CHAPTER 1

General introduction
HUMAN PAPILLOMAVIRUS (HPV)

HPV types
Since the discovery of the link between cervical cancer and HPV in 1984 by professor Harald zur Hausen and his team, HPV has been the subject of extensive research. HPV is a small, non-enveloped deoxyribonucleic acid (DNA) virus which belongs to the family of Papillomaviridae. The circular, double-stranded viral genome is approximately 8000 base pairs in length. The genome encodes 6 early proteins for virus replication (E1, E2, E4, E5, E6 and E7) and 2 late proteins (L1 and L2), which are the viral structural proteins. HPVs are strictly epitheliotropic and can be subdivided into mucosal and cutaneous types according to their affinity for one of these epithelia. To date, more than 200 different types of HPV have been identified on the basis of DNA sequence data showing genomic differences. Approximately 40 HPV types are known to infect the genital mucosa. HPV types are subdivided into low-risk and high-risk types, according to their oncogenic potential: 12 types of mucosal HPV are known to cause cervical cancer and therefore considered high-risk (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59; IARC class 1). Several other HPV types have been classified as ‘possibly high-risk’ (HPV68; IARC class 2A) or ‘probably high-risk’ (HPV26, 53, 66, 67, 70, 73 and 82; IARC class 2B). Together, the latter group accounts for a minority (about 3%) of cervical cancers. Low-risk HPV (lrHPV) types are associated with the development of benign lesions such as genital condylomata accuminata, cutaneous warts, papillomas of the respiratory tract, as well as a subset of cervical low-grade premalignant lesions. HrHPV can be detected in virtually all squamous cell carcinomas and 94-100% of adenocarcinomas of the cervix. Oncogenic potential differs greatly between hrHPV types. HPV16 is the most common and most carcinogenic HPV type, causing more than 60% of cervical carcinomas worldwide; it is followed by HPV18, which is responsible for another 10%. Besides being responsible for cervical carcinogenesis, hrHPV types also play a causal role in the development of a subset of cancers of the anus, vulva, vagina, penis and oropharynx. In the following paragraphs, the life cycle of HPV is described in the context of the female cervix, as this is the field in which the most extensive HPV research has been performed.

Productive HPV infections
According to the classical view, HPV infections are initiated by viral entry into the basal cells of the squamous epithelium of the cervical transformation zone, which are accessible through common microtraumata of the epithelial layer. Recent research of Herfs et al. however suggests that a population of embryonal cuboidal cells, so called squamo-columnar junction cells which are characterized by specific biomarkers, are the main target of HPV infection. Viral entry requires binding of virions to heparin sulfate proteoglycans (HSPGs) on the cell surface, resulting in a conformational change, followed by site-specific enzymatic cleavage of the L2 protein by furin, a cell-encoded proprotein convertase. The conformational change resulting from the proteolytic cleavage facilitates virus internalization. The normal life cycle of HPV
is highly dependent on the differentiation process of the infected squamous epithelium. In undifferentiated basal epithelial cells, the HPV genome is present in episomal form at low copy numbers and expressed at low levels. During host cell differentiation, the expression of viral genes increases. Although differentiated epithelial cells normally are in a non-dividing state with an inactive replication machinery, the expression of the viral genes E6 and E7 facilitates the vegetative replication of the viral genome to high copy numbers. In the upper epithelial layers, new viral particles are assembled and released from shedding terminally differentiated epithelial cells. This state of productive hrHPV infection can lead to mild or moderate cellular atypia, histologically classified as cervical intraepithelial neoplasia (CIN) 1 or 2, so-called productive CIN lesions. Due to a combination of cell-mediated immune responses and innate immunity, productive hrHPV infections are usually cleared, leading to regression of these CIN lesions.

Transforming HPV infections
In a minority of cases, hrHPV infections are able to evade immune surveillance, persist during many years and lead to the development of high-grade cervical premalignant lesions, morphologically classified as CIN2 and CIN3. CIN3 lesions and a subset of CIN2 lesions are clinical manifestations of so-called transforming hrHPV infections, characterized by a deregulated and abundant expression of the viral oncogenes E6 and E7 in proliferating cells. The mechanisms behind the deregulated expression of E6 and E7 have not yet been fully clarified; genetic and epigenetic alterations of the viral genome, such as methylation of viral DNA, may play a role. E6- and E7-driven transformation is effectuated through different pathways. The most important interaction of E6 is with the protein encoded by the tumor suppressor gene p53, leading to degradation of p53 through the ubiquitin-dependent proteolysis pathway. Consequently, p53-mediated apoptosis and cell cycle arrest are inhibited, resulting in cell cycle progression despite the presence of DNA damage. In addition, E6 has been shown to contribute to immortalization of host cells through activation of telomerase. E7 is known to bind and degrade the Retinoblastoma protein (pRb), thereby facilitating entry into S-phase of the cell cycle even in the absence of mitogens, leading to uncontrolled proliferation. In addition, it makes cells insensitive to the cyclin-dependent kinase inhibitor p16INK4A (further referred to as p16) that normally can block cell cycle entry by preventing inactivation of pRb. Furthermore, E6 and E7 are increasingly acknowledged as modulators of epigenetics in the host cell. One of these epigenetic mechanisms is DNA methylation, a process which entails the covalent binding of a methyl group to a cytosine and is regulated by the activity of DNA-methyltransferase (DNMT) proteins. DNA methylation is crucial for the regulation of many physiological and pathological processes. In the context of promoter regions, hypermethylation of CpG islands generally results in transcriptional repression. During transforming hrHPV infections, E6 and E7 interfere with DNA methylation patterns by increasing DNMT activity, both indirectly through E6-induced suppression of p53 and by direct E7-induced stimulation of DNMT1 and DNMT3b. The silencing of several specific (tumor suppressor) genes as a result of promoter methylation has been shown to contribute
to transformation and malignant progression of cervical cells. Besides DNA methylation, other epigenetic processes such as chromatin remodeling and histone modification can be affected by the overexpression of E6 and E7.

In summary, through complex interactions with several host cell proteins, the products of the HPV oncogenes, E6 and E7, impair the usual mechanisms for cell cycle control and DNA repair. As a consequence, cells with DNA damage and chromosomal instability are able to survive and proliferate. The resulting accumulation of (epi)genetic errors in the host cell genome affecting both oncogenes and tumor suppressor genes is considered responsible for malignant progression, and ultimately the development of cancer.

Natural history of HPV infections in women

Genital infection with HPV is very common; it is estimated that 80% of sexually active women will experience an HPV infection at some point in life. Most women get infected with HPV shortly after their sexual debut. Risk factors for acquiring an HPV infection include the lifetime number of sexual partners, young age at sexarche and smoking. HPV prevalence in women is strongly dependent on age and geographical region. All over the world, HPV prevalence consistently peaks in women ≤25 years of age, with a decrease in older age groups. In the Netherlands, the HPV prevalence peaks to 24% in women aged between 20 and 25 years and declines to below 4% in women above the age of 45 years. In women aged 30-60 years, the group targeted by organized cervical cancer screening in the Netherlands, the overall HPV prevalence is 5 - 8%. Cervical cancer development is considered a rare complication of a persistent hrHPV infection. The majority of hrHPV infections is transient and remains unnoticed due to the lack of symptoms. Of all women who get infected with HPV, 80% clear the virus spontaneously within a period of 1-2 years. In most of the remaining 20% of women, non-progressive CIN1 or CIN2 may develop, most of which will eventually regress following viral clearance. These lesions should be differentiated from the minority of hrHPV infections which will persist for longer time and may lead to transforming infections and the development of high-grade precursor lesions (CIN3 and a subset of CIN2) with a risk of progression to cancer. In these cases, viral clearance may still occur and lead to spontaneous regression of associated lesions. Eventually, only about 1-3% of all women who get infected with hrHPV may ultimately develop cervical cancer (Figure 1).
Natural history of genital HPV infections in men

HPV infection in men can lead to the development of anogenital condylomata accuminata and cancers of the penis, anus and oropharynx. Unlike cervical cancer, which is in virtually all cases causally associated with persistent HPV infection, only 88% of anal cancers, 30-50% of penile cancers\(^2\) and approximately 24-60% of oropharyngeal cancers\(^3\) are attributable to an infection with HPV. For oropharyngeal cancers, increasing HPV-attributable fractions have been observed over the past 20 years.\(^4\) Each year approximately 39,000 males are diagnosed with a cancer type that results from persistent HPV infection.\(^3\)

Reported prevalences of HPV infections in men vary widely between 1% and 93%.\(^3\) Variations in the reported prevalences are likely to reflect differences in the applied sampling techniques and HPV assays, the sampled populations and the anatomical site(s) used for sampling. Recent large studies described a global male HPV prevalence of 21.0%\(^4\), a prevalence of 52.8 - 65.2% in North and Latin American men\(^1\) and of 30.9% in European men.\(^2\) In general, these prevalences seem higher than those in women, who have a global HPV prevalence of 10.4%, ranging from 8.1% in Europe and 22.1% in Africa.\(^2\) Similar to women, HPV16 is the most common type detected in men.\(^3\)

In great contrast with women, age-specific HPV prevalence curves in men are relatively flat, indicating that there is no association of male HPV prevalence with age.\(^3\) This might result from a higher rate of reinfection in men compared to women, potentially reflecting a lower likelihood...
of seroconversion after natural infection in men compared to women.\textsuperscript{38} The incidence of genital HPV infection in men is estimated at 38.4 per 1000 person months.\textsuperscript{44} The median duration of HPV infection in men is 7.52 months for any HPV and 12.19 months for HPV16.\textsuperscript{44} Although some studies observed that the median duration of an HPV infection appears comparable between men and women\textsuperscript{44,45}, other studies observed a shorter clearance time in males and found that HPV infections are less likely to persist in men than in women.\textsuperscript{46} The male genital sites most likely to test HPV positive are the penile shaft, followed by the glans or coronal sulcus and scrotum, whereas HPV detection is lowest in the urethra.\textsuperscript{47,48} Like in women, risk factors for acquiring HPV in men are the lifetime and recent number of sexual partners, sexual frequency and smoking. Circumcision has a protective effect against acquiring HPV and circumcised men exhibit a faster clearance of HPV infection compared to uncircumcised men.\textsuperscript{49} In men, the prevalence of low-risk HPV types is substantially higher than that of high-risk types, in contrast to women who carry an equal proportion of both types.\textsuperscript{37} Men who have sex with men have high HPV prevalences compared to men who have sex with women. However, the natural history of genital HPV infections does not differ significantly between these groups, with similar 12-months cumulative incidences estimated at 21-25\% and similar intervals needed for viral clearance.\textsuperscript{50}

**Sexual transmission of HPV**

HPV transmission typically occurs through direct skin-to-skin contact, including genital-to-genital contact. The most common route of sexual HPV transmission is through penetrative sex, but there is one publication describing female virgins acquiring HPV through non-penetrative sexual acts.\textsuperscript{51} Furthermore, some studies have indicated that vertical transmission might occur during vaginal delivery.\textsuperscript{52} Exposure to HPV is determined by risk factors that are similar to those of many sexually transmitted diseases; factors influencing susceptibility and infectivity are much less clear.\textsuperscript{53} The transmissibility of HPV is rather high, which is illustrated by high type-specific concordances between heterosexual couples.\textsuperscript{54} In a large meta-analysis, 26\% of all couples and 63\% in couples who both tested genital HPV-positive had concordant HPV types.\textsuperscript{55} Female-to-male transmission is consistently found to be more prevalent than male-to-female transmission of HPV, possibly resulting from higher seroconversion rates among women compared to men, and/or higher viral loads in women.\textsuperscript{56,57} This is consistent with the observation that genotype-specific HPV load in one partner is associated with the risk of new detection of that genotype in the other partner\textsuperscript{58} and indicates that methods aiming to reduce viral load (such as condom use) might be useful in preventing HPV transmission. High rates of concordance shortly after intercourse suggest that some of the detected HPV DNA in the genital area reflect viral DNA depositions from a partner and not established HPV infections.\textsuperscript{56} Several large studies have observed a lack of condom use as a risk factor for HPV acquisition.\textsuperscript{59–61} A Dutch study showed that regression of HPV-associated flat penile lesions of male partners of women with CIN was accelerated by condom use.\textsuperscript{62} Furthermore, condom use has been shown to promote HPV clearance and regression of CIN lesions in female partners.\textsuperscript{53}
Presence of HPV in semen

The detection of HPV in semen was first described in 1986. Since then, several studies have reported the presence of HPV in semen, with prevalences ranging from 2% to 31% in asymptomatic men, 3-36% in men seeking fertility evaluation and 16-26% in semen donors. A meta-analysis by Laprise et al. resulted in a pooled HPV prevalence of 10% in semen of healthy male populations and of 16% in fertility clinic attendees. The exact localization of HPV in the different components of semen is unclear. Some studies have reported that HPV particles can bind to spermatozoa, especially to distinct sites at the equatorial region of the sperm head, whereas the presence of HPV DNA has also been described in exfoliated cells present in semen.

The relevance of HPV presence in semen in terms of alterations of fertility is a matter of controversy. Several studies have described relations between the presence of HPV in semen and alterations of seminal parameters such as sperm motility, sperm count and the number of morphologically normal sperm cells; however, other researchers failed to find these associations.

Some in vitro and animal studies have shown negative effects of semen-transferred HPV on embryonic development and hatching. An in vitro study has shown that HPV16-transfected human spermatozoa are able to penetrate hamster oocytes, enabling viral gene transcription. There are indications that seminal HPV positivity might be related to decreased pregnancy rates and the occurrence of spontaneous abortions in the female partner. These studies, addressing truly clinical reproductive outcomes in relation to the presence of HPV in semen, have however been obtained in small cohorts.

CLINICAL MANIFESTATIONS OF GENITAL HPV INFECTION

Clinical manifestations of genital HPV infection in women

In women, the most important consequence of HPV infection is the development of cervical cancer and its precursor stages. Other (less prevalent) female HPV-related cancer types are beyond the scope of this thesis and will therefore not be further discussed.

Epidemiology of cervical cancer

Cervical cancer is an important public health problem. With an estimated 528,000 new cases and 266,000 deaths per year, cervical cancer is the fourth most common cancer among women worldwide, and seventh overall. The majority of the global cervical cancer burden (85%) occurs in the developing world, where it accounts for 12% of all female cancers. The highest incidence rates are found in Sub-Saharan Africa, Melanesia, South Asia and parts of Central and South America (Figure 2). The high variability in cervical cancer incidence and mortality rates around the world results from a disproportionate availability of adequate preventive programs, which include
both primary prevention (prophylactic vaccination) and secondary prevention (cervical screening) strategies.

In the Netherlands (16.9 million residents), a country with a well-organized HPV vaccination and cervical cancer screening program, each year approximately 730 women are diagnosed with cervical cancer and approximately 230 women die as a result of this disease.\textsuperscript{86,87} In contrast to other gynecological cancers, cervical cancer has its peak incidence in relatively young women (35-45 years).\textsuperscript{88} The 5-year survival rate in women diagnosed at an early stage (FIGO I or II) is 64-98% and markedly lower (7-38%) in women diagnosed at FIGO stage III or IV.\textsuperscript{88}

\textbf{Cervical cancer: pathogenesis}

Persistent infection with hrHPV is a necessary etiological factor for the development of almost all cervical cancers.\textsuperscript{89} Cervical cancer originates in the uterine cervix, which is the lower part of the uterus, partly protruding into the vagina (Figure 3).\textsuperscript{90} The cervix consists of two parts: the ectocervix (the outer part of the cervix on the vaginal side), which is lined with squamous epithelium, and the endocervix (the inner part of the cervical canal on the uterine side) which is covered with glandular columnar epithelium. The place where these two types of epithelia meet is referred to as the squamo-columnar junction (SCJ). As a result of hormonal changes, starting from puberty, metaplastic squamous epithelial cells replace endocervical epithelial cells, causing the junction to migrate proximally. The dynamic and macroscopically visible region between the former and new SCJ is traditionally called the transformation zone (TZ). Until recently, metaplastic cells in this zone were thought to be the origin of cervical cancer and its precursor lesions. Recent studies have, however, described a discrete population of embryonic cuboidal epithelial cells, located at the SCJ, which are hypothesized to be the cells most susceptible to oncogenic transformation by hrHPV and responsible for the development of cervical cancer.\textsuperscript{10}
Classified according to histological subtype, squamous cell carcinomas (SCCs) are the most common (80%), followed by adenocarcinomas (AC; 15%). The remaining 5% of cervical cancers comprise neuro-endocrine and clear-cell carcinomas.

Precursor lesions of cervical cancer

The development of cervical SCCs occurs through premalignant stages characterized as cervical intraepithelial neoplasia (CIN). CIN lesions are classified morphologically as CIN1 (mild dysplasia), in which atypical cells are limited to the lower one third of the epithelial layer, CIN2 (moderate dysplasia), in which the lower two thirds of the epithelium contain atypia and CIN3 (severe dysplasia or carcinoma in situ), in which more than two thirds, up to the entire epithelial layer, comprise atypical cells. When atypical cells invade the basal membrane, lesions are defined as carcinoma.

All CIN lesions have the ability to regress, persist or progress. The likelihood of progression to cervical cancer increases with the histological severity, the duration of existence and the size of the lesion, while the opposite is true for the likelihood of spontaneous regression. According to statistical models 1.6% of early-onset CIN2/3 lesions progress to cervical cancer within 10 years. For older, large CIN3 lesions, 10 year cancer incidence rates of 20% have been described, while progression
rates are estimated at 30-50% within a period of 30 years.\textsuperscript{54,56} Approximately 33% of CIN3 lesions are thought to exhibit spontaneous regression.\textsuperscript{92} CIN2 lesions are estimated to progress in 5-30% and to regress spontaneously in 26-74% of cases.\textsuperscript{3,15,30,31,97–107} For CIN1 the described rates of progression are 3-11%, with a spontaneous regression rate of 34-93%.\textsuperscript{3,15,24,31,97,99,108,109} The current morphological CIN classification does not distinguish between progressive and non-progressive lesions. Especially CIN2 comprise a heterogeneous group of lesions with a highly variable clinical course. Traditionally, CIN2 and CIN3 are considered precursors of cervical cancer and in most cases detection of these lesions is followed by excisional treatment, to prevent progression to cancer. Treatment is usually done by large loop excision of the cervical transformation zone (LLETZ) or conisation of the cervix. As most CIN2 and CIN3 lesions will, however, never progress to SCC in the absence of treatment, the current practice leads to considerable overtreatment.

Histological differentiation between CIN2 and CIN3 is challenging and subjective, which is illustrated by a high inter-observer variability in the diagnosis of these lesions.\textsuperscript{110} The reproducibility of CIN2 and CIN3 diagnosis can be increased by additional immunological staining of the p16 protein in histological specimens.\textsuperscript{111} Furthermore, chromosomal profiling studies using array-based comparative genomic hybridization have shown that CIN2/3 lesions associated with a preceding HPV infection of <5 years have significantly less chromosomal aberrations compared to CIN2/3 lesions associated with HPV infection with a duration of ≥5 years.\textsuperscript{112–114} In addition, recent studies have shown that the methylation levels of promotor regions of certain tumour suppressor genes in CIN2/3 lesions are associated with the duration of the associated HPV infection and that these methylation levels are extremely high in cervical cancer. These findings have led to the concept that methylation levels of promotor regions of tumour suppressor genes could aid in distinguishing between CIN2/3 lesions that carry a low risk of short term progression to cancer (so-called ‘early’ lesions) and CIN2/3 lesions with a high risk of short term progression to cancer (so-called ‘advanced’ CIN2/3 lesions).\textsuperscript{29} Especially in young women, who are likely to mainly have harmless, regressive lesions, this differentiation is essential to reduce over-treatment.

In contrast to SCCs, evidence on the existence and diagnostic relevance of precursor lesions of cervical ACs is limited.\textsuperscript{115} The only reproducible precursor stage of ACs is adenocarcinoma in situ (AIS), which is considered the glandular counterpart of CIN3 and forms an indication for conisation.\textsuperscript{116,117}

**Clinical manifestations of genital HPV infections in men**

Similar to HPV infections in women, genital HPV infections in men are usually symptomless and self-limiting. There are however various known clinical manifestations, which will shortly be discussed. The most prevalent manifestation of HPV infection in males is the development of condylomata acumminata, with a global incidence of 160 to 289 cases per 100.000 person-years.\textsuperscript{118} The vast majority (90%) of condylomata is caused by HPV6 and HPV11. Although benign and not associated with any mortality, the presence of condylomata acumminata is an important
source of psychosocial distress, as well as a reason for frequent consumption of medical care.

More modest manifestations of genital HPV infections in men are so-called flat penile lesions: small well-demarcated flat defects of the penile epithelium, which can only be visualized by whitening through the application of acetic acid. These lesions have been hypothesized to be reservoirs of HPV, contributing to the spread of HPV during sexual intercourse.

A very rare manifestation of genital HPV infections in men is penile cancer, a disease that mainly occurs in elderly men (mean age 60 years). The global incidence of penile cancer is estimated at 26,000 cases per year, with a wide variation depending on socio-economic and religious factors. Approximately 30-50% of penile cancers is causally associated with HPV infection; these are often cancers with a warty or basaloid histology. HPV-related penile cancers are likely to be preceded by a precancerous condition called penile intraepithelial neoplasia (PIN); however, in contrast to CIN, little is known of the natural history of PIN and guidelines for monitoring or treatment of PIN do not exist, probably because the time from PIN to cancer exceeds 30 years. Anal cancer, a disease which is in most cases attributable to HPV infection (88%), has an incidence of 30,400 cases per year globally. Anal cancer is more common in women than in men from the general population. Among men, the incidence of anal cancer is highest in those having sex with men (MSM) and especially individuals who are infected with HIV. Anal cancer is assumed to be preceded by a precursor stage called anal intraepithelial neoplasia (AIN), particularly high-grade AIN.

**PRIMARY PREVENTION OF HPV-ASSOCIATED LESIONS: PROPHYLACTIC VACCINATION**

Primary prevention of HPV-associated lesions intends to prevent healthy people from getting persistently infected with HPV through prophylactic vaccination. Prophylactic HPV vaccines contain virus-like particles (VLPs) that consist of HPV-L1-proteins expressed in yeast or bacteria. These HPV-L1-proteins spontaneously fold like viral capsids, but do not contain viral DNA.

 Intramuscular vaccination induces the production of high levels of immunoglobulins (IgG), which are able to bind to subsequently encountered HPV particles, thereby preventing viral access into epithelial cells. Although VLPs are produced in a type-specific manner, cross-reactivity to other HPV-types has been demonstrated.

Currently, three different prophylactic HPV vaccines are commercially available. A bivalent vaccine (Cervarix, GSK) is directed against HPV16 and HPV18, the most carcinogenic HPV types. A quadrivalent vaccine (Gardasil, Merck) additionally protects against HPV6 and HPV11, low-risk types responsible for the majority of genital warts. Recently, a nonavalent vaccine (Gardasil-9, Merck) has been approved, containing VLPs against HPV6, -11, -16, -18, -31, -33, -45, -52 and -58.
Due to the duration of cervical carcinogenesis and the availability of cervical cancer prevention through excisional treatment of CIN2/3 lesions, it would be neither feasible nor ethical to study the effect of HPV vaccination using cancer incidence as an endpoint. For this reason, intermediate endpoints such as the incidence of persistent vaccine-type associated HPV infections and the incidence of CIN2/3 have been regularly used as alternatives.

In large randomized controlled trials, HPV vaccines have been shown to be highly effective in the prevention of vaccine-type related CIN2, CIN3 and AIS, especially among HPV-naïve women (efficacy 93-100%). Moreover, different degrees of protection against CIN lesions associated with non-vaccine HPV types were found for the different vaccine types. As the efficacy of vaccination is considerably lower (20-34%) in women who previously experienced an HPV infection, vaccination is best performed in HPV naive women, i.e. prior to the onset of sexual activity.

Many countries have implemented routine HPV vaccination programs. In the Netherlands, vaccination with the bivalent HPV vaccine is offered to twelve-year-old girls as part of the national vaccination program since 2010. Since 2014, vaccination with two doses (instead of three) has been approved. Two recent studies suggest that vaccination with one dose might also be effective in the prevention of cervical cancer. The participation rate in the HPV vaccination program in the Netherlands is slowly rising, but remains relatively low (61.0% in 2016) compared to the uptake of other routine childhood vaccinations (92.0 - 94.8%).

Until recently, programs of prophylactic HPV vaccination were focused on women, aiming to prevent cervical cancer. The introduction of HPV vaccination programs in girls and young women has been shown to not only reduce HPV infections and CIN lesions in this group, but also to reduce the number of HPV infections and condylomata accuminata in boys and men. These findings indicate that unvaccinated males may benefit from herd protection; it should be noted that this was only found in settings where vaccination coverage in females is sufficiently high (at least 50-55%). Recent large randomized controlled trials have evaluated the effects of HPV vaccination with the three available vaccine types in boys and young men (age 9 -26 years), showing a similarly high immunogenicity and similar effects to those in girls in terms of reduction of HPV infections and related lesions. These trials have led to the approval of all three vaccines with the indication of preventing anal cancer in men and condylomata accuminata (for the two vaccines that also target HPV6 and HPV11).

Whether it is worthwhile to vaccinate boys against HPV remains a matter of debate. The incremental benefit of male vaccination is expected to be in the prevention of anal cancer in men having sex with men, as this group is insufficiently covered by female vaccination. In addition, vaccination of boys is assumed to improve cervical cancer prevention in women. Another argument in favor of gender-neutral vaccination is its added effect on the future occurrence of oropharyngeal cancer, which is expected to be considerable if 60% of girls are vaccinated but is estimated to become small if uptake in girls reaches 90%. It has been estimated that, in the current setting in the Netherlands with 60% vaccine uptake in girls, the number of boys who would need to be vaccinated to prevent one cancer in men will be about four times as
high as the number of girls needed to prevent one cervical cancer.\textsuperscript{138} Vaccination of 40\% of boys combined with vaccination of 60\% of girls is expected to yield the same gain in life years as an increase in girl vaccination from 60\% to 80\%. It is highly likely that gender-neutral vaccination is cost-effective under current vaccine costs and uptake in the Netherlands.\textsuperscript{139} Recently, Australia was the first country to implement a (school-based) HPV vaccination program for both girls and boys.\textsuperscript{140}

Because of the duration of development of cervical cancer after acquiring an HPV infection (23.5 years)\textsuperscript{141} and the relatively late implementation of vaccination in the Netherlands, the earliest effects of vaccination on cervical cancer incidence are expected to become evident in the future 15 to 20 years. Suboptimal vaccine coverage of young adults and the limited availability of the vaccines in low-income countries will restrict the beneficial effect of the vaccines worldwide. Therefore, screening will remain an essential part of cervical cancer prevention in the coming decades.

SECONDARY PREVENTION OF HPV-ASSOCIATED LESIONS: CERVICAL CANCER SCREENING

General aspects of screening
The slow development of cervical cancer through well-recognizable premalignant stages, which can be effectively treated, offers the possibility of secondary prevention by screening.\textsuperscript{142,143} The efficacy of cervical cancer screening depends on several factors: the clinical performance of the applied screening test, the participation rate of invited women and the adequacy of treatment and follow-up regimens for women with abnormal test results.\textsuperscript{144}

The clinical performance of a screening test is expressed by its sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The clinical sensitivity reflects the probability that the test result is positive in a person with the disease. Thus, screening with a highly sensitive test limits the number of false-negative results, thereby reducing the harms of false reassurance such as a delayed diagnosis. The clinical specificity reflects the probability that the test is negative in the absence of disease. Consequently, a test with a high specificity limits the number of false-positive results, reducing the harms of unnecessary follow-up tests such as additional anxiety and stress. The predictive values of a test are influenced by the prevalence of a disease in the sampled population. The PPV of a test reflects the probability that the disease is indeed present in a person who tested positive. Conversely, the negative predictive value of a test reflects the probability that a person with a negative test result truly does not have the targeted disease.

In practice, the search for an ideal screening tool is usually a trade-off between the abovementioned characteristics. The sensitivity and negative predictive value represent the safety of a screening test (correct diagnosis of persons with the disease and correct reassurance
of those without). However, in many cases, a high sensitivity and high NPV are at the cost of a loss in specificity and PPV, representing unnecessary anxiety, referral and treatment of women without disease.

In contrast to cervical cancer, other HPV-related cancers do not meet the criteria to make these diseases eligible for screening. In contrast to HPV tests in women, genital HPV tests for men are only performed on clinical indication and in research settings. The following paragraphs will focus on cervical cancer screening.

Cervical cancer screening in the Netherlands

Recently, cervical cancer screening in the Netherlands has been subject to major changes. In January 2017, a new improved HPV-based screening program has been launched. The following paragraphs will first describe the previous, cytology-based screening program (performed until December 2016), followed by the recently implemented, HPV-based screening program including the rationale behind the new approach and remaining challenges.

Cytology-based screening

For decades, cervical cancer screening has been primarily based on the cytomorphological examination of exfoliated cells from the endocervix, ectocervix and squamo-columnar junction. This ‘Pap test’ was named after a Greek gynecologist called Dr. Papanicolaou, who was the first to describe cervical cytology as a method to detect the presence of cervical (pre-
cancer in so-called Pap smears.\textsuperscript{146} His finding resulted in worldwide implementation of cytology-based cervical cancer screening. In the Netherlands, cervical cancer screening was introduced in the early seventies. After a start with local pilot programs a nationwide screening program was implemented in 1988 for women aged 34 to 54 years. In 1996, the program was restructured by extending the screening interval from three to five years and by increasing the target population to women aged 30-60 years. Preservation of cervical cells can be done by two different methods: formerly, cells were smeared directly from the brush onto the microscopy glass and sprayed with a fixative (conventional cytology), whereas nowadays cells are collected in a vial with a liquid preservative to be used for later concentration of cells on a microscopy glass (liquid-based cytology). The latter method enables the production of multiple slides of high quality and the performance of additional (molecular) tests.

Cervical cytology slides are read by specialized cytotechnicians under supervision of a cytopathologist and classified according to the CISOE-A classification, reporting on the composition of the specimen, signs of inflammation, abnormalities of squamous cells, other cells, endometrial cells, endocervical cells and a separate rating of the adequacy of the sample.\textsuperscript{147} The results of the CISOE-A classification can be summarized in a Pap score (ranging from Pap 1 to Pap 5) and can be translated into the Bethesda 2001 classification, which is used internationally (Table 2).\textsuperscript{147} In the previous screening program, borderline to mild dyskaryosis (BMD; Pap scores Pap 2 and Pap 3a1), which equals ASC-US/ASC-H/LSIL, was considered an equivocal result that required repeat testing after 6 and 18 months. Women who had a repeated abnormal cytology at either of these occasions were referred to a gynecologist. During the last years in this program, HPV triage was recommended for the first repeat smear of women with BMD, in case this smear revealed normal cytology. When negative for HPV, these women were dismissed from further follow-up, whereas HPV positives were invited for repeat cytology at 18 months. A \textgeq BMD cytology result (Pap score Pap 3a2 or worse), which equals \textgeq HSIL, was considered a direct indication for referral to a gynecologist.

In the Netherlands, annually approximately 750,000 women are invited for cervical screening, with a rather stable participation rate of approximately 65%.\textsuperscript{148}

Effects and limitations of cytology-based screening

The introduction of cytology-based screening has led to a significant reduction in cervical cancer incidence and mortality.\textsuperscript{151-153} Participation in screening has been estimated to reduce the risk of cervical cancer with 66 - 80%.\textsuperscript{154-156} In the Netherlands, the mortality due to cervical cancer has decreased with 64% since 1980 to a current mortality rate of approximately 200 women per year (cijfersoverkanker.nl).\textsuperscript{86} The incidence of cervical cancer in the Netherlands has declined significantly between 1990 (world standardized incidence rate (WSR) 7.59/100.000) and 2001 (WSR 5.20/100.000), followed by a slight rise in 2002-2007 (WSR 6.38 /100.000).\textsuperscript{86} Since 2007, cervical cancer incidence has remained stable,\textsuperscript{86,157} indicating that the maximum impact of the (previous) screening program might have been reached. The main factors restricting a
further decrease in the cervical cancer burden are the suboptimal diagnostic accuracy of Pap cytology\textsuperscript{152,158,159} and the limited attendance to screening.\textsuperscript{160} Pap cytology has a limited sensitivity for high-grade CIN and cancer (50-70\%).\textsuperscript{152} To reach sufficient program safety, cytology testing needs to be repeated at least every five years. Secondly, cytology is a subjective assay with a limited reproducibility, leading to a variable accuracy. Consequently, the performance of Pap cytology is dependent on the level of training and expertise of cytologists, which explains large global differences in the efficacy of cytology-based screening.\textsuperscript{152} Furthermore, Pap cytology is a labor-intensive test, unsuited for high-throughput screening.\textsuperscript{152} Although computer-assisted screening systems are known to reduce the time needed for cytomorphological analysis, they can only make a preselection of cell groups of interest. Thus, the interpretation of cell morphology remains a human task.\textsuperscript{161} Another limitation of the success of cytology-based screening is the suboptimal attendance of invited women. In the Netherlands, approximately 35\% of eligible women does not respond to the invitation to participate in the cervical screening program.\textsuperscript{148} The main reasons for non-attendance are organizational barriers and emotional problems with the collection of a cervical scrape by a physician.\textsuperscript{162} Of note, the group of non-attendees harbors a disproportionate part of the burden of cervical premalignant disease: 50\% of all cervical cancers is diagnosed among never-screened or under-screened women.\textsuperscript{160} Therefore, attracting these women to screening is expected to have considerable impact on cervical cancer incidence and mortality.\textsuperscript{163–167}

**HPV testing versus cytology**

Several large randomized clinical trials have convincingly demonstrated that screening by a primary hrHPV test has a superior sensitivity for detection of ≥CIN2, ≥CIN3 and cervical cancer compared to screening by cytology.\textsuperscript{26,168–176} As a consequence, hrHPV testing has a significantly higher long-term NPV, indicating that a negative hrHPV test result provides a better reassurance against the presence or development of ≥CIN3 and cervical cancer compared to a normal Pap cytology result.\textsuperscript{169–171,174,175,177,178} Consequently, primary HPV testing enables safe extension of the applied screening intervals,\textsuperscript{174,179–181} up to intervals of ten years in screen negative women aged ≥40 years.\textsuperscript{181} In addition, in contrast to cytology, a hrHPV test is a molecular and thus objective assay with a high reproducibility.\textsuperscript{182} Following this evidence, several countries have recently replaced primary cytology by primary HPV testing or are planning to make this change in the near future.\textsuperscript{183–185} Not every commercially available HPV test is suitable for use in cervical cancer screening. The applied assay needs to have an optimal balance between the detection of clinically relevant HPV infections (leading to ≥CIN2) and the detection of transient (irrelevant) infections. GP5+/6+ polymerase chain reaction (PCR) with enzyme-immunoassay (EIA) readout and the Hybrid Capture 2 assay have been extensively validated in large clinical trials and are therefore considered reference assays.\textsuperscript{186} Several other HPV assays have been validated for screening by equivalent performance according to criteria formulated by an international consortium.\textsuperscript{187}
Another advantage of hrHPV testing is its applicability to self-sampled material. Several devices for self-collection of a cervico-vaginal sample, for example a cervico-vaginal lavage device or a small brush for vaginal sampling have been evaluated. HrHPV testing on self-sampled material enables reliable HPV-testing, with a similar accuracy to HPV-testing on cervical scrapes, given that a clinically accurate combination of self-sampling device and PCR-based HPV assay is used. Offering non-attendees a self-sampling device for collecting cervico-vaginal material for HPV testing has been shown effective in attracting a substantial part (30%) of these non-responding women into screening. However, self-collected cervico-vaginal material does not yield sufficient amounts of cervical indicator cells to enable adequate cytomorphological evaluation.

**Table 2. Classification of cervical cytology (adapted from Bulk and Bulkmans et al.)**

<table>
<thead>
<tr>
<th>Description</th>
<th>NILM</th>
<th>atrophy</th>
<th>ASC-H</th>
<th>ASC-US</th>
<th>LSIL</th>
<th>HSIL</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bethesda 2001</td>
<td>Unsatisfactory for evaluation</td>
<td>Normal</td>
<td>Borderline</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
<td>Carcinoma in situ</td>
</tr>
</tbody>
</table>

**HPV-based cervical cancer screening in the Netherlands**

Since January 2017, the Dutch cervical cancer screening program has changed significantly. An overview of the previous and current screening program is shown in Figure 4. Most importantly, primary Pap cytology testing has been replaced by primary hrHPV testing. Like before, eligible women are invited to visit their general practitioner for collection of a cervical scrape. The scrape material is tested for the presence of hrHPV; in case of hrHPV positivity, a reflex Pap cytology test is performed on the remaining liquid-based scrape material. Women who test both hrHPV-positive and cytology positive (≥BMD / ≥Pap2) are referred to a gynecologist for colposcopy. Women who test hrHPV-positive and cytology negative (NILM / Pap1) are invited for a follow-up cytology test after 6 months. If this 6 month cytology test is positive, women are referred for colposcopy; if negative they are referred to the next screening round (HPV-positive and repeat cytology negative).

An additional change in the screening program is the extension of the screening interval. Currently, women are invited for screening at the ages of 30, 35, 40, 50 and 60 years, provided that they are screen-negative at the age of 40 and/or 50 years. Women who test hrHPV-positive at the age of 40 or 50 are invited for screening at the age of 45 or 55 years, respectively. Only
women who test hrHPV-positive at the age of 60 are offered an extra screening round at the age of 65 years.

A third improvement in screening is the implementation of self-sampling. Women who do not respond to the invitation for a cervical scrape are offered the possibility of self-collection of a cervico-vaginal specimen at home using a brush. This specimen is sent to a laboratory (by general mail) and tested for hrHPV. Women who test hrHPV-positive are invited for a cervical scrape at the general practitioner to enable Pap cytology testing, following the same referral schedule as those for women who had the primary hrHPV test on a cervical scrape.

Colposcopy

Women who test high-risk HPV-positive and have a positive triage cytology test are referred to a gynecologist for colposcopy. Colposcopy is a technique to visualize the cervical transformation zone with magnification. During colposcopy, acetic acid (3-5%) is applied directly to the cervix, which can lead to whitening and the appearance of specific patterns (punctuation, mosaic and atypical vessels) associated with the presence of HPV-induced dysplasia.\textsuperscript{197} The average diagnostic sensitivity of colposcopic impression, distinguishing low- from high-grade lesions and cancer, has been estimated at 50-70\%\textsuperscript{198-201} and is even lower in case of small CIN3 lesions.\textsuperscript{202} Suspected high-grade lesions are sampled with one or more punch biopsies. In case the transformation zone is not completely visible due to retraction into the cervical canal (for example in postmenopausal women or women using hormonal contraceptives), an endocervical curettage sample may be obtained to rule out the presence of (pre)malignant disease in this area. Biopsies or endocervical curettage specimens are analysed by a pathologist for diagnosis or exclusion of CIN.

Treatment of high-grade CIN

Guidelines on the treatment of CIN are currently under debate. Until recently, both CIN2 and CIN3 were considered high-grade premalignant lesions which require treatment by large loop excision of the transformation zone (LLETZ) or conisation. Also, several clinics perform direct LLETZ treatment in women with a \textgreater BMD cytology result, eliminating the step of histological confirmation by colposcopy-directed biopsy (‘see and treat’ approach).\textsuperscript{203} Recent studies have indicated that at least a substantial part of CIN2 lesions result from non-transforming HPV infections and carry a very low risk of short-term progression,\textsuperscript{29} justifying a policy of close follow-up instead of excisional treatment for such women. In current Dutch clinical practice, virtually all women diagnosed with CIN3 are treated by LLETZ or conisation.\textsuperscript{116} For women with CIN2, the decision to treat or to perform follow-up is made on an individual basis, taking into account the size of the lesion, the patient’s age and future child wish, her own preference, her likelihood to adhere to follow-up visits and other clinical characteristics.\textsuperscript{116}
Figure 4. Overview of the previous and current screening program in the Netherlands (adapted from Bosgraaf et al.)

hrHPV: high-risk human papilloma virus; NILM: negative for intraepithelial lesion or malignancy; BMD: borderline to mild dyskariosis; CIN: cervical intraepithelial neoplasia
New techniques for optimal management of hrHPV-positive women

An important challenge in HPV-based screening is the 1-4% lower specificity of a hrHPV test for high-grade CIN compared to primary Pap cytology. Most women who test hrHPV-positive have harmless productive infections, which will be cleared spontaneously. Only a fraction (less than 20%) of hrHPV-positive women harbor a transforming infection that may lead to the development of premalignant disease with a high short-term risk of progression to cancer. Therefore, referral of all hrHPV-positive women to a gynecologist for colposcopy would induce significant over-referral and over-treatment, leading to unnecessary anxiety, excessive costs and increased risks of (obstetric) complications. Consequently, to ensure the success of HPV-based screening, a feasible so-called triage test is necessary to identify only those hrHPV-positive women with clinically relevant cervical premalignant disease who are in need of treatment. Currently, there is no consensus on the optimal management of hrHPV-positive women. Although in the Netherlands Pap cytology has been selected as the most extensively studied and therefore most advocated triage test, this strategy has several important drawbacks. The main limitation of Pap cytology is its subjective nature, which results in a variable accuracy and a suboptimal sensitivity for ≥CIN3. To ensure sufficient safety of HPV-based screening programs with cytology triage, repeated Pap cytology tests are necessary, leading to the problem of loss to follow-up. Moreover, prior knowledge of the hrHPV status influences cytology reading, leading to an increase in the number of false-positives. As an alternative to Pap cytology, several other strategies have been suggested for triage of hrHPV-positive women and will be shortly introduced here. A more detailed overview and comparison of available triage strategies for hrHPV-positive women is provided in Chapter 8 (general discussion).

A strategy of recent interest is p16/Ki-67 dual-stained cytology, which refers to the combined immunostaining of the proteins p16\[\text{INK4A}\] and Ki-67 in cytological specimens. Simultaneous expression of p16 and Ki-67 in one cell is indicative for cell cycle dysregulation as can be seen in a transforming hrHPV infection. Recent studies among hrHPV-positive women have shown that p16/Ki-67 dual-stained cytology has a higher specificity compared to Pap cytology, at a similar sensitivity.

Another molecular triage marker is the specific detection of HPV16 and/or HPV18, representing the most prevalent and most oncogenic HPV types. Although the detection of HPV16/18 positive women enables the selection of a high-risk population, the residual ≥CIN3 risk of hrHPV-positive women who are infected with non-HPV16/18 types is too high to justify safe referral to the next screening round. As a solution, the combination of HPV16/18 genotyping with Pap cytology has been suggested. This combined strategy has been shown to enable safe triage of hrHPV-positive women without the necessity of repeat testing, however with increased referral rates.

As a fully molecular alternative, the detection of mRNA of HPV oncogenes E6 and/or E7 has been evaluated for triage of hrHPV-positive women. Based on the concept that it is not the presence of hrHPV as such, but the activity of the hrHPV oncogenes E6 and E7 which is responsible for
malignant transformation, several studies have evaluated the accuracy of tests detecting E6/E7 mRNA of 5 hrHPV types. These studies described a specificity compared to cytology, however at a suboptimal sensitivity.\textsuperscript{225–227} Furthermore, host gene methylation analysis has been recently suggested as a triage strategy for hrHPV-positive women. Methylation of promoter regions of specific host (tumor-suppressor) genes, which can be assessed by methylation-specific PCR, has been associated with the presence of cervical cancer and high-grade CIN.\textsuperscript{228} Methylation levels of several genes have been shown to correlate with histological severity and the duration of preceding hrHPV infection.\textsuperscript{114} An advantage of using a fully molecular triage test is the possibility of screening and triage in a single step (on a self-collected sample), without the necessity of a physician-taken scrape.

**OUTLINE OF THIS THESIS**

**Part one: how to improve the triage of high-risk HPV-positive women**

Part one of this thesis describes the evaluation of various strategies for colposcopy triage of hrHPV-positive women. We designed a multicenter prospective cohort study, shortly addressed as the COMETH trial (comparing methods for triage of hrHPV-positive women). This study was performed at the gynecology outpatient departments of six Dutch (secondary and tertiary) hospitals. Participants were asked to self-collect a cervico-vaginal lavage sample, on which a hrHPV test was done. Only women who tested hrHPV-positive on this self-sample were included into the further study. All hrHPV-positive women were invited for collection of a physician-taken liquid-based cervical scrape and a colposcopy-directed biopsy, thereby assuring a histological diagnosis for each hrHPV-positive participant. On the liquid-based cervical scrape samples of these hrHPV-positive women, we performed different triage tests: Pap cytology, p16/Ki-67 dual-stained cytology, HPV DNA genotyping, HPV E7 mRNA detection and \textit{FAM19A4} methylation analysis. In addition, we performed \textit{FAM19A4} methylation analysis on the cervico-vaginal lavage samples to compare its performance to that of \textit{FAM19A4} methylation analysis in cervical scrapes. The goal of this study was to compare the ability of these triage strategies to adequately detect women with high-grade cervical intraepithelial neoplasia and cervical cancer.

In **Chapter 2**, we evaluate the accuracy of a septivalent E7 mRNA test in comparison with the accuracy of Pap cytology and HPV DNA genotyping in cervical scrapes for detection of ≥CIN2 and ≥CIN3 among hrHPV-positive women.

In **Chapter 3**, we compare the accuracy of p16/Ki-67 dual-stained cytology with the accuracy of Pap cytology, with or without HPV16/18 genotyping, in cervical scrapes for detection of ≥CIN2 and ≥CIN3 among hrHPV-positive women.
Chapter 1

In Chapter 4, we evaluate the accuracy of FAM19A4 methylation analysis in comparison with the accuracy of Pap cytology and HPV16/18 genotyping in cervical scrapes for detection of ≥CIN2 and ≥CIN3 among hrHPV-positive women. In addition, the accuracy of several combinations of triage markers is assessed.

In Chapter 5, we describe the accuracy of FAM19A4 methylation analysis, with or without HPV16/18 genotyping, in self-collected cervico-vaginal lavages compared to its analysis in physician-taken cervical scrapes.

Part two: the prevalence and clinical relevance of HPV presence in semen

The second part of this thesis focuses on genital HPV infections in men and especially on the detection of HPV in semen. To study the prevalence and relevance of HPV presence in semen, we designed two cohort studies which were shortly addressed as the MOTION studies.

In Chapter 6, a study on the presence of HPV in semen of healthy volunteers is presented (MOTION 1). In a cohort of 213 healthy volunteers (young men recruited at a Dutch university) we collected one penile scrape and three consecutive semen samples from each participant. We performed penoscopy on each participant to study the presence of flat penile lesions and other HPV-related penile lesions. Penile scrapes specimens and semen samples were tested for the presence of both high-risk and low-risk HPV using PCR. In addition, HPV genotyping was performed. Goals of this study were to estimate the prevalence of HPV in semen in Dutch healthy males and to explore the relationship between seminal HPV positivity and HPV infections of the penile epithelium, also at genotype-specific level. Furthermore, the presence of flat penile lesions was studied in relation to HPV positivity of penile epithelium and semen.

In Chapter 7, we further explore the relationship of seminal HPV positivity with functional semen parameters (MOTION 2). In a Dutch academic fertility clinic, we recruited a cohort of 430 male partners of couples who were seeking fertility evaluation. Each participant provided one semen sample, on which a functional semen analysis was performed according to standards of the World Health Organization (WHO). In this analysis, the volume, sperm concentration, Furthermore, each semen sample was tested for the presence of both high- and low-risk HPV types by PCR.

Chapter 8 provides a general discussion, summarizing the results of chapter 2 to 7 in light of the currently available evidence.
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General introduction