possibly’ cancer-causing, i.e. HPV 68 and 73 (IARC class 2A) and several other types are considered as probable high-risk, i.e. HPV 26, 53, 66, 67, 70 and 82 (IARC class 2B).

Not all hrHPV types have similar oncogenic potential. HPV 16 causes more than 50% of cervical cancers worldwide, followed by HPV 18 (approximately 10-15%). Besides the causal relation between HPV and cervical cancer, HPV is associated with cancers located in other parts of the genital tract, for example anal (90%), penile (55%) and vulvar cancers (50%), as well as SCCs in the head and neck region (HNSCCs).

2.1. Prevalence of HPV infections

About 80% of sexually active women become infected with a genital HPV infection during their life. However, most infections will pass unnoticed. The prevalence of HPV greatly varies worldwide, and is related to the corresponding risk of cervical cancer. The highest prevalence is observed in South America and Africa and the lowest in Asia and Europe. Since the transmission of HPV in the genital tract is mainly through sexual contact, HPV prevalence is highest in women after starting sexual contact. In the Netherlands, prevalence is highest in women between 18 and 24 years of age (20%), whereas it decreases with increasing age to under 3% in women over 45 years of age. In the Netherlands, the prevalence is around 5% in women (30-60 years) who participate in cervical screening. Of all HPV infections, at least 80% is believed to have a transient behavior; these are cleared by the immune system before any precancerous lesion may become manifest. Of the 20% infections that persist, the majority will develop into a non-progressive, low-grade CIN lesion (CIN1), whereas only a minority will progress into high-grade CIN lesions with a risk of progression to cervical cancer. Thus, HPV infections are very common, but only a small percentage (1-3%) of infected women will eventually develop cervical cancer. At the same time backwards steps also take place, with concomitant HPV clearance and regression of CIN lesions.
2.2. HPV mediated cervical carcinogenesis

The normal viral life-cycle is dependent on the differentiation programme of the infected squamous epithelium, with viral replication taking place in differentiated, non-dividing cells. The viral proteins E1 and E2 are both functionally involved in replication of the viral genome. However, for viral DNA synthesis components of the host cell DNA replication machinery are pivotal. Normally, this replication machinery is not active in these differentiated epithelial cells, but some early viral proteins, in particular E7, can re-activate this machinery. This makes these differentiated cells replication competent allowing viral replication. In this type of infection, referred as productive infection, new virus particles are formed and released from terminally differentiated cells. Productive infections with hrHPVs can become manifest as CIN1 and CIN2 lesions, which generally do not develop into more severe precursor lesions (i.e. CIN3) and cervical cancer. Alternatively, a so-called transforming infection may arise, which have a potential risk for progression to cervical cancer and are associated with CIN2, CIN3 and cervical cancer (Figure 4). This indicates that CIN2 represents a heterogeneous group of lesions representing both productive and transforming infections. In transforming infections the viral oncogenes E6 and E7 reveal an improperly increased expression in the proliferating cell fraction of the squamous epithelium. The E6 protein binds with the p53 tumor suppressor gene product, thereby impairing p53-mediated apoptosis and cell cycle arrest. E7 binds with the protein encoded by the retinoblastoma tumor suppressor gene (pRb), thereby disturbing the G1/S checkpoint and continuous triggering transition to S-phase by activating E2F transcription factor family members. Moreover, complex formation of E6 and E7 with other cellular proteins is also considered to contribute to the virus-mediated transformation process. The exact mechanisms for deregulation of E6 and E7 proteins are not completely elucidated. Possible explanations are integration of viral DNA in the host cell or methylation of E2 binding sites, since E2 is a transcriptional regulator of E6 and E7. The continuous overexpression of viral oncoproteins E6 and E7 in proliferating basal cells of the epithelium results in genomic instability, leading to additional (epi)genetic alterations with

Figure 4. Schematic presentation of progression model cervical carcinogenesis [Adapted from Snijders et al 2006]13. *Activation of oncogenes, loss of tumour suppressor gene functions.
an increased expression of E6 and E7 proteins. This overexpression of oncogenes E6 and E7 in combination with additional (epi)genetic changes can ultimately result in malignant transformation. After initial HPV infection, premalignant lesions can develop within 2-3 years. Only 1-3% of all HPV positive women will develop a transforming infection. Only in case of viral persistence an advanced high-grade precursor lesion of cervical cancer may develop after a long time period. Together with persistence of a transforming infection, additional (epi)genetic changes are necessary for further progression towards cervical cancer, which may occur in a minority of cases. This last step, from high-grade CIN lesion to cervical cancer, may last 10-30 years. The fact that cervical cancer develops via premalignant CIN lesions, which can be detected and effectively be treated, offers the possibility for screening to prevent cervical cancer.

3. PREVENTION OF CERVICAL CANCER

There are two ways for the prevention of cervical cancer. As primary prevention, which attempts to prevent healthy people from developing a disease in the first place, prophylactic HPV vaccination has been introduced. As secondary prevention, which is defined as preventing cancer by detecting and treating women with asymptomatic subclinical, precursor lesions, screening by cytology is the current strategy. With cytology based screening, women with abnormal cells are referred to the gynaecologist or closely followed by a repeat cytology testing.

3.1. Primary prevention: prophylactic HPV vaccination

Currently available HPV vaccines are based on virus like particles (VLPs) consisting of HPV L1 capsid proteins that have been self-assembled into particles in specific cell culture systems. Two prophylactic vaccines have been developed commercially: one bivalent vaccine that consists of VLPs of HPV16 and HPV18 (Cervarix®, GSK) and one quadrivalent vaccine, containing VLPs of both (high-risk) HPV16 and HPV18 and low-risk types HPV6 and HPV11 (Gardasil®, MSD). The outside of the VLPs is geometrically and antigenically nearly identical to that of a real HPV viron, but the HPV DNA is absent. Therefore, VLPs have no infectious potential. VLPs prevent the attachment of HPV to the epithelium by inducing high-levels of anti-HPV-16 and -18 immunoglobulin G antibodies, thus preventing subsequent HPV infections. The protection is type specific, but cross-reactivity against HPV45 and HPV31 has been documented. Since the prophylactic vaccines have not shown to be effective in persons who were already infected by HPV before vaccination, it is important to vaccinate girls before their sexual debut. This can lead to a prevention of the majority of cervical cancers because HPV16 and HPV18 are responsible for 70% of the cervical cancers worldwide. Many countries have started HPV vaccination campaigns, targeting mostly young girls. In the Netherlands, HPV vaccination of girls aged twelve years old was implemented in the national immunization programme in 2010, preceded by a catch-up vaccination campaign among girls aged 13-16 years in 2009. However, so far the participation rate of the Dutch vaccination programme has been suboptimal (58.1% in 2013). In addition, even with optimal coverage, additional secondary screening will still be needed in the foreseeable future because of non-HPV 16 and 18-related cervical cancer.
3.2. Secondary prevention: cervical screening programme

3.2.1. General aspects

Cervical cancer is a preventable disease, because the period of preclinical symptoms (CIN lesions) to cervical cancer is 10-30 years and women with CIN lesions can be treated effectively. In 1968, Wilson and Jungner were the first who stated that for an effective screening programme ten criteria should be fulfilled (Table 1).46 The ten old principles have been expanded by new principles, which are universally applicable and unambiguous.

<table>
<thead>
<tr>
<th>Table 1. Principles and practice of screening for disease</th>
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<tbody>
<tr>
<td>1. The condition should be an important health problem.</td>
</tr>
<tr>
<td>2. There should be a treatment for the condition.</td>
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<tr>
<td>3. Facilities for diagnosis and treatment should be available.</td>
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<tr>
<td>4. There should be a latent stage of the disease.</td>
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<td>5. There should be a test or examination for the condition.</td>
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<td>6. The test should be acceptable to the population.</td>
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<td>7. The natural history of the disease should be adequately understood.</td>
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<td>8. There should be an agreed policy on whom to treat.</td>
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<td>9. The total cost of finding a case should be economically balanced in relation to medical expenditure as a whole.</td>
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<tr>
<td>10. Case-finding should be a continuous process, not just a &quot;once and for all&quot; project.</td>
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</table>

[Adapted from Wilson and Jungner et al 1968]46

In a screening setting, screening tests could be negative (indication of test that there is no disease) or positive (indication of test that there is an underlying disease). The test could be true-positive (TP) or true-negative (TN), which indicates that the test result reflects the true condition, or the test could be false-positive or false-negative. A false-positive (FP) test indicates a positive test result in individuals without underlying disease, whereas a false-negative (FN) result means a negative test result in women with disease.

To evaluate the performance of tests that are used in cervical screening, certain test parameters are used like sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Sensitivity is the proportion of women with underlying (preclinical) disease tested as positive and is calculated by the formula TP/(TP + FN). Sensitivity can vary between 0-100%; a test with 100% sensitivity will recognize all patients with the disease by a positive test result. Specificity is the proportion of truly negative test results in women without underlying disease (calculated by TN/(TN + FP)). Specificity can vary between 0-100%; a test with 100% specificity will, on the basis of a negative result, accurately exclude disease in all healthy individuals. However, these test parameters are not so practical for the individual patient; predictive values have more intuitive appeal. The positive predictive value (PPV) is the proportion of women with a positive test result who have underlying disease (TP/(TP+FP)), whereas the negative predictive value is the proportion of women with a negative test result who do not have underlying disease (TN/
(TN+FN)). PPV and NPV of screening tests are indirectly influenced by the prevalence of a disease, in this case the prevalence of precursors of cervical cancer in the studied population.

The success of screening depends on sensitivity of the test, the participation rate of individuals who should be screened, the possibility of accurate diagnosis, availability of follow-up tests and treatment of women with preclinical disease. The disadvantages of screening consist of unnecessary referrals for colposcopy (false positive test results) and subsequent unnecessary treatments, as well as a delay of diagnosis caused by a low sensitivity (false negative test results).

### 3.2.2. Current screening in the Netherlands: Cytology as screening test

The PAP smear was introduced as cervical screening tool in the Netherlands in the early sixties, followed by regional organized screening programmes with cervical cytology in the beginning of the 1970s. However, a nationwide screening programme was only introduced in 1988, by targeting women between 35 and 54 years of age with a cervical smear three-yearly. Since the coverage and efficiency of this programme turned out to be suboptimal, the screening programme was restructured in 1996. This resulted in an extended screening period and lengthened intervals by inviting all women between 30-60 years once every five years.

The original PAP-classification ranges from PAP1 to PAP5 (inappropriate smears are classified as PAP0). However, along with the introduction of nationwide screening in the late eighties, the PAP classification was expanded by the division of the old PAP3 class into Pap 3a and Pap 3b. Further modification of the national screening programme resulted in a further detailed classification of PAP3a1 (‘mild dyskaryosis’), PAP3a2 (‘moderate dyskaryosis’), while the PAP3b equaled ‘severe dyskaryosis’. With the fusion of carcinoma in situ with severe dyskaryosis, the old PAP4 classification effectively became obsolete (‘suspected of carcinoma in situ or CIS’). Throughout the Netherlands, the PAP5 class was maintained in case of suspected (micro-invasive) cancer. Up to 1996, all women with abnormal cytology (i.e. ≥PAP2) were followed up intensively by either repetitive cytology or referral for colposcopy. This led to an increase in burden of follow-up and colposcopy capacity.

The PAP classification was improved into the more detailed CISOE-A nomenclature. Since this new classification, a decrease of equivocal results (PAP2 and PAP3a1) from 11.3% in 1990 to 2.6% in 2000 was observed. The CISOE-A classification can be converted into the Bethesda nomenclature that is used in other countries (Table 2). Briefly, the CISOE-A classification scores for five parameters that evaluates all components of the smear which includes information on specimen composition, inflammatory characteristics and the adequacy of the smear. The abbreviation CISOE-A stands for Composition, Inflammation, Squamous epithelium, Other abnormalities and endometrium, Endo-cervical columnar epithelium, and Adequacy of the smear.
Table 2. Classification of cervical cytology. (Adapted from Bulk 2004 and Bulkmans 2004)49,50

<table>
<thead>
<tr>
<th>CISOE-A</th>
<th>CO</th>
<th>S1, E1-2, O1-2</th>
<th>S2-3, O3, E3</th>
<th>S4, E4-5</th>
<th>S5, O4-5</th>
<th>S6, O6, E6</th>
<th>S7, E7</th>
<th>S8-9, O7-8, E9</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP</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>
</tr>
</tbody>
</table>

**Description**
- Inadequate
- Normal
- Borderline
- Mild
- Moderate
- Severe
- Carcinoma in situ
- Carcinoma

**Bethesda 2001**
- Unsatisfactory for evaluation
- Negative Atrophy
- ASC-H
- ASC-US
- LSIL
- HSIL
- SCC
- AGC
- AGC favour neoplastic
- AIS
- AC

CISOE-A, C composition, I inflammation, S squamous epithelium, O Other abnormalities and endometrium, and E endo-cervical columnar epithelium; ASC-H, atypical squamous cells cannot exclude HSIL; ASCUS, 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.

Currently, for cytology testing, a cervical scrape is taken from the transformation zone of the cervix. The cervical cells are directly smeared on a glass slide (conventional cytology) or suspended in liquid present in a vial with liquid (liquid-based cytology) before a slide is made with a monolayer of cervical cells. In the Netherlands, the majority of screened women (96.5%) have a normal cytology result and are invited for the next screening round after five years.51 Women with an abnormal smear are advised according to the level of abnormality: women with a PAP2 or PAP3a1 result (borderline or mild dyskaryosis, BMD) are advised to get a follow-up smear after 6 and 18 months and referred for colposcopy only in case of a persistent abnormality at either 6 or 18 months. Women with PAP3a2 or worse (>BMD) are directly referred for colposcopy. In the Netherlands, annually around 800,000 women are invited for cervical screening at the physician.51 Of the invited women, approximately 478,000 (64%) attended screening in 2012.52

4. IMPROVEMENT OF CERVICAL CANCER SCREENING

Cytology-based cervical screening programmes have proven to be effective in decreasing the incidence of and mortality from cervical cancer.53,54 However, currently the decrease of incidence has leveled off and a plateau has been reached in the Netherlands (Figure 5). The two main reasons for missing cervical cancers despite a screening programme are the relatively high number of women with false negative cytology results (low sensitivity)55-57 and the relatively low participation rate to the screening programme (~65% per screening round).58 Due to the low sensitivity of cytology (50-70%) frequent testing is required.56 Moreover, cytology has low reproducibility, which leads to variable accuracy.55,59 By repeat cytology testing the amount of false positive test results increases over the total screening period. Therefore, cervical screening could be improved by a better primary screening test.

4.1. HPV testing versus cytology

Since an infection with HPV is a prerequisite for development of cervical cancer, several
randomised controlled trials have studied whether HPV testing could replace cytology as a primary screening tool.\textsuperscript{33,60-66} These studies have shown that HPV testing detects 30% more CIN2+, and 20% more CIN3+ in women aged 30 years and older. Moreover, four trials have shown longitudinal data about CIN3+ diagnosis at a subsequent screening round (after 3-5 years).\textsuperscript{60,64-66} All studies observed approximately 50% lower CIN3+ incidence rates in women with a negative HPV test at baseline compared to those with a normal cytology result. A meta-analysis of these studies showed a 57% lower risk of CIN3+ in the next screening round in women who tested HPV negative compared to women who tested cytology negative.\textsuperscript{67}

In keeping with these data, the Dutch Ministry of Health has decided to replace cytology by HPV testing as a primary screening tool in the cervical screening programme of the Netherlands in 2016.\textsuperscript{51,68} Moreover, other European countries consider replacing cytology testing by HPV testing as primary screening tool in the near future. In addition, a recent study of pooled data of the four abovementioned randomized trials have shown a lower risk (60-70%) of cervical cancer in HPV negative tested women compared to cytology negative tested women.\textsuperscript{69}

It should be added that the HPV test to be used in a screening setting should be clinically validated, i.e. should display a good balance between detecting clinically relevant (i.e. disease related) infections compared to transient (i.e. clinically irrelevant) HPV infections. Too sensitive HPV tests would lead to an excessive follow-up of women with transient HPV infections or HPV positive women without clinically meaningful lesions (CIN2+). The guidelines that have been prepared by an international consortium indicate that the CIN2+ sensitivity should be not less than 90%, and the clinical specificity not less than 98% of that of a clinically validated reference assay.\textsuperscript{70} In addition, these criteria emphasize the importance of a high intra- and inter-laboratory reproducibility of the HPV test in order to fulfill the clinical validation criteria as described by Meijer et al.\textsuperscript{70}
4.2. Improving screening attendance by offering self-sampling

As mentioned above, one of the drawbacks of current screening programmes involves the suboptimal participation rate of invited women (~65%). Since more than half of all women diagnosed with invasive cervical cancer have not adequately been screened before, it is important to reach these so-called non-attendees.\textsuperscript{18,71} Offering self-sampling of cervico-vaginal material for hrHPV testing (further referred to as ‘HPV self-sampling’) to non-attendees has shown to attract these women into screening.\textsuperscript{72,73} HPV self-sampling allows women to sample themselves at home for self-collecting cervico-vaginal material, which they have to send to the laboratory for hrHPV testing. A large variety of collection devices have been used as self-collection devices, including swabs, brushes, tampons or cervico-vaginal lavage devices.\textsuperscript{74-76} Studies using interview surveys have shown that women prefer self-collection over clinician-collection; the mentioned advantages of self-sampling were privacy, ease, time and place of sampling. Only a small percentage of women preferred a physician-taken sample because they did not understand the provided user instructions or felt insecure about using the self-sampling device in the right manner.\textsuperscript{74,76-79} In countries with an organised screening programme, offering HPV self-sampling to non-attendees has resulted in a higher response rate than offering a recall invitation for cytology at the physician.\textsuperscript{72,73,80-85} In several studies, up to 39.0% of the invited non-attendees returned their self-sampled (cervico-) vaginal material to the laboratory for hrHPV testing. Moreover, self-sampling might also facilitate access to cervical screening in low- and middle income countries without standard clinician-based screening facilities.\textsuperscript{86-88} These data support the value of HPV self-sampling as an alternative cervical cancer screening tool or as a way to increase screening compliance, under the condition that a validated combination of self-sampling device and HPV test are used to reach clinical accuracy comparable to HPV testing on a physician taken smear in terms of detection of high grade CIN2.\textsuperscript{89}

In the Netherlands, from 2006 to 2008, two large cohort studies were conducted among non-attendees of the regular cervical screening programme registered in 2005 and 2006.\textsuperscript{73,80} These PROHTECT (Protection by Offering HPV testing on self-sampled Cervico-vaginal specimens Trial) studies showed that ~30% of non-attendees submitted a self-collected sample for hrHPV testing. However, a triage test in HPV positive women is needed because HPV testing also detects women with transient infections without underlying disease. Cytological examination on self-sampled material is not feasible because this material only contains few intact cervical indicator cells in a background of vaginal cells, resulting in an unacceptable low sensitivity for cytology on self-sampled material.\textsuperscript{90-92} Therefore, an additional visit to the physician is needed for cytology triage in women tested HPV positive on their self-sampled material. However, from the PROHTECT trials we have learned that the requirement for a physician visit and subsequent triage algorithm for self-sample hrHPV positive women is complicated and may restrain non-attendees from submitting a self-sample. Alternative triage tools that could be directly applied to self-sampled material (i.e., HPV16/HPV18 genotyping or molecular biomarkers) could play an important role to further identify women with CIN2+, without an extra visit to the physician for triage testing.\textsuperscript{93-95}
4.3. Management of HPV positive women

HPV testing as primary screening tool has a lower specificity than cytology for CIN2+ due to the detection of transient HPV infections. Therefore, triage of HPV positive women in primary screening is necessary to control the number of colposcopy referrals, treatments, and costs by referring only those HPV positive women with clinically meaningful lesions (i.e. CIN2, CIN3, and cancer). The most widely studied triage tool in HPV positive women is cytology. Two large cohort studies in the Netherlands have shown that cytology triage at baseline, with or without HPV16/18 genotyping, with repeat cytology testing at six or twelve months in triage test negative women was the preferred triage algorithm in HPV positive women. Another study has shown that cytology in combination with HPV16/18 genotyping might be an attractive triage strategy. However, in the latter study women who tested HPV16/18 negative with normal cytology at baseline, were still advised to undergo a repeat test after one year. In future screening, triage by cytology will probably be replaced by other more objective triage markers since cytological assessment on cervical smears is subjective with a large intra- and inter-observer variability.

Cytology can be made more objective when cells are dual stained for p16 and Ki-67 by immunocytochemistry. The p16/ki67 dual staining is particularly interesting since it is considered a surrogate marker for cell cycle deregulation in transforming hrHPV infections. HPV infected cells in CIN2+ lesions generally show high p16 expression in proliferating (i.e. Ki-67 positive) cells due to viral oncprotein E7 overexpression in these transforming infections. Since p16/Ki-67 double positivity is considered not to be present in normal or reactive cervical cells without a transforming HPV infection it is indicative for the presence of CIN2+. Indeed, p16/Ki-67 dual-staining of cervical smears has shown promising results compared to triage by sole cytology in HPV positive women. However, with this triage test cyto-pathological experience is still required. Presently, non-morphological molecular markers have gained most attention, since it has been shown that these are promising alternative triage markers and applicable to both cervical smears and self-sampled material.

4.4. Molecular methylation markers

Specific (epi)genetic alterations of host cell genes are necessary for the development of cervical (pre)cancer from persistent HPV infections. Biomarkers for lesions representing progressive transforming HPV infections are of value. An example of such a biomarker is a molecular methylation marker. Methylation involves the covalent binding of a methyl-group (CH3) at the carbon-5 position of a cytosine located 5’ of a guanine, to generate a 5-methylcytosine. The function of methylation is believed to be an overall genetic stability and provision of chromosomal integrity, and the coordination of the genome into inactive and active regions with regard to gene transcription. In normal tissues, tumor suppressor genes with CpG islands in their promoter region are generally unmethylated, whereas methylation in the CpG islands of these promoter regions can result in gene silencing and therefore be functionally involved in (cervical) carcinogenesis (See Figure 6).
Figure 6. Schematic representation of promoter methylation in a normal cell and a cancer cell, respectively. [Adapted from Esteller 2007 and http://medschool.ucsf.edu/]. In normal cells transcription factors bind to the gene promoter and enable gene transcription, whereas in cancer cells transcription factors cannot bind to the methylated gene promoter and inhibit gene transcription.

Quantitative methylation-specific PCR (MSP) is one of the used techniques to detect methylation in the promoter region of a gene. With this technique, bisulphite treatment of the DNA converts unmethylated cytosine to uracil, whereas methylated cytosines preceding guanine (CpG) are not converted. After bisulphite treatment, (real-time) PCR is performed using primers specific for the methylated DNA.

Since it has been shown that methylation-mediated inactivation of several tumor suppressor genes is involved in cervical carcinogenesis, methylation analysis of several of these genes have been studied in cervical (pre)cancers (reviewed by Wentzensen et al.). Human genes that have been described in more than five studies on cervical (pre)cancers are APC, CADM1, CDH1, CDH13, CDKN2A, DAPK1, FHIT, GSTP1, HIC1, MGMT, MLH1, RARB, RASSF1, TERT and TIMP3. However, frequencies of methylation of several genes largely varied while other genes were rarely or not methylated in SCCs or ACs. Besides studies about host cell methylation, HPV DNA methylation has also been studied. These studies have recently been reviewed elsewhere.

Only a limited amount of host cell methylation markers have more thoroughly been studied as triage tool in smears of HPV positive women, such as the marker panel JAM3-EPB41L3-TERT-C13orf18, combinations of SOX1, PAX1, LMX1A and NKX6-1, and bi-marker panel CADM1-MAL. The latter marker panel has shown to be equally discriminatory for CIN3+ as cytology or cytology with HPV16/18 genotyping, when applied to cervical scrapes of hrHPV positive women. Moreover, methylation analysis is feasible on self-sampled specimens. Recently, a study revealed that combined promoter methylation of bi-marker panel MAL/miR-124-2, is an alternative triage marker to detect CIN3+ on self-collected cervico-vaginal lavage material of
women with a hrHPV positive self-sample. Therefore, methylation marker analysis on cervical material or self-sampled material can be a promising triage tool for HPV positive women that can improve cervical screening algorithms. When methylation marker testing is used as triage tool in screening settings, it is important that at least all invasive cancers and advanced high-grade CIN lesions are detected. Noteworthy, Bierkens et al. showed for CADM1 and MAL genes that promoter methylation levels increase with duration and degree of cervical CIN disease and are extremely high in cervical cancers. This suggests that methylation marker testing is especially useful in detection of more advanced high-grade lesions and cervical cancer. In keeping with this hypothesis, methylation marker analysis could be a promising triage tool that particularly detects women with cervical cancer and advanced transforming CIN (with a high short term progression rate) with high sensitivity. However, further validation of methylation markers is needed before these markers can safely be used in cervical screening.

5. AIM AND OUTLINE OF THIS THESIS

Despite the long period (10-30 years) between initial HPV infection and development of cervical cancer and the well organised cytology based screening programme in the Netherlands, the incidence of cervical cancer in the Netherlands remains stable, partly due to the relatively low participation rate. HPV self-sampling seems a feasible alternative to attract the non-attendees into screening. The aim of this thesis is to gain more insight in effectiveness of HPV self-sampling and to improve cervical screening among non-attendees by self-sampling. Moreover, we evaluated the optimal triage strategy for women tested HPV positive.

The studies described in this thesis aimed at answering the following questions: How accurate is HPV self-sampling and what is the impact on population attendance in cervical cancer screening? (Chapter 2: High-risk HPV testing on self-sampled versus clinician-collected specimens: a review on the clinical accuracy and impact on population attendance in cervical cancer screening)

Does replacement of the first generation of a cervico-vaginal lavage device by an ergonomically improved, second generation lavage device result in a higher response rate when using this device among non-attendees of regular screening? And is the clinical performance of the second generation lavage device similar to its preceding version? (Chapter 3: A second generation cervico-vaginal lavage device shows similar performance as its preceding version with respect to DNA yield and HPV DNA results)

Is the performance of a brush-based lavage device similar to that of a cervico-vaginal lavage device? Are there differences in attendance rate of non-attendees of screening and HPV-positivity rate when using these self-sampling methods? (Chapter 4: Comparative performance of novel self-sampling methods for high risk HPV detection in 30,130 women not attending cervical screening: a randomized trial)
What are women’s reasons for non-attendance to the cervical screening programme? And what are the reasons of non-responders to participate in screening when HPV self-sampling is offered? *(Chapter 5: Reasons for non-attendance to cervical screening and preferences for HPV self-sampling in Dutch women)*

Is methylation marker triage feasible directly on self-sampled material of HPV positive women? And is direct methylation marker testing similar to cytology testing on an additional physician-taken smear in the detection of CIN2+? *(Chapter 6: Triage by methylation-marker testing versus cytology in women who test HPV-positive on self-collected cervicovaginal specimens (PROHTECT-3): a randomised controlled non-inferiority trial)*

What is the performance of triage testing on self-sampled material of HPV positive women at varying methylation thresholds for test positivity, with and without additional HPV16/18 genotyping? *(Chapter 7: Methylation marker analysis and HPV16/18 genotyping in high-risk HPV positive self-sampled specimens to identify women with high grade CIN or cervical cancer)*

Is methylation marker testing of additional value to cytology testing on cervical scrapes of HPV positive women? *(Chapter 8: Follow-up of high-risk HPV positive women by combined cytology and bi-marker CADM1/MAL methylation analysis on cervical scrapes)*

In *Chapter 9* we present an overview of the arguments to implement HPV testing as a primary screening tool for cervical screening. In *Chapter 10* we provide a general discussion of the results presented in this thesis.
REFERENCES

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