General introduction and thesis outline

HPV infection and anogenital cancer

Infection with the human papillomavirus (HPV) can cause a variety of anogenital cancers such as cervical cancer, anal cancer and vulvar cancer, and other cancers such as oropharyngeal cancer. Other HPV's cause genital warts and are only rarely found in cancers [1, 2]. Over 200 different types of HPV have been identified[3], of which 14 alpha types are recognised as carcinogens and are called high-risk (hr)HPV types [4]. Low-risk HPV types are known to cause genital warts and laryngeal papillomatosis [5]. hrHPV is found in virtually all squamous cell cervical carcinomas and almost 90% of anal carcinomas, with HPV16 and HPV18 accounting for over 70% of cervical carcinomas[6, 7] and HPV16 alone detected in almost all HPV-positive anal cancers[8, 9]. HPV is also the most common sexually transmitted infection, with a lifetime risk of a hrHPV infection around 80% in women [10, 11].

HPV and development of cervical cancer

With <1% of HPV infections leading to cervical cancer, the development of a cervical carcinoma is the most well-known but rare complication of a hrHPV infection. Most infections are cleared by the host immune system within 2 years and do not produce detected dysplastic lesions at all[12, 13]. Several determinants have been found to influence the progression of oncogenic HPV infection to a dysplastic lesion, such as smoking, and contraceptive use [14-16]. If not cleared by the immune system, cervical cancer develops through a series of stages and a complex process of different events including chromosomal loss, somatic gene activation and suppression, somatic mutation, viral integration and methylation[17]. hrHPV virions enter the basal cell layer of the transformation zone of the cervical epithelium through a micro-laceration or specifically susceptible cells at the squamocolumnar junction, such as, in the endocervix, reserve cells or the region of immature metaplasia. The development of cancer after hrHPV infection usually takes about 15 years and is a complex process driven by the genes of hrHPV, but progression is variable and occasionally is very rapid [18].

HPV and prevention of invasive cervical cancer

There are two current approaches to preventing cervical cancer which have been largely implemented in many wealthy, developed countries and to a limited degree in low and middle income countries (LMIC) across the world. Secondary prevention through cervical screening was first developed in the 1950's and implemented more effectively in an organised way in the 1980's in most HIC (higher income countries) countries and used cytology to detect women with precursor lesions. The introduction of screening programmes has greatly reduced cervical cancer incidence in the developed world[20-24], but these programmes are costly, require multiple screening rounds and have the side effect of overdiagnosis and overtreatment of lesions which will never progress to cancer[25].

Interpretation of cytology is subjective and variable, depending on individual skill in interpreting microscopic changes in individual cells. Searching for a molecular alternative, a hrHPV test, which detects the causative agent of cervical cancer and precursors, was found to have a higher sensitivity for detection of high-grade cervical intraepithelial neoplasia (CIN2+), also known as high-grade squamous intraepithelial lesions (HSIL) but also has a lower specificity than cytology, detecting many transient infections rather than those progressing to cancer [26, 27].

Primary prevention is achieved via HPV vaccination of either girls and women only or including boys. Three vaccines are available that all protect against at least HPV16/18, thus reducing about 50% of CIN2+ and at least 70% of all cervical carcinomas[28].

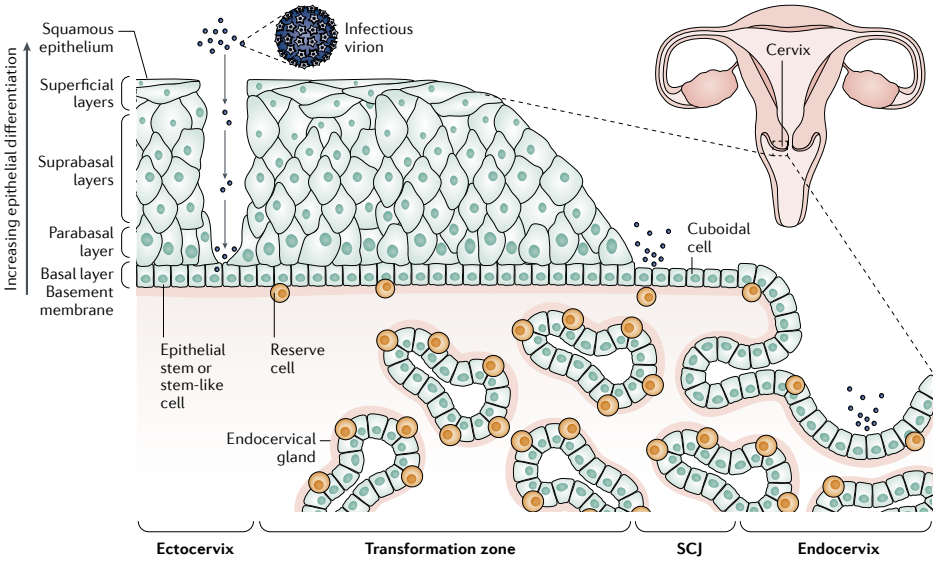

Figure 1. Cervical epithelium and the transformation zone. Schematic representation of the ectocervix, transformation zone, squamocolumnar junction (SCJ) and the endocervix. Human papillomavirus virions enter the basal layer through a micro-laceration in the squamous epithelium. Adapted from Schiffman et al. [19]

HPV and secondary prevention through screening

The usually slow progression from cervical hrHPV infection to cancer offers usually a large window for detection and treatment of precursor lesions, which is what large population-based screening programmes aim to do to prevent cervical cancer. First cervical cancer screening programs were cytology-based and involved the cytological evaluation of cervical smears containing squamous and columnar epithelial cells from

the transformation zone collected by a physician. Cytological assessment however, requires a subjective interpretation and lacks sensitivity for the detection of high-grade precursor lesions as well as requiring triage of uncertain, borderline cytological abnormalities (ASC-US). The development of highly sensitive and reproducible laboratory-based hrHPV tests has led to the replacement of cytology screening by hrHPV screening in several higher income countries with established screening programmes, such as The Netherlands, Australia and more recently the UK. This development is also likely to be more cost-effective than cytology in vaccinated populations[29]. The use of hrHPV as a screening tool could be applied in LMICs where there is no or very limited screening and the requirements of training and adequate sampling for cytology render this an unsuitable approach. In these countries it is being evaluated against other direct approaches to screening such as visual inspection of cervix with acetic acid (VIA) [30, 31].

Since the late 90's several large randomized controlled trials (RCTs) have been performed that compared hrHPV testing and hrHPV testing plus cytology (co-testing) with cytology alone. The most important studies have been carried out in Canada, Italy, the Netherlands, England and Sweden[32-35 36]; countries with different screening programs, different outcome measures and different hrHPV tests. Both baseline and follow-up results from the large RCTs showed similar results: the hrHPV-based screening offers better detection of HSIL and offers longer protection in case of a hrHPV negative screening result [37, 38]. HPV-based screening provides 60–70% greater protection against invasive cervical carcinomas compared with cytology and can start from the age of 30 years with screening intervals of at least 5 years. In addition, primary hrHPV screening can be performed on self-collected material. The introduction of self-sampling, which is much more suited to hrHPV screening than to cytology, into the screening program is expected to result in a higher participation rate, offering a better protection against cervical cancer for former non-responders of the screening programs[39-43]. Currently, the only self-sampling tool implemented in a screening program is a brush which collects cervicovaginal cells from the vagina (Evalyn Brush, Rovers, Oss, The Netherlands) [44]. The search for less invasive and more user-friendly self-sampling methods is ongoing and might include the use of urine samples for hrHPV-screening.

hrHPV-testing as a screening test has a lower specificity compared to cytology and therefore requires a triage test for hrHPV-positive women to avoid unnecessary referral for colposcopy of women without clinically relevant disease[45]. The most frequently used triage strategy currently is cytology with or without HPV16/18 genotyping[34, 46, 47] but the search for a better strategy is ongoing and largely focusses on the use of molecular markers such as methylation of human tumour suppressor genes [48]. The need to perform

cytology as a triage test and the requirement for good morphological preservation also limits the development of further approaches to self-sampling.

HPV and primary prevention through vaccination

Prophylactic HPV vaccination can, as well as reducing cervical cancer incidence, also reduce the burden of other HPV-related cancers such as vulvar, vaginal, penile, anal and oropharyngeal cancers [49, 50]. By including antigens for low-risk HPV types, HPV6 and HPV11, vaccination can also help prevent genital warts [51]. Since 2006, three HPV vaccines were approved: the quadrivalent Gardasil (HPV6/11/16/18) [52, 53], the bivalent Cervarix (HPV16/18) [54] and the nonavalent Gardasil 9 (HPV6/11/16/18/31/33/45/52/58) [55]. All have been found highly effective when given in a three-dose or two-dose schedule, and new studies show comparable effectiveness for one dose [56]. Many countries have implemented a prophylactic HPV vaccination programme, most of them offering vaccination to young girls in three doses. Several countries, including Australia and Austria, have implemented a gender-neutral approach, also vaccinating boys. Exact target populations and coverage levels vary between countries [57]. Group-protection has been seen in populations with a high vaccination coverage, with lower infection rates and precursor lesion incidence in both the vaccinated and unvaccinated population [58].

HPV vaccination is expected to reduce the lifetime risk of cervical cancer by 52% in vaccinated populations compared to unvaccinated populations, with uptake rates of about 80% in girls and 76% in boys. With these preventive approaches, elimination of cervical cancer is within reach. In Australia, one of the first countries to introduce a national HPV vaccination programme, simultaneous use of both hrHPV-based screening and HPV vaccination is believed to lower the cervical cancer incidence below the threshold of four new cases per 100 000 women annually, within the next 20 years [59]. Achieving this requires high vaccination coverage of both girls and boys with the 9vHPV vaccine, whether or not there is cervical screening every 5 years. The HPV Faster approach proposes to extend the routine vaccination programmes to women of up to 30 years of age, paired with at least one HPV-screening test at the age of ≥30, aiming to accelerate the decline in cervical cancer incidence [60].

Prevention of anal cancer

HPV does not only infect the cervical epithelium, it infects other anogenital sites and the oro-pharyngeal mucosa[61]. Persisting infections in these anatomic sites can lead to precursor lesions and/or cancer. HPV infections of the anal epithelium however, have received far less attention over the years due to both the social taboo on sexually-transmitted anal disease, and the much lower incidence and prevalence of anal cancer in the general population[62]. Anal cancer is more prevalent among high-risk subpopulations such as

men who have sex with men (MSM) [63], immunocompromised patients such as those living with HIV and women with a history of cervical, vulvar or vaginal hrHPV-related disease [64]. The incidence of anal cancer dramatically increased among HIV positive MSM in the antiretroviral therapy (ART) era, although the exact relationship between ART and anal cancer remains uncertain [65]. It is possible that earlier HIV control by ART results in lower cumulative risk, that is counterbalanced by longer survival and the increasing risk of anal cancer development with age. Anal cancer incidence is also increasing in the general population, and the disease is only now getting more attention in research.

Similarities and differences between anal and cervical cancer

One of the most important similarities between cervical- and anal cancer, is that most hrHPV lesions are found at the morphologically similar transformation zones in both sites. This has led to most of the tools developed over decades to assess cervical lesions being directly extrapolated to the anus. Years of research on cervical cancer led to the development of widely implemented and very successful screening programs, aiming to detect women at risk of precursor lesions of cervical cancer through the identification of abnormal squamous cells or the presence of hrHPV in cervical smears. The problems of cervical cancer screening with cytology largely focus on the subjectivity of cytological interpretation of specimens and its poor sensitivity. Problems with the more 'upstream' hrHPV test are decreased specificity and the need for a triage test to focus the referral rate for colposcopic examination.

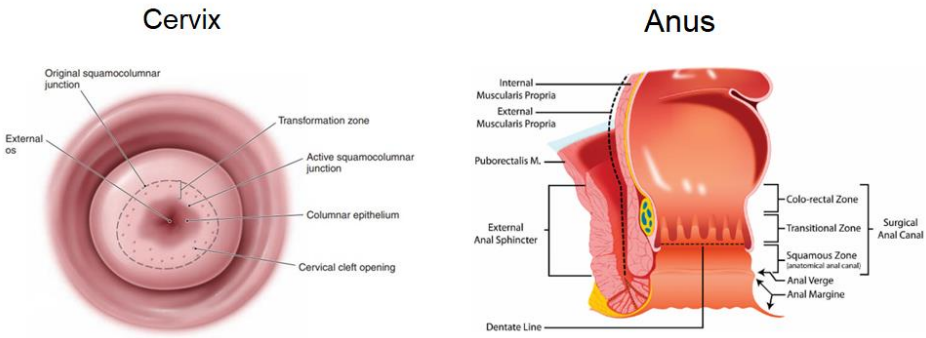

Figure 2. The anatomy of the cervix and its transformation zone and the anatomy of the anal canal with its transformation zone.

Anal cancer screening faces the same challenges but is in addition more complicated by the anatomical location of the anal mucosal transformation zone. The circular folded nature of the surface and the hypocellularity of the resulting anal cytological specimens form challenges for a proper diagnosis. Sensitivity for the detection of high-grade precursor lesions through cytological examination is low and repeat sampling is needed [66]

[67]. Guidelines for anal cancer screening are available but are much more limited and are different from those for the cervix [68]. Individuals at increased risk, like HIV positive MSM, are advised to annually undergo digital anal rectal examination (DARE), looking for hard lumps or masses, which can be further examined by high-resolution anoscopy (HRA) in a similar way to colposcopy with biopsy sampling of lesions suspected of high-grade anal intraepithelial neoplasia (HGAIN) dysplasia [69].

The anal canal appears to be very frequently infected with HPV, either through receptive anal intercourse or autoinoculation from cervical or vulvar infection, and might even function as a reservoir for HPV infection [70]. Despite the high prevalence of anal HPV infection with a high frequency of multiple infections and multiple lesions of different grades of AIN, the incidence of anal cancer is relatively low compared with cervical cancer per HPV infection, while the same HPV types are involved in the development of cervical and anal cancer, with a major role for HPV 16 [71]. Several factors including influences of hormones such as estrogen, the large differences in the microbiome colonizing cervix and anal canal, differences in microanatomy or a difference in local immune response among others might partially explain this difference in risk of development of cancer after hrHPV infection [72].

Among HIV positive MSM, the incidence of anal cancer exceeds the cervical cancer incidence found among women before screening was introduced [73].

Treatment of anal precancer is more difficult than cervical precancer [74, 75]. Since excision of the entire transformation zone as done in treatment of cervical HSIL is not possible, targeted ablation is the treatment of choice in anal precancerous lesions. The recurrence rate after ablation of anal HSIL is high and new HSIL in proximity of the earlier treated lesion are found frequently [76]. Therefore it is uncertain whether screening for and treatment of anal HSIL will reduce the incidence of cancer in the same way as screening for and treatment of cervical HSIL.

Histological classification of anogenital precursor lesions

Histopathology is important in distinguishing lesions that are likely to progress to cancer and require treatment, from self-limiting and regressing lesions. The large majority of lesions that are detected on either cervical or anal biopsy are productive lesions which produce and shed viral particles without signs of cellular transformation [77, 78]. These lesions might show morphology which is hard to distinguish from transforming lesions, but have a high chance of spontaneous regression. Regression rates of CIN2 lesions have been reported in several studies and vary between younger women (<25 years) and

older women from 30-50% to 70%, with lesions caused by HPV16 being less likely to regress [79].

Transforming lesions are characterized by viral oncogene E6 and E7 overexpression in dividing cells and may lead to cancer if not treated [2]. Which hrHPV infections lead to productive infections that will never progress and which infections lead to progressive transforming lesions and warrant treatment is not clear. Hypotheses that have been formulated discuss the site of infection and the type of epithelial cells that are infected. In the cervix, cells at the squamo-columnar junction (SCJ), localized between ectocervical squamous epithelium and endocervical glandular epithelium, in the zone of active squamous metaplasia, are thought to be highly susceptible to transforming HPV infection while productive infections are thought to arise from squamous epithelium from the ectocervix [80 80]. The exact nature of these cells remains uncertain.

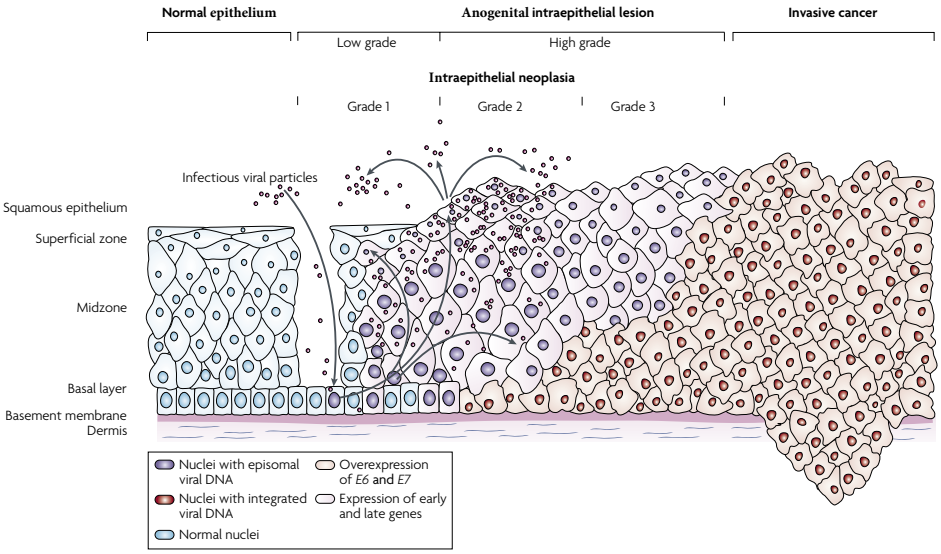

Figure 3. The development of invasive anogenital cancer through intraepithelial neoplasia (-IN) grade 1, grade 2 and grade 3 on histology after HPV infectious particles enter the basal layer of the squamous epithelium. Adapted from Woodman et al. [18]

In both cervical and anal biopsies, dysplastic lesions are currently classified by agreed cytologic and histomorphological criteria based on observable microscopic cellular changes. The classification describes the stage of lesion development on the biological continuum through which an hrHPV infection develops into cancer according to the concept: hrHPV persistence, hrHPV-mediated epithelial transformation, development of precancerous lesions and finally invasive cancer [81 82]. For histology, two schemes are used to describe severity of a precancerous lesion: intraepithelial neoplasia (-IN; anal

called AIN and cervical called CIN) grade 1-3 [83], and squamous intraepithelial lesion (SIL), low-grade or high-grade. Lesions that have similar histologic features as -IN 1 are called low-grade SIL (LSIL), and lesions with similar histologic features as -IN2 and -IN3 are called high-grade SIL (HSIL). This broad grouping was done since histologic distinction between -IN2 and -IN3 is poorly reproducible and has led to all -IN2+ lesions are treated [84, 85 86]. However, neither of these schemes directly reflect cellular transformation and the schemes do not distinguish accurately between productive and transforming lesions. Thus, histology as a sole diagnostic method does not provide adequate information to categorize lesions according to biologic behaviour, and malignant potential. Therefore, molecular markers that would make diagnoses more accurate, reflect cellular transformation, and are more reproducible among pathologists would have a significant impact on management of women with CIN/SIL and men and women with AIN/SIL.

Ideally, these markers would in an early stage identify hrHPV infections that will persist and eventually cause a transforming lesion if not treated. Overexpression of HPV E6 and E7, seen in transforming infections, leads to chromosomal instability and increased susceptibility to accumulation of alterations in cancer genes of the host cell increasing cancer risk. However, no reliable antibodies are currently available for HPV E6 and E7 overexpression, or other specific markers that distinguish between cells that have undergone malignant transformation by hrHPV and cells in which HPV will not cause dysplasia.

Molecular markers with the ability to differentiate between productive and transforming lesions that have been identified so far, are either focussed around the detection of the associated genotype, viral genes involved in productive infection, the overexpression of viral oncogenes seen in transforming infection, or the identification of viral or cellular changes required for the progression of transforming infection to cancer.

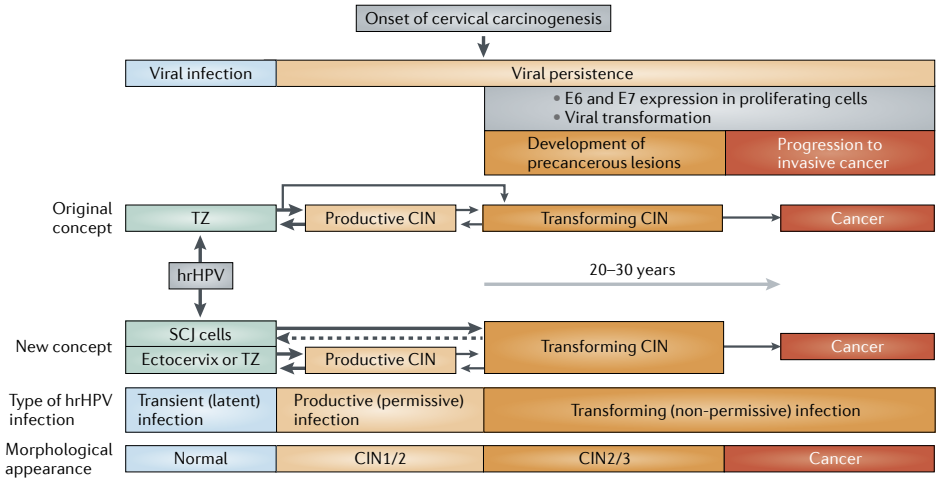

Figure 4. Human papillomavirus-mediated cervical carcinogenesis. After infection, viral persistence with overexpression of viral oncogenes E6 and E6 and viral transformation as a result can lead to the development of productive and transforming precancerous lesions and invasive cervical cancer. Adapted from Steenbergen et al. [17]

Biomarkers for transforming HPV-infections: p16INK4a and Ki-67

p16^{INK4a} (p16) is a cyclin-dependent kinase inhibitor and an integral component of normal cell cycle control. It inhibits phosphorylation of the cyclin D-dependent kinase 4 and 6 complex, which hyperphosphorylates the pRB gene product, resulting in the inactivation of pRB and the release of bound E2F transcription factors. Increased free levels of E2F lead to activation of S-phase progression genes which pushes the cell into the S-phase of the cell cycle. Increased levels of p16 result in cell cycle arrest and induction of senescence which protects the cell against genomic damage as a result of oncogenic stress such as oncogene activation or aging of the cell. Enhanced expression of HPV E7 also leads to oncogenic stress and in addition inactivates the essential pRB-mediated control functions of the cell cycle, leading to continued proliferative activity despite high levels of p16 expression [87]. This makes p16 a highly valuable biomarker for clinical detection of high-grade precursor lesions, used as a surrogate marker for the overexpression of hrHPV E7 [88-90].

p16 has become an important tool in diagnosing histological HSIL, as there is a strong correlation with diffuse strong p16 positive staining in the lower third of the epithelium or more with HSIL[87, 91]. Even though scoring p16 staining has an excellent interobserver reproducibility[92], there is currently insufficient evidence to support or discourage its use as a standalone marker to prospectively determine high-grade versus low-grade disease. Its current place in diagnostic medicine was described in the the Lower Anogenital Squamous Terminology (LAST) recommendations, which recommends its use

when the H&E morphologic differential diagnosis is between precancer (–IN 2 or –IN 3) and a mimic of precancer[84]. Strong and diffuse block-positive p16 results support a categorization of precancer (HSIL), but any identified p16 positive area must meet H&E morphologic criteria for a high-grade lesion to be reinterpreted as such. At the same time, recommendations against the use of p16 IHC as a routine adjunct to histologic assessment of biopsy specimens with morphologic interpretations of negative, -IN1, or -IN3 are made. The natural history of p16-positive –IN 1 and p16-negative –IN 3 are uncertain, and so the use of p16 to upgrade an unequivocal -IN1 or downgrade an unequivocal –IN 3 is not recommended. In addition, without interpretation of morphology, p16 cannot differentiate between CIN2 and CIN3 and cannot separate transforming from productive infections, which leads to the lack of important information that holds implications for progression risk[93]. p16 is therefore an important marker in daily practice for diagnosing HSIL, but there is still a need for objective, reproducible markers that show distinguishing expression patterns between productive lesions with a higher chance of spontaneous regression and transforming lesions which might progress to cancer and should be treated.

Biomarkers for productive HPV-infections: HPV E4

HPV E4 is an accessory protein which is highly expressed in productive HPV infections and marks the initiation of the late stage of the virus life cycle[94]. HPV infects the basal layer through a micro laceration and first maintains itself episomally at a low copy number. As epithelial cells differentiate, mature and migrate towards the surface of the epithelium, high-risk-HPV expresses E6 and E7 proteins that stimulate cell proliferation and ensure that cells in the superficial cell layers are retained in the cell cycle. Overexpression of E6 and E7 stimulates the synthesis of cellular proteins that are necessary for S-phase entry, allowing the replication of viral episomes and initiation of the productive viral phase. In LSIL/-IN cases, overexpression of E6 and E7 is restricted to the lower layers of the epithelium, still allowing for a productive virus life cycle in the upper epithelial layers. In the superficial layers of the epithelium, the hrHPV infected cells elevate viral replication by an increase in late promotor activity and accessory proteins (E1, E2, E4), after which the infectious virions are assembled and shed. Late proteins (L1 and L2, but also E4 which is more abundant) of the virus can only be expressed in terminally differentiated squamous epithelial cells which are capable of replicating the HPV-particles[94, 95]. Therefore, the detection of the late gene products E4 and L1 has been suggested as a marker for productive HPV-infections.

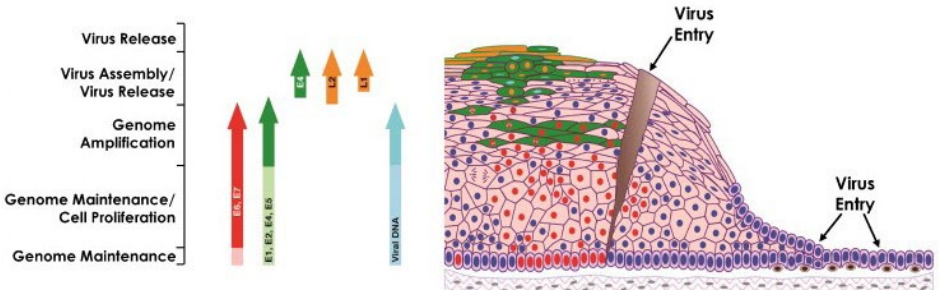

Figure 5. The high-risk human papillomavirus life cycle. After infection, different viral genes are expressed, leading to genome replication and transcription, control of cell growth and differentiation, reactivation of cell division, inhibition of apoptosis and epithelial differentiation, viral assemblage and release. Adapted from Doorbar et al. [95]

When E6 and E7 overexpression is not restricted to the lower parts of the epithelium, but extends into the upper two thirds of the epithelium or even in the full thickness, as is seen in persisting HPV infections resulting in HSIL/-IN2+ lesions, this is associated with increased risk of transforming the phenotype of the cell which then no longer allows for initiation of the productive phase of the HPV life cycle. p16 is a protein which is activated in cells overexpressing viral oncogenes E6 and E7 and is therefore useful as a marker of transforming infection when diffusely present [96]. This marker is widely used in histology to identify transforming infections classified as HSIL/CIN2+ lesions which in current practice are eligible for treatment [84, 97]. However, far from all HSIL/CIN2+ lesion will progress to cancer and by setting the treatment threshold at HSIL/CIN2, most likely many self-limiting productive infections are overtreated [98-100]. With a set of complementary markers that can both identify the transforming aspect of the lesion (p16), but can also show viral production in the upper layers of the epithelium (HPV E4), persisting transforming infections with a chance of progression to cancer could be distinguished from productive HPV infections in which spontaneous regression is more likely.

Biomarkers for transforming HPV-infections: methylation of cellular genes

Where E6 and E7 initially endorse replication of viral episomes and initiation of the productive viral phase in differentiated cells, long duration of their overexpression in dividing cells can lead to oncogenic events that drive the progression from transforming precancer to cancer through human chromosome instability. This instability allows for accumulation of aberrations that can result in loss of function of human tumour suppressor genes or activation of human oncogenes[17, 101]. Human DNA methylation is among these aberrations and involves the addition of a methyl group (CH_3) on specific cytosine nucleotides located 5′ of a guanine to generate a 5-methylcytosine [102]. These so-called CG dyads are connected by a phosphodiester bond (p), forming a CpG site. Methylation of CpG sites often occurs in CpG islands which are regions with multiple

CpG's allocated in the gene promoter. The DNA methyltransferases (DNMTs) responsible for CpG methylation can be activated by hrHPV E6 via p53 and directly by E7[103, 104]. The addition of multiple methyl groups on a given CpG island will make the DNA sequence less accessible for proteins or even entirely transcriptionally inert, leading to temporary or permanent changes in expression of genes and in addition, DNA instability.

Over the last years, many studies have focussed on DNA methylation of cellular genes as a molecular marker of cervical cancer and precancer. These mostly involved methylation of human tumour suppressor genes which were identified as markers of other human cancers. Hypermethylation patterns of these genes were studied in cervical cancer cell lines, cervical tissue samples and cervical scrapes. To date, over 100 human genes have been proposed as possible cervical cancer markers and the list is getting longer. Markers are often presented as a panel of multiple tumour suppressor genes. This clustering is done in the search for a highly sensitive and specific marker for disease detection, aiming to identify all cervical cancer and all precancer that the screenings programs target for (CIN2+/HSIL). The use of methylation markers in anal disease is not yet as advanced as in cervical disease. Studies so far mostly have been exploratory, using a set of markers identified as of value in cervical disease, on anal tissue specimens [106, 107]. First results show that a methylation marker panel, including ZNF582, can identify anal cancer and high-grade AIN with a cancer-like methylation pattern[108].

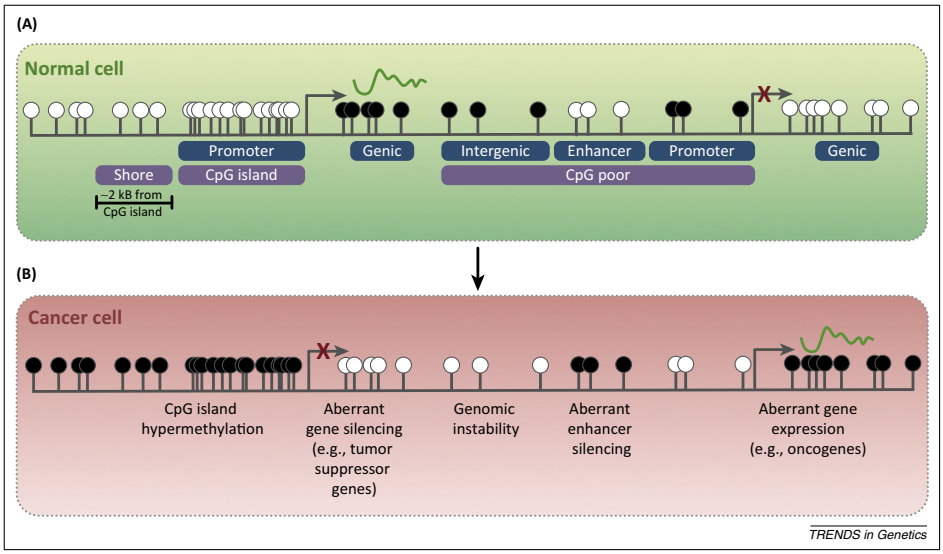

Figure 6. DNA methylation in a normal cell and the changes in a cancer cell. CpG islands in the promotor region of tumor suppressor genes unmethylated and expressed in normal cells **(A)** (green). In cancer cells **(B)**, CpG islands are prone to DNA hypermethylation, which results in aberrant gene silencing of tumor suppressor genes. Adapted from Stirzaker et al. [105]

The most frequently used technique to detect hypermethylation is through bisulphite conversion of DNA during which unmethylated cytosines only are conversed to uracil, followed by quantitative methylation-specific polymerase chain reaction (qMS-PCR). Hypermethylation of multiple genes can be detected in one test using a multiplex PCR, and such a test can be performed on tissue samples as well as liquid-based samples such as physician-taken cervical scrapes and self-collected cervicovaginal samples. Several marker combinations have been studied extensively in triage populations, mostly consisting of hrHPV-positive women: JAM3/EPB41L3/TERT/C13ORF18, JAM3/C13ORF18/ANKRD18CP[109-112] and several CADM1, MAL and miR124-2 and FAM19A4 combinations[113-122]. Asian studies more often focussed on SOX1 and PAX1 in several combinations[123, 124], and single markers among which POU4F3 have shown promising results in smaller studies but have not yet been confirmed by large prospective follow-up studies[125, 126]. To improve the performance of methylation marker panels, combinations with the detection of viral hypermethylation and hrHPV genotyping show potential. As an example, studies have explored the combination of hypermethylation of EPB41L3 and L1 and L2 regions of HPV16/18/31/33 [127]. And FAM19A4/miR124-2 in combination with HPV16/18 genotyping, showed a higher sensitivity of 84.7% and a decreased specificity of 54.9% for the detection of ≥CIN3 compared to methylation testing only [118].

A limitation of studies of methylation of cellular genes done so far is that hypermethylation levels and diagnostic performance of methylation of most of the identified genes vary between studies. This may not only have to do with storage and test characteristics but may also represent differences between populations and heterogeneity of cervical cancer and precancer development or other unknown factors. New techniques and platforms for discovery of new marker genes allow for agnostic profiling studies that specifically focus on cervical carcinogenesis. Genome-wide methylation profiling using next-generation sequencing of methyl-binding domain-enriched DNA studies are now being conducted to identify novel markers which can be used for screening or triage purposes[128].

Another use of methylation markers may be found in the identification of precursor lesions with a higher risk of progression to cancer. Increasing levels of hypermethylation have not only been correlated to increasing grade of cervical intraepithelial neoplasia, but also to duration of hrHPV infection (>5 years) [129]. Early productive viral infections with CIN1 or CIN2 lesions as a result have a low risk of progression and can be distinguished from more advanced transforming CIN (CIN2-3/HSIL) by methylation levels [130]. Advanced transforming CIN has a higher chance of progression to cancer and detection of increased methylation of tumour suppressor genes could be used as an indication for

treatment while absence of hypermethylation could allow for close follow-up, preventing unnecessary treatment of women of reproductive age.

HPV genotype

The development of anal and cervical HSIL requires the persistence of a type-specific hrHPV infection over months and years. How this is defined and detected is important for determining the strength of association between hrHPV infection, its persistence and development of HSIL [131]. However, hrHPV positive smears are often positive for multiple hrHPV genotypes when genotyped with a sensitive test [132, 133]. In serial swabs, genotyping is important for the identification of genotype specific persistent infections. Determining the genotypes present in a single sample is also important for risk stratification as underlying lesion severity varies with the associated HPV type, with non-HPV16/18 types accounting for a high percentage of ≤LSIL of which many will regress without interference [46, 134]. In addition, knowing the role of each HPV genotype in cervical and anal cancer and precancer development can help select types to be included in vaccines and in genotyping assays for triage.

Detection of the causative HPV genotype of the lesion: laser capture microdissection

In the presence of multiple HPV infections, determining which genotype is the causative genotype of the worst underlying lesion can only be determined when genotyping the biopsy material, firstly genotyping the entire biopsy in a whole tissue section PCR (WTS-PCR) and in case of multiple HPV infections on biopsy level, genotyping only the worst lesion present in a biopsy [135]. The most precise approach to do this lies in laser capture microdissection PCR (LCM-PCR) [136], in which a microbeam UV laser micro-dissection system is used to excise and transfer a selected region to a tube, after which it is genotyped using an analytically sensitive HPV genotyping assay (SPF10-PCR-DEIA-LiPA25version1) [137]. It is known that each HPV genotype involved in a multiple infection is associated with an independent CIN or AIN lesion [135, 138], and knowing the causative HPV genotype of the worst lesion is important for risk stratification, as different genotypes entail different risks of cancer development [139]. In both cervical and anal cancer, HPV 16 is the most important oncogenic HPV genotype and is found in 60% of cervical and over 90% of anal cancer [140].

With the increasing use of HPV genotyping as a triage test on smears and self-samples after a screening-positive result, it is meaningful to study if the HPV genotypes present on screening samples are representative of the HPV genotype causing the worst underlying, high-grade lesion. In addition, HSIL lesions in HIV positive MSM can be caused by not only hrHPV but also lrHPV infections, and clinically benign appearing warts can har-

bour hrHPV infection with HSIL foci [141]. In the search for improved tests for the selection of patients and distinguishing biomarker expression patterns of transforming HPV infections with a higher risk of progression to cancer, identification of the causative genotype of the lesion could play an important role. The pattern of genotypes will change when HPV vaccinated women and men are screened.

AIMS AND OUTLINE OF THIS THESIS

Identification of molecular differences that can distinguish between productive lesions which might regress spontaneously and advanced transforming lesions with a higher risk of progression to cancer could help identify those patients that require treatment and differentiate those patients in which close follow-up would be more appropriate. This would help to avoid overtreatment which is costly, unpleasant and has its own risks. This thesis aimed to identify biomarker expression patterns corresponding with different grades of cervical and anal precursor lesions in cytological and histological specimens, proposing an important tool for diagnosis and clinical management of patients at risk of cervical and anal cancer.

This thesis consists of three parts. The first part consists of Chapters 2, 3 and 4 and focusses on cervical cancer screening and triage. Chapter 2 compares the sensitivity of hrHPV and genotyping in self-collected urine samples in the morning and later on during the day, brush-based self-samples, and clinician-taken smears for the detection of CIN2+ in a cytology-screened colposcopic referral population. Chapter 3 investigates hrHPV detection with genotyping and methylation of FAM19A4/miR124-2 for detection of CIN3+ in women from a similar population. Chapter 4 describes the study of whether HPV genotypes detected on self-samples represent the hrHPV type causing the worst cervical lesion, and whether any differences in hypermethylation of FAM19A4/miR124-2 exist between CIN lesions caused by different hrHPV types.

The second part of this thesis describes how the use of immunohistochemical markers can be used to identify differences in biomarker expression patterns between different grades of anal intraepithelial neoplasia. Chapter 5 studied the immunohistochemical staining patterns in AIN, adding a novel marker for initiation of the productive phase of the HPV life cycle (HPV E4) to those for cell cycle activity (Ki-67) and transforming activity of HPVE7 gene (p16). In Chapter 6, we focused on immunohistochemical markers HPV E4 and p16, using them on a set of anal biopsies aiming to improve the definition of HGAIN and show differences between lesions graded as AIN2 and AIN3.

The final part of this thesis describes the use of combinations of different molecular markers in a study of cytology-screened women, involving both cytology and histology samples. In Chapter 7, immunohistochemical staining patterns of markers HPV E4 and p16 in cervical biopsies are correlated to results of methylation markers FAM19A4/miR124-2 on smears. We aimed to describe biomarker expression patterns of women with different grades of CIN and negative controls, combining the results of immunohistochemistry and methylation. In the general discussion described in Chapter 8, findings from all chapters are discussed and future perspectives are outlined.

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