Improving Cervical Cancer Prevention by HPV Self-sampling, Colposcopy and Biomarkers

Romy van Baars

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Improving Cervical Cancer Prevention by HPV Self-sampling, Colposcopy and Biomarkers

Verbetering van preventie van baarmoederhalskanker met behulp van HPV zelfafname, colposcopie en biomarkers

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Zit het je eens tegen en vind je iets niet fijn
Ook regen is een zegen, die moet er soms ook zijn

Theodoor van Baars

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General introduction

GENERAL INTRODUCTION

1. CERVICAL CANCER AND CERVICAL INTRAEPITHELIAL NEOPLASIA

1.1 Cervical cancer

The worldwide incidence rate of cervical cancer is around 500 000 per year with a mortality rate of around 270 000 women per year. [1, 2] With these figures, it represents the third most common cancer among women worldwide, after breast and colorectal cancer. However, the incidence varies widely among countries; with an average incidence of 452 000 new cases per year in developing countries versus 76 000 new cases per year in developed countries (Figure 1). [2] This lower incidence of cervical cancer in developed countries is at least partially the result of organized cervical cancer screening programs, which lead to earlier detection of cervical cancer and treatable premalignant stages. [3] Since the introduction of the national organised screening program in the Netherlands in the 1970s, the cervical cancer incidence and mortality have significantly reduced (see paragraph 3.2.1). Between 1999 and 2009 around 700 new cases of cervical cancer were diagnosed yearly with approximately 200-250 deaths. [4] The peak cervical cancer incidence is between 40 and 44 years. [4] The overall 5-year survival rate is 66% in the Netherlands. [4]

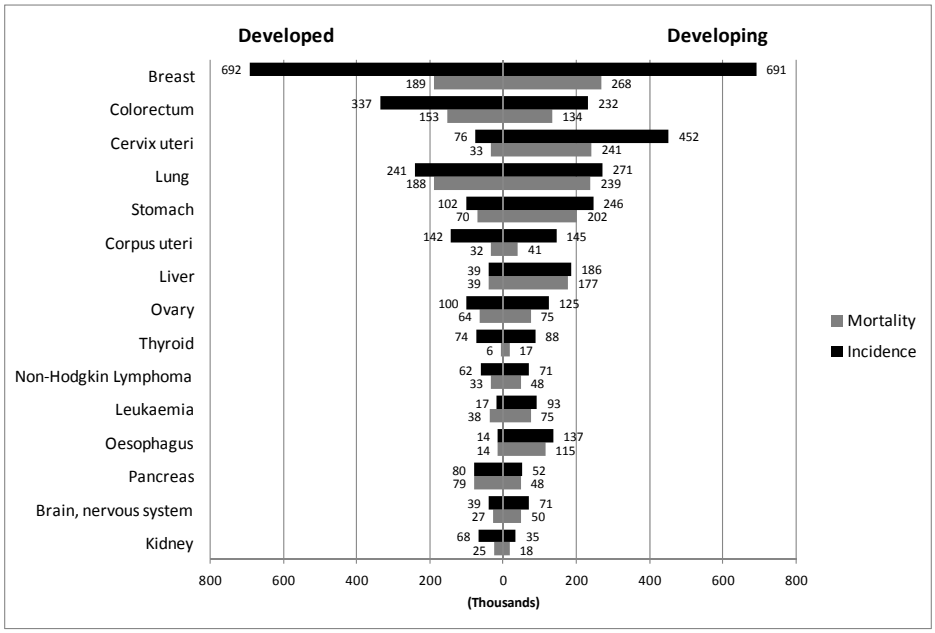

Figure 1. Estimated number (per 1000) of new cancer cases (incidence) and deaths (mortality) in women in developed and developing countries worldwide in 2008 [Adapted from GLOBOCAN 2008][2]

1.2 Precursor lesions of cervical cancer

The cervix is the lower part of the uterus. It consists of the endocervix covered with glandular columnar cells and the ectocervix covered with squamous epithelium. The transition area

where the glandular cells meet the squamous cells is called the squamocolumnar junction (SCJ). During puberty, a metaplastic change starts, resulting in transformation of columnar cells into metaplastic squamous epithelium. This manifests in a shift of the SCJ from the ectocervix to the endocervix. The area between the old and the new SCJ is called the transformation zone (TZ). Because this area is assumed to be particular vulnerable for neoplastic changes, it is thought that most cervical cancers originate from this site. [5]

Histologically, cervical cancers can be classified into different subtypes. The two most common subtypes are squamous cell carcinoma (comprising approximately 80% of cervical cancers), and adenocarcinoma (comprising approximately 10-20% of cases).

It is thought that cervical squamous cell cancer arises from precursor lesions, histologically recognisable as cervical intraepithelial neoplasia (CIN). CIN lesions are classified into three groups: CIN1 (mild dysplasia) with morphological changes up to the lower one third of the squamous epithelium, CIN2 (moderate dysplasia) with morphological changes until two thirds, and CIN3 (severe dysplasia, including carcinoma in situ) with morphological changes in more than two thirds of the epithelium (Figure 2). CIN2 and CIN3 together are also called high-grade CIN (HG-CIN). CIN1 is also referred to as low-grade CIN. The precursor lesion of the adenocarcinoma is adenocarcinoma in situ (AIS).

If these precursor lesions are not treated they may finally develop into cervical carcinoma. According to national guidelines CIN2 is the treatment threshold (see paragraph 3.3). [6] Despite that histological grading of CIN is such an important determinant for clinical management, histological grading is subject to substantial inter- and intra-observer variability. [7-10] Particularly, the reproducibility of diagnosing CIN1 and CIN2 is low, [10, 11] leading to extensive follow-up and overtreatment of falsely diagnosed high-grade lesions.

Recently, the theory of cervical squamous cancer development from squamous TZ epithelium has been questioned and a discrete, single layered embryonic cell population with unique morphology (cuboidal), expressing junction cell specific genes, has been proposed as origin of most cervical cancers. [12, 13]

2. HUMAN PAPILLOMAVIRUS AND CERVICAL CARCINOGENESIS

Cervical cancer is caused by a persistent infection with a high-risk human papillomavirus (hrHPV). [15-18] HPV can be detected in 90-100% of SCC and 80-90% of the adenocarcinomas. [19] HPV is a highly infectious sexual transmitted virus and it is assumed that the life-time risk to acquire a genital HPV infection is around 80%. [20] HPV is frequently found in young women (around twenty years of age), [21] but most infections are cleared by the immune system in 1-2 years and only around 10-20% progress to CIN (see Figure 3). [22-24] If left untreated, 30-50% of the CIN3 lesions may progress to cancer. [25]

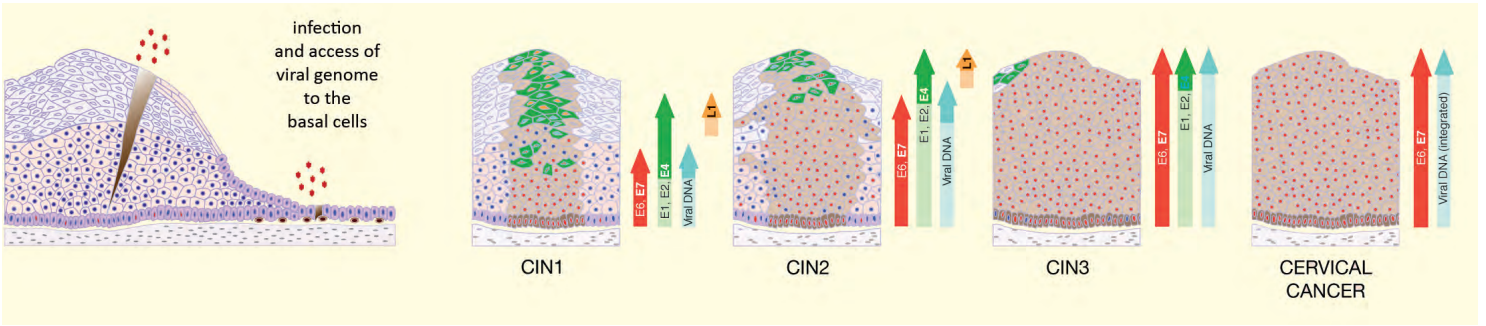

Figure 2. Changes in expression patterns of the HPV proteins that accompany progression to cervical cancer

Micro lacerations in the skin or mucosa permit the HPV virus to enter the basal cells of the squamous epithelium. Infection starts an ordered expression pattern of the viral genes that lead to virus synthesis and release (productive infection or CIN1). Deregulation of viral gene expression occurs with increasing lesion grade. Furthermore, there is an increasing risk of viral genome integration into the host cell chromosome. Persistent deregulated gene expression and viral DNA integration can eventually result in accumulation of secondary genetic changes and development of cancer. Proliferating cells are indicated by the presence of red nuclei. Cells expressing the HPV E4 protein are shown in green and cells expressing HPV L1 are shown in yellow [Adapted from Doorbar *et al*. 2012]. [14]

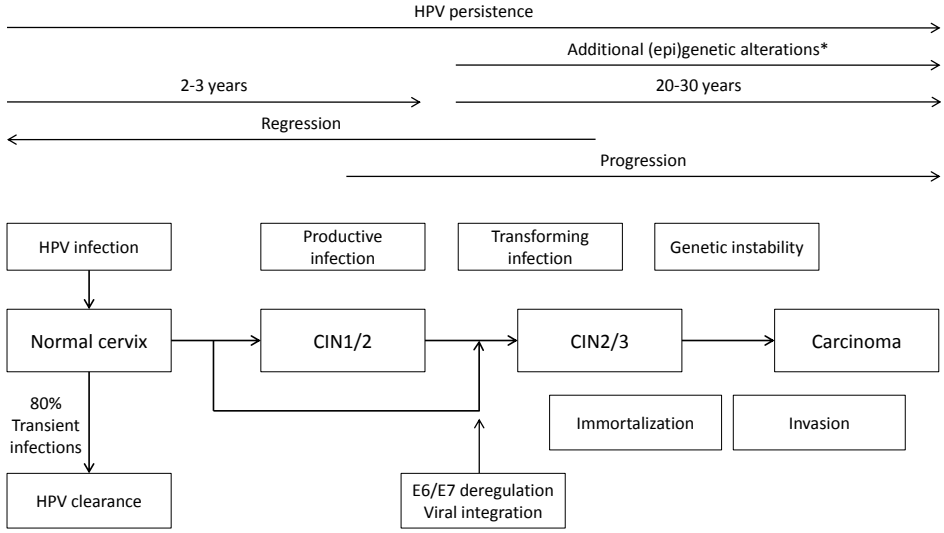

Figure 3. Progression model of cervical cancer [Adapted from Snijders *et al*. 2006] [40]
* Activation of oncogenes, loss of tumour suppressor gene function

HPV is a small double-stranded, circular DNA virus belonging to the family of Papillomaviridae. [26] The HPV genome consists of three regions: the long control region (LCR) without coding potential, a so-called early region, encoding proteins essential for viral genome replication (E1, E2, E4, E5, E6 and E7), and a late region, encoding the viral capsid proteins (L1 and L2). Micro lacerations in the skin or mucosa permit the virus to enter the basal cells of the squamous epithelium. Infection starts an ordered expression pattern of the viral genes that can lead to viral assembly in the upper layers of the squamous epithelium, and virions are released from shedded terminally differentiated cells and may infect adjacent tissue (productive infection or CIN1). [14] During progression from normal epithelium to cervical cancer, normal regulation of the HPV life cycle is lost and the viral gene expression becomes perturbed (see Figure 2). [27] The extent of cells with E6 and E7 expression is increased proportional to CIN grade. The immortalizing and transforming potential of E6 and E7 make them the most important viral oncoproteins. The HPV E6 protein inhibits the function of the tumour suppressor protein p53 and contributes to the activation of the enzyme telomerase, preventing chromosome shortening, leading to an increased life-span of the infected cell. [28] The HPV E7 protein binds to the tumour suppressor protein retinoblastoma (pRB), finally resulting in uncontrolled cell division. In normal situations, p53 would induce apoptosis in response to unregulated cell proliferation and DNA damage. By E6 induced p53 degradation this apoptosis mechanism is blocked which results in accumulation of chromosomal changes of cervical cells. [28, 29] Furthermore, there is an increasing risk of viral genome integration into the host cell chromosome. Persistent deregulated viral oncogene expression and chromosomal instability eventually result in accumulation of secondary genetic changes and development of cancer (see Figure 3). [30, 31]

Currently, more than 120 HPV types have been identified. HPV types are divided into the so-called low-risk types and high-risk HPV types (hrHPV). Low-risk types cause in particular condylomata acuminata (genital warts) en hrHPV is related to a high risk of malignant transformation of the infected epithelium, causing CIN and cancer. [32] Based on epidemiological evidence, the World Health Organization, International Agency of Research on Cancer (IARC) has classified 12 HPV types as high-risk types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59); with additional types (HPV68, 73) being considered as possibly carcinogenic types. [14, 33-35] Based on phylogenetic relationships also several other HPV types probably belong to the hrHPV types. [36, 37]

HPV16 is the most common HPV genotype in squamous cell carcinoma, adenocarcinoma and HG-CIN worldwide. The second most prevalent HPV genotype in HG-CIN and cervical cancer is HPV18. [19, 38, 39]

3. CERVICAL CANCER PREVENTION

3.1 Primary prevention

Two prophylactic HPV vaccines are available for primary prevention of cervical cancer. The bivalent vaccine (Cervarix, GSK) protects against the two most important HPV types (e.g. HPV16 and 18), which together cause approximately 70% of cervical cancers. [41] The quadrivalent vaccine (Gardasil, Merck) also prevents against infections with HPV6 and 11, which together cause almost all condylomata acuminata (genital warts). [42]

Because of practical and social reasons, vaccine coverage in developed countries is variable. In the Netherlands, false stories on social media led to fear of vaccination in young girls and have resulted in a suboptimal vaccine uptake of only 50%. [43] Vaccination is almost not available in developing countries, since the vaccines are expensive, require cold storage and transport and have to be administered in (2 to) 3 doses in 6 months.

3.2 Secondary prevention

The main intent of secondary prevention is to identify the women with cervical precancer by screening and accordingly treat them to prevent cervical cancer.

3.2.1 Cytology screening

Cervical cytology screening programs have significantly reduced cervical cancer incidence and mortality.[2, 44]

In the Netherlands women between 30 years and 60 years receive every 5 years an invitation to attend the national cervical screening program for a so-called Pap-smear. The purpose of this test is to detect potentially precancerous changes in order to treat them before progression to cancer. In the Netherlands, the CISOE-A classification (in Dutch KOPAC-B) is currently used to grade. Internationally, the Bethesda classification is used. [45] Women with moderate dyskaryosis

or worse cytology result are directly referred for colposcopy (see paragraph 3.2.4). Women with borderline or mild dyskaryosis are followed in the screening program by repeat cytology at 6 and 18 months and are referred for colposcopic examination if the repeat test is abnormal.

Although effective, cytological screening has several limitations. The first limitation is the low sensitivity to detect cervical lesions. This limited sensitivity is caused by inadequate sampling in which abnormal cells are not obtained from the cervix, and interpretation errors in which the few abnormal cells are not identified between the multitudes of normal cells. [46-48] Cytology has a sensitivity to detect CIN2+ of 50-70%. [47] The second limitation is the moderate specificity of approximately 95%, [49] leading to a substantial over-referral rate of women with minor abnormalities who do not have underlying high-grade disease. Thirdly, the incidence of cervical cancer has declined since the introduction of cytology-based cervical screening, but no change has been observed in the incidence of cervical adenocarcinoma, [49, 50] suggesting that cytology fails to detect adenocarcinoma and its precursor lesions. Finally, the attendance to the screening program is limited. [51] As a consequence, there is a need of improvement of primary screening.

3.2.2 HPV screening

Several trials have shown that HPV testing has a higher sensitivity for cervical precancer and cancer detection than cytology (~95%), although it is less specific. [52-57] Due to this higher sensitivity, HPV negative women have a lower 5 year risk of CIN3 and cancer than Pap negative women. [52] Therefore, screening based on the detection of HPV DNA will further improve the effectiveness of the screening program. [53-57] Moreover, the screening interval for HPV negative women might be extended.[49, 58-60] In the Netherlands primary screening with hrHPV testing will be implemented in 2016 as a way to improve cervical cancer prevention. In the newly proposed screening algorithm women will receive 5 invitations to the screening program instead of 7, at age 30, 35, 40, 50 and 60. To retain the number of colposcopies and thus the costs and unnecessary distress for women within acceptable limits, triage testing of hrHPV positive women is necessary. In the new screening algorithm, hrHPV positive women will receive triage testing by cytology and in case of abnormal cytology will be referred to colposcopy. If cytology is normal a repeat HPV test and cytology after 6 months is advised.

3.2.3 HPV Self-sampling

In the Netherlands, only 65% of the invited women participate in the cytological screening program. [51] Over half of all cervical cancers arise in women who do not respond to the invitation to the cervical screening program. [61] Previous studies have shown that providing the opportunity of self-sampling for HPV testing (HPV self-sampling) can improve the attendance of women to the cervical screening program, by re-attracting those who currently do not attend the screening program. [62-65] Also in countries without an organized nationwide screening program HPV self-sampling could be a good option for screening. Several studies have

shown that self-sampling combined with HPV testing can have a similar sensitivity for HG-CIN as HPV testing on a cervical scrape obtained by a physician, although this likely depends on the self-sampling device and HPV testing method used (this thesis, **Chapter 2 and 3**). [66-69] However, self-sampled material is not suitable for cytomorphological analysis, and therefore cytology cannot be used as a direct triage method on self-samples. [70] Interestingly, non-morphology-based, objective molecular markers such as promoter methylation analysis of tumour suppressor genes (see paragraph 4.2) have shown to be alternative triage tools for hrHPV positive women that are feasible on self-samples and clinically perform similarly as cytology triage testing on a physician-collected cervical scrape. [71-73]

3.2.4 Colposcopy

Colposcopy is performed after an abnormal Pap-smear and is the current standard for visualisation of cervical lesions after application of an acetic acid solution (3-5%). Reid and Scazi developed a scoring system using varying colposcopic characteristics that gynaecologists use to evaluate the severity of the lesion: the Reid colposcopy index. [74] These colposcopic characteristics are acetowhitening, lesion margins, punctation, mosaicism, the presence of atypical vessels and lesion size (Figure 4). The grade of the characteristics is used to identify the area on the cervix that most likely represents the worst lesion. [75-78] This area is biopsied and the biopsy is send to the pathologists for histopathological diagnosis. A limitation of colposcopy is that is has a sensitivity of only 50-70% to detect CIN2+. [79-83] Gains in sensitivity can be obtained by increasing the number of biopsies (this thesis, **Chapter 5**). [84] Moreover, a previous study performing random biopsies of regions without visual abnormalities has shown that 23-37% of CIN2+ lesions were detected in such a biopsy only. [85] Another limitation of colposcopy is that not always the entire transformation zone can be visualised. Endocervical curettage is required when the transformation zone is not visualised. [86]

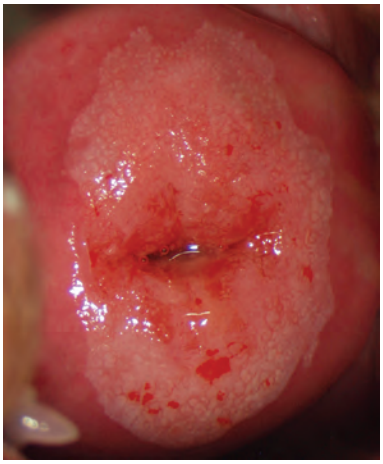

Figure 4. Colposcopic image of a cervix with clear acetowhitening and coarse mosaicism

3.3 Treatment

Further clinical management is depending on the histology result of the biopsy taken during colposcopy. Because 60% of CIN1 lesions regress spontaneously, [22, 87, 88] women diagnosed with CIN1 or without CIN are followed-up by cervical cytology 6, 12 and 24 months after colposcopy. If these three smears are normal, women can return to the regular screening program. According to the national guidelines all women with CIN3 and almost all women with CIN2 receive treatment. [6] Whether treatment will be performed and the type of treatment depends on the patients age, potential child wish, the visualisation of the transformation zone, the severity and the extension of the lesion. Nowadays, the most common procedure is a loop electrosurgical excision procedure (LEEP). This procedure can be performed under local anaesthetics at the outpatient clinic. A cold-knife conisation is performed when the biopsy showed AIS or when a micro-invasive carcinoma is suspected. Because of the destruction of the tissue cryotherapy is not indicated in the Netherlands. In case of vaginal extension of the lesion (vaginal intraepithelial neoplasia; VAIN) laser evaporisation can be used. More extensive operations such as hysterectomy or trachelectomy are used for cervical cancer if necessary in combination with radiotherapy and/or chemotherapy.

4. MOLECULAR BIOMARKERS

More insight into the pathogenesis of cervical cancer has led to the discovery of various biomarkers that could be useful at several levels; from screening to diagnostic workup. As indicated above, in an HPV-based screening program triage testing is needed to compensate for the lower specificity of the HPV test, and in the Netherlands triaging by cytology at baseline and repeat cytology after 6 months has been advised by the Health Council to the Minister of Health. [89] However, it is expected that cytology having its drawback of subjective reading, will face growing competition from other, objective biomarkers, some of which are applicable to self-sampled specimens. Molecular biomarkers that are currently under study include hrHPV genotyping, methylation of tumour suppressor genes and immunohistochemical staining of proliferation marker MCM, viral markers E4 and L1 and transformation marker $p16^{INK4a}$. [90] The latter markers might also be of value to increase the reproducibility and consistency of diagnosing CIN, thereby reducing extensive follow-up and overtreatment of falsely diagnosed high-grade lesions.

4.1 HrHPV genotyping

Considerable evidence exist that the absolute risk for cervical precancer and cancer varies substantially among specific HPV genotypes. Studies have shown that HPV16 has the highest oncogenic potential [32] and HPV16 and 18 have found to be associated with the highest risk of the development of CIN3 and ICC. [19, 39, 91] Furthermore, HPV18 and 45 are associated with a lower difference in age between diagnosis of CIN3 and cancer.[39] Therefore, hrHPV genotyping

might be useful for the risk assessment and triage of HPV positive women, particularly those who have normal cytology. A recent post-hoc analysis of the POBASCAM trial showed that HPV16/18 genotyping was involved in two out of three eligible triage strategies, but these strategies had a lower positive predictive value (PPV) compared to the PPV obtained by triage with baseline cytology and repeat cytology after 6 months, which might lead to potential overtreatment resulting from the increase in referral rate. [92]

4.2 Methylation markers

The presence of a hrHPV infection alone is, though essential, not sufficient to develop a CIN lesion or cervical (pre)cancer. Particularly, progression from HG-CIN to invasive cancer is a long-lasting process (20 to 30 years) [93] that is believed to depend on accumulation of various crucial additional genetic and epigenetic alterations (Figure 3). [40] Indeed, a longer duration of a preceding hrHPV infection is associated with increased levels of chromosomal alterations in HG-CIN lesions. [94] Genetic alterations such as DNA copy number gains and losses can result in the activation of oncogenes and inactivation of tumour suppressor genes, respectively. [95] Epigenetic alterations are reversible changes in the gene function without changes in the DNA sequence. DNA methylation is an epigenetic process whereby a methyl group is covalently bound to a cytosine in a CpG dinucleotide. CpGs are enriched in so-called CpG islands of the promoter regions of many genes and hypermethylation in these islands contributes to a structural change of the chromatin and usually abrogation of the transcription of the gene. If such hypermethylation events involve promoter regions of tumour suppressor genes, this will lead to inactivation of such genes and therefore contribute to cell transformation. Hypermethylation of CpG islands in the promoter region of tumour suppressor genes has been recognized as a common molecular change in cancer,[96, 97] including cervical cancer. [98] Moreover, similarly as for chromosomal aberrations, methylation levels of several genes like cell adhesion molecule 1 (CADM1) and T-lymphocyte maturation associated protein (MAL) are increased proportional to degree and duration of underlying cervical disease. [99]

DNA methylation of several host cell genes that might be feasible for triage of hrHPV positive women has been described in literature (reviewed by Wentzensen *et al.* [98]), and combined methylation testing of CADM1 and MAL [100, 101] has successfully been analysed in a training/validation set approach. [100] In this thesis (**Chapter 7**), we further explored CADM1 and MAL methylation in women having multiple cervical lesions.

4.3 Immunohistochemical markers

4.3.1 Biomarker for transforming infections: p16^{INK4a}

HrHPV E6 and E7 proteins have a central role in the cascade of events leading to malignant transformation by among others inactivating the tumour suppressor genes p53 and pRB, respectively (see paragraph 2). Therefore, detection of E6 and E7 overexpression in the proliferating cell compartment of cervical epithelium could serve as a marker to differentiate

women with productive HPV infections from those with transforming infections that may develop into true precancer. Nowadays, there are no highly effective and specific monoclonal antibodies for HPV E6 or E7 available.

An alternative, however, is staining for the cyclin dependent kinase-4 inhibitor $p16^{INK4a}$, which is considered a surrogate marker for increased E7 expression in proliferating cells. The $p16^{INK4a}$ expression pattern has been extensively investigated by immunohistochemistry in cervical (pre) malignancies. [102-105] In normal tissue $p16^{INK4a}$ expression is hardly detectable, and if so, it only involves isolated cells. Strong, diffuse $p16^{INK4a}$ immunohistochemical staining in proliferating cells is found in cervical cancers and high-grade CIN lesions. Therefore, $p16^{INK4a}$ overexpression is now widely accepted as a sensitive and specific biomarker for HPV induced HG-CIN [106, 107] and $p16^{INK4a}$ immunohistochemistry has shown to improve diagnostic accuracy of CIN grading, and reduce interobser variability. [105, 108] Moreover, when applied in a dual stain format combined with the proliferation marker ki-67, $p16^{INK4a}$ immunocytochemical staining of liquid-based cytology slides has shown to be a promising candidate triage test for hrHPV positive women.[109-111] Despite its high sensitivity, $p16^{INK4a}$ staining has its limitation: $p16^{INK4a}$ overexpression in the basal and parabasal cell layers can also been seen in a substantial number of hrHPV positive, histologically typical CIN1 lesions, and clinical management of these lesions remains unclear.

4.3.2 Biomarker for cell proliferation: MCM

Minichromosome maintenance (MCM) proteins are DNA helicases that are crucial for DNA replication and confine replication to once per cell cycle. [112] Several studies have shown MCM to be a proliferation marker. [113-116] In normal squamous epithelium MCM proteins are limited to the (para)basal proliferating cell layers and are absent in differentiated cells. However, MCM expression is increased in cervical dysplasia. [117, 118] A drawback is that proliferation markers such as MCM cannot differentiate between proliferation due to benign conditions such as inflammation, metaplasia and epithelial repair or due to hrHPV induced neoplasia (this thesis, **Chapter 6**).

4.3.3 Biomarkers for productive HPV-infections: E4 and L1

The viral proteins L1 and E4 are expressed in the late stage of the HPV replication cycle and are involved in viral packaging and support genome amplification, respectively. [119, 120] As can be seen in Figure 2, they are only expressed in terminally differentiated HPV-infected squamous cells of the intermediate or superficial cell layers. [27] With increasing lesion grade cell differentiation is lost. Consequently, the expression of these viral genes is progressively lost in transforming HPV infections, proportional to increased E6 and E7 expression. Therefore, E4 and L1 have been suggested as markers of the onset of the productive stage in the viral life cycle and low-grade CIN. [121, 122] The inclusion of a viral protein marker appears to help in the discrimination between HPV and non-HPV induced CIN1 and may help in the subdivision of lesions that fall into the equivocal CIN2 category (this thesis, **Chapter 6**).

5. AIM AND OUTLINE OF THIS THESIS

Current screening algorithms suffer from several shortcomings at different levels. Anticipating on new developments and insights, we have explored, in this thesis, alternative methodologies aiming at improvements at various steps, including sampling for primary testing, secondary (i.e. triage) testing, and diagnostic (colposcopy and pathology) work-up steps.

HrHPV DNA testing will be implemented as primary screening tool in the Netherlands. Furthermore, hrHPV self-sampling will be offered to non-responders. Liquid-based self-samples are not ideal to sending by mail and their application might be more challenging. Therefore, we evaluated in **Chapter 2** the clinical sensitivity and sensitivity for CIN2+ of HPV testing using a solid-state sample carrier, the FTA cartridge, for dry transport of self-collected brush samples. In **Chapter 3** we subsequently investigated acceptability of a novel self-sampling device, the Evalyn Brush, and its suitability as a dry transport system compared to concurrently physician-obtained samples for the detection of hrHPV.

Clinical management of women with abnormal cytology relies on colposcopy. During colposcopy a biopsy of the area on the cervix with visual characteristics that most likely represents the worst lesion, is taken. HPV16 has been previously suggested to cause more definite visual abnormalities than other hrHPV types. In **Chapter 4** we studied the visual appearance of the cervix using colposcopic characteristics combined with hrHPV genotyping to predict CIN2+. Previous studies have shown that the sensitivity of colposcopy to detect CIN2+ is 50%-70%. In **Chapter 5** the benefit of collecting a second lesion-directed biopsy and an additional biopsy of visual normal appearing tissue (non-directed biopsy) to improve cervical precancer detection, was investigated.

Clinical management is further determined by CIN-grading, but reproducibility of CIN-grading is low. In **Chapter 6** we investigated whether combining biomarkers of productive HPV infection and transformation (E4, MCM and $p16^{INK4a}$) provided a more reliable categorisation of CIN than histopathology.

Chapter 7 dealt with a thorough analysis of candidate methylation triage biomarkers MAL and CADM1 in relation to heterogeneity of CIN lesions in women who presented with multiple lesions of different histological grade on their cervix. Here, in order to find out to what extent methylation analysis of cervical scrapes are representative for underlying lesions, results at lesion level from different biopsies were compared with that obtained from the cervical scrape.

In **Chapter 8** we studied the presence of hrHPV and the expression of $p16^{INK4a}$, CK7 and CK17 in different types of cervical epithelium and we describe the potential importance of hrHPV infected atypical immature metaplastic cells as precursor for cervical carcinomas.

The findings of this thesis are discussed in the general discussion (**Chapter 9**) and summarized in **Chapter 10**.

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chapter 2

Clinical evaluation of high-risk HPV detection on self-samples using the Indicating FTA-elute solid-carrier cartridge

Romy van Baars*, Daan T. Geraets*, Inmaculada Alonso, Jaume Ordi, Aureli Torné, Willem J.G. Melchers, Chris J.L.M. Meijer, Wim G.V. Quint

*Both authors contributed equally

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ABSTRACT

Background: High-risk human papillomavirus (hrHPV) testing in cervical screening is usually performed on physician-taken cervical smears in liquid-based medium. However, solid-state specimen carriers allow easy, non-hazardous storage and transportation and might be suitable for self-collection by non-responders in screening and in low-resource settings.

Objectives: We evaluated the adequacy of self-collected cervicovaginal (c/v) samples using a Viba-brush stored on an Indicating FTA-elute cartridge (FTA-based self-sampling) for hrHPV testing in women referred to a gynecology clinic due to an abnormal smear.

Study design: 182 women accepted to self-collect a c/v sample. After self-sampling, a physician obtained a conventional liquid-based cervical smear. Finally, women were examined by colposcopy and a biopsy was taken when clinically indicated. Self-samples required only simple DNA elution, and DNA was extracted from physician-obtained samples. Both samples were tested for 14 hrHPVs by GP5+/6+-EIA-LQ Test and SPF_{10}-DEIA-$LiPA_{25}$.

Results: Both assays detected significantly more hrHPV in physician-collected specimens than in self-collected samples (75.3% and 67.6% by SPF_{10}; 63.3% and 53.3% by GP5+/6+, respectively). The combination of physician-collected specimen and GP5+/6+ testing demonstrated the optimal balance in sensitivity (98.0%) and specificity (48.1%) for CIN2+ detection in this referral population. A test system of FTA-based self-collection and SPF_{10} hrHPV detection approached this sensitivity (95.9%) and specificity (42.9%).

Conclusions: These results show that the clinical performance of hrHPV detection is determined by both the sample collection system and the test method. FTA-based self-collection with SPF_{10} testing might be valuable when a liquid-based medium cannot be used, but requires further investigation in screening populations.

BACKGROUND

The incidence of cervical cancer has been reduced by implementation of national cytology-based screening programs. The incorporation of high-risk human papillomavirus (hrHPV) testing in primary screening is expected to further improve cervical cancer screening. HrHPV testing has a higher sensitivity than cytology for detecting high-grade cervical intraepithelial neoplasia (CIN) and cervical cancer.[1, 2]

In current practice, hrHPV testing is generally performed on cervical smears stored in liquid-based medium. However, solid-carrier collection systems could be suitable alternatives, allowing storage and easy transportation at room temperature. Furthermore, solid-carriers are nonflammable, non-hazardous and can therefore be posted by regular mail.

Solid-state sample carriers might be of use for women who do not attend cervical cancer screening programs, but might respond to the option of self-sampling. Thus, the effectiveness of cervical screening programs could be enhanced by increasing the participation rate.[3] Several studies showed 30% of the non-responders do respond to a screening invitation if offered the option of self-sampling for hrHPV testing.[3-6]

Additionally, solid specimen carriers could also be applied to HPV testing in low-resource settings. When access to refrigeration is limited or absent, solid carriers might offer a convenient alternative for liquid-based storage of samples.

A novel solid-state carrier, the Indicating FTA-elute cartridge (FTA) combined with the Viba-brush might constitute a suitable self-collection and storage system of c/v specimens prior to hrHPV testing (FTA-based self-sampling).[7-9] In the previous FTA-based self-sampling studies, no relation was made with the histological outcome.

OBJECTIVES

The clinical sensitivity and specificity of a hrHPV test is defined by all the different steps in the diagnostic chain: sample collection system (sampling, storage, and processing) and test method (amplification and read-out). The aim of this study was to evaluate the suitability of this novel FTA-based self-collection method for hrHPV testing in terms of sensitivity and specificity for CIN2+. HrHPV positivity by the FTA-based self-collection method was compared with physician-collected cervical samples stored in liquid-based PreservCyt medium. Both specimens were tested by the GP5+/6+-EIA followed by the LQ Test, and by the SPF_{10}-DEIA-$LiPA_{25}$ version 1. For each combination of sample collection and test method, we determined the clinical sensitivity and specificity for high-grade CIN.

STUDY DESIGN

Clinical specimen collection

Between January 2010 and August 2010 clinical specimens, i.e. physician-collected (PC) cervical smears and c/v self-collected (SC) samples, were collected from 182 women visiting the gynecological outpatient clinic of the Hospital Clinic in Barcelona, Spain. All women had been referred because of an abnormal Pap smear (ASC-US+) detected at local health centers on average 3 months prior to the study visit (range: 1.5–6 months). The median age of the participants was 34 years (range 16–76 years).

Prior to colposcopic examination, women were asked to self-collect c/v material. Women received a self-sampling kit and verbal instructions from the physician. The sample was taken by the Rovers Viba-Brush (Rovers Medical Devices, Oss, The Netherlands), and subsequently applied to an Indicating FTA-elute cartridge (GE Healthcare, Buckinghamshire, United Kingdom) and air-dried. The FTA cartridge contains an indicating dye that changes from purple to white when material of the c/v swab sample was applied to the cartridge. The FTA matrix is chemically treated with proprietary reagents to lyse cells upon application and become non-infectious, allowing safe and easy transport. FTA cartridges were stored for 2–15 months (median: 4 months) and transported at room temperature. Following self-sampling, a trained gynecologist obtained a cervical scrape using the Rovers Cervex-Brush (Rovers Medical Devices). The brush was collected in 20 ml PreservCyt medium (Cytyc Corp., Boxborough, MA, USA) and cytologically examined. All samples were tested for hrHPV.

Finally, colposcopy was performed and biopsies were taken if the colposcopic impression was abnormal. The biopsy specimens were fixed in 10% formalin and paraffin-embedded. H&E sections were examined by a gynecological pathologist and classified as normal, CIN1, CIN2, CIN3 or cervical cancer. The local ethical committee approved the study. Informed consent was obtained from all participating women.

Processing of specimens

DNA was isolated from 250 µl PC specimen in PreservCyt medium using the QIAamp MinElute Virus Spin kit (Qiagen, Hilden, Germany). As recommended by the manufacturer, DNA was eluted in 100 µl buffer AVE from the kit.

DNA from the FTA cartridges was eluted as described previously with minor modifications.[9] Four 3 mm punches were taken from each FTA cartridge using a sterilized perforator (Miltex GmbH, Rietheim-Wielheim, Germany) and transferred into a microcentrifuge tube. The punches were washed in sterile water and vortexed three times for 15 s. The water was removed by a sterile fine-tip pipette. DNA elution was performed with 70 µl distilled water at 95 °C for 30 min. Next, the sample was removed and pulse-vortexed six times for 5 s. Finally, the specimen was centrifuged for 30 s and the eluted DNA was transferred to a new Eppendorf tube.

HrHPV detection and genotyping

All samples were tested with two HPV assays at DDL Diagnostic Laboratory, Rijswijk, The Netherlands: the HPV SPF_{10} PCR-DEIA-$LiPA_{25}$ version 1 algorithm (Labo Bio-medical Products BV, Rijswijk, The Netherlands) was carried out as described previously.[10, 11] The second assay was the standardized and clinically validated GP5+/6+-EIA kit (Diassay BV, Rijswijk, The Netherlands); this test was performed according to the kit insert. HrHPV-positive samples by the GP5+/6+-EIA were subsequently genotyped by the digene HPV Genotyping LQ Test (LQ Test; Qiagen) according to the manufacturer's instructions.[12]

Statistical analysis

In this study, comparisons for hrHPV detection and genotyping in both systems were limited to the 14 common hrHPV types, i.e. HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

The two-tailed McNemar's test was used for mutual comparison of positivity rates. The level of agreement was determined using Cohen's kappa statistics.

The clinical sensitivity and specificity for detection of biopsy-proven CIN2+ (CIN2, 3, invasive cervical cancer) of each combination of sampling procedure and HPV test were computed. The clinical sensitivity was compared using a non-inferiority score test in accordance with previously defined guidelines. We applied a sensitivity threshold for CIN2+ of ≥90% relative to that of GP5+/6+ performed on physician-collected specimens.[13, 14] Confidence intervals were calculated, and the level of statistical significance was set at 0.05. All analyses were performed using SPSS version 15.0.

RESULTS

Cytology results and histological diagnoses

The cytological results on physician-collected (PC) specimens obtained during the study visit in the 182 women enrolled were squamous cell carcinoma (n=2), HSIL (n=59), ASC-H (n= 4), LSIL (n=54), ASC-US (n=9), and normal (n=54). Colposcopically directed biopsies were taken from 166 women for histological analysis. Histological diagnoses were squamous cell carcinoma (n=2), CIN3 (n=27), CIN2 (n=20), CIN1 (n=36) and normal (n=81).

Overall hrHPV detection in PC samples versus SC samples

In the total population, 137/182 (75.3%) PC specimens tested positive for hrHPV by SPF_{10}. HrHPV-positivity by GP5+/6+ was lower (117/182; 64.3% ($p<0.001$)). Significantly more PC specimens (137/182; 75.3%) than SC specimens (123/182; 67.6%) were hrHPV-positive by SPF_{10} ($p=0.003$). The hrHPV test results in SC and corresponding PC samples demonstrated a substantial agreement of 89.0%, resulting in a kappa of 0.733 (95% CI: 0.625–0.841).

GP5+/6+ also detected significantly more hrHPV in PC specimens (117/182; 64.3%) compared to SC samples (97/182; 53.3% (p<0.001)). The agreement in GP5+/6+-positivity between the two collection methods was substantial as well (82.4%; k=0.642, 95% CI: 0.532–0.751).

HrHPV detection in relation to high-grade CIN

High-grade CIN (CIN2+) was detected in 49 (26.9%) patients. SPF_{10} performed on PC specimens demonstrated a sensitivity of 100% (95% CI: 90.9–100%) and a specificity of 33.8% (26.0–42.6%) for CIN2+ (Table 1) in this referral population. The same assay on FTA-based SC samples showed a lower sensitivity of 95.9% (84.9–99.3%) but a higher specificity of 42.9% (34.4–51.7%). SPF_{10} was negative in the SC specimen of two women diagnosed with CIN3.

The sensitivity of GP5+/6+ for detection of CIN2+ on PC and FTA-based SC samples was 98.0% (87.8–99.9%) and 87.8% (74.5–94.9%), with a specificity of 48.1% (39.4–56.9%) and 60.2% (51.3–68.4%), respectively. GP5+/6+ was negative in the PC specimen of one woman with CIN3 and in the SC specimen of three women with CIN2 and three with CIN3. According to the non-inferiority score test parameters described before,[13, 14] the clinical sensitivity for CIN2+ by GP5+/6+ testing on this series of FTA-based SC samples was inferior to GP5+/6+ performed on PC samples (p=0.538). The clinical sensitivity for detecting CIN2+ of SC samples was also inferior to the PC samples (p=0.084) by the more sensitive SPF_{10} test.

Table 1 also shows the clinical sensitivity and specificity for the detection of CIN3+ (n=29) of the four combinations of two collection methods and two hrHPV tests. No major differences were observed between the detection of CIN3+ and CIN2+.

The clinical sensitivity for CIN2+ of PC samples tested with SPF_{10} was non-inferior to PC samples tested with GP5+/6+ (p=0.006), but the clinical specificity was inferior (p=0.999). Of the four evaluated combinations, GP5+/6+ on PC specimens demonstrated the optimal balance in clinical sensitivity and specificity. That clinical performance was closely approached by the test system comprising self-collection and hrHPV detection by SPF_{10}.

The non-inferiority score test could not decisively determine if the clinical sensitivity for CIN2+ of SPF_{10} performed on SC samples was inferior to that of GP5+/6+ carried out on PC samples (p=0.051). The other two combinations of collection and test method did not perform as well as GP5+/6+ on PC specimens in detecting CIN2+. The combination of SC samples and GP5+/6+ testing lacked sensitivity, while SPF_{10} analysis of PC samples lacked specificity.

HrHPV genotypes identified in PC and SC samples

All HPV-positive specimens by SPF_{10}-DEIA and GP5+/6+-EIA were genotyped by $LiPA_{25}$ and the digene HPV Genotyping LQ Test (LQ Test), respectively. The five most prevalent genotypes identified in PC and SC samples according to LQ Test were HPV16, 31, 18, 51 and 56 (Table 2). In total, LQ Test found a significantly higher prevalence of these genotypes in PC samples (n=103) compared to SC specimens (n=83; p=0.001). Similarly, $LiPA_{25}$ detected these types significantly more often in PC specimens (n=124) than in SC samples (n=110; p=0.034).

Table 1. Sensitivity and specificity for CIN2+ and CIN3+ of PC samples in PreservCyt and FTA-based SC samples tested by GP5+/6+ and SPF_{10}

			CIN2+						CIN3+					
			Sensitivity			Specificity			Sensitivity			Specificity		
Sample collection by	Storage medium	Test	*n*	%	95% CI	*n*	%	95% CI	*n*	%	95% CI	*n*	%	95% CI
Woman	FTA	GP5+/6+	43/49	87.8%	(74.5%-94.9%)	80/133	60.2%	(51.3%-68.4%)	26/29	89.7%	(71.5%-97.3%)	83/153	54.2%	(46.0%-62.3%)
		SPF_{10}	47/49	95.9%	(84.9%-99.3%)	57/133	42.9%	(34.4%-51.7%)	27/29	93.1%	(75.8%-98.8%)	57/153	37.3%	(29.7%-45.5%)
Physician	PreservCyt	GP5+/6+	48/49	98.0%	(87.8%-99.9%)	64/133	48.1%	(39.4%-56.9%)	28/29	96.6%	(80.4%-99.8%)	64/153	41.8%	(34.0%-50.1%)
		SPF_{10}	49/49	100%	(90.9%-100%)	45/133	33.8%	(26.0%-42.6%)	29/29	100%	(85.4%-100%)	45/153	29.4%	(22.5%-37.4%)

Table 2. The most prevalent HPV types identified in PC and SC samples by genotyping of GP5+/6+ are shown on the left. The prevalence of the same genotypes according to SPF_{10} is demonstrated on the right.

Genotype	Detected by GP5+/6+ in[a]:				Detected by SPF_{10} in[a]:			
	PC and SC sample	*PC sample only*	*SC sample only*	*none of both samples*	*PC and SC sample*	*PC sample only*	*SC sample only*	*none of both samples*
HPV16	43	13	2	124	49	11	2	120
HPV31	11	4	1	166	17	4	3	158
HPV18	6	5	1	170	8	4	3	167
HPV51	8	2	2	170	13	4	2	163
HPV56	7	4	2	169	11	3	2	166
Total	75	28	8	799	98	26	12	774

PC, physician-collected sample; SC, self-collected sample

[a] Frequencies indicated here include presence of HPV types in both single and multiple infections

DISCUSSION

Overall, hrHPV detection was significantly lower in FTA-based SC specimens compared to PC samples. A test system consisting of SC samples and SPF_{10} approached the performance of PC samples and GP5+/6+ in terms of sensitivity and specificity for CIN2+ lesions. The non-inferiority score test could not decisively determine if the clinical sensitivity of SPF_{10} performed on SC samples was inferior.

The higher hrHPV positivity by SPF_{10} is caused by infections characterized by a low viral load that are not associated with CIN2+.[13] However, the sensitivity and specificity for CIN2+ of SPF_{10} in combination with a less sensitive FTA-based self-collection method approached that of GP5+/6+ on physician-collected swabs. This underlines that clinical performance of a hrHPV test is defined by all different steps of the diagnostic chain, e.g. the sample collection system (sampling, storage, and processing) as well as test method (amplification and read-out). Also the clinical performance will be affected by the population studied and the prevalence of hrHPV and of high-grade CIN. This specific combination of FTA-based self-collection and hrHPV testing by SPF_{10} should also be investigated for its diagnostic performance, effectiveness and efficiency in larger screening populations representing the women for whom it might be of most use: non-responders to screening and screening in low-resource settings.

The difference in hrHPV detection observed between SC samples and PC samples was also observed on the genotype level by the LQ Test and $LiPA_{25}$. The overall number of genotypes detected in SC samples was significantly lower than in PC samples. Previous studies found that c/v self-samples are representative for hrHPV types that infect the cervix.[15-18] Therefore, the lower number of HPV genotypes detected in self-collected c/v specimens does not seem to be related to the anatomical site from which material was collected, but might be a consequence of the collection device that was used in our study.

Our findings differ from those of three previous studies [8, 9, 19] that have evaluated FTA-based self-sampling. However, in these studies, the collection method (by patient or physician) or storage medium (solid FTA or liquid-based) to which self-sampling was compared differed. Lenselink et al., Gustavsson et al. and de Bie et al. showed that the Viba-brush applied to the FTA cartridge represents a convenient transport carrier, but their study groups consisted of a lower number of patients and a low amount of HPV positive samples (n=28, 34 and 32, respectively).[8, 9, 19] In the study of Gustavsson et al. the physician-collected sample and the self-collected specimen was applied to the FTA cartridge and thus compared the collection methods in the same storage medium. De Bie et al. compared the same collection method (e.g. self-collection) in different storage media (e.g. FTA cartridge and PreservCyt). In this study for the first time two variables, collection method and collection medium were introduced. The lower detection of hrHPV in FTA-based SC samples in our study could be due to insufficient collection of material by the patient, improper transfer of material from the brush

onto the cartridge, or a limited DNA binding capacity. This requires further investigation by e.g. DNA quantification by qPCR.

This study had several limitations. The small size of the study population may account for the overlapping confidence intervals of the clinical sensitivity and specificity of the different collection devices and tests. Another limitation is that the self-collection method was evaluated on a referral population of women with an abnormal smear. The clinical specificity determined in this referral population is not necessarily representative for women attending a cervical screening cohort. Thirdly, the self-sample was always collected before the physician-taken sample, creating a bias in favor of the SC samples. Self-collection by FTA might even be less sensitive.

The role of self-collection of cervical samples is to improve screening coverage. Self-sampling might be especially useful in primary screening of women otherwise not screened, e.g. non-responders or for women in low-resource countries without a screening program.[20, 21] Different self-sampling devices have been studied and shown to be as suitable for hrHPV detection as physician-sampling.[20, 22, 23] Brush-based self-sampling is highly accepted for HPV detection.[18, 24-26] Because of the high acceptability and the high agreement in hrHPV detection with physician-taken smears, the implementation of self-sampling in primary screening may potentially reduce cervical cancer incidence.[27, 28]

In low-resource settings with limited access to refrigeration or where samples need to be sent by post, the FTA cartridge might provide a convenient alternative for liquid-based storage. Cervical self-collection for hrHPV testing using FTA as a solid sample carrier seems suitable in combination with the SPF_{10} assay.

In conclusion, in this study the hrHPV detection on FTA-based self-samples was lower compared to conventional liquid-based cervical specimens collected by a physician. However, self-collection by FTA-based self-sampling combined with SPF_{10} hrHPV testing showed a clinical performance close to that of GP5+/6+ on physician-taken samples in this cohort of women referred because of an abnormal smear. This combined collection and test algorithm might therefore be valuable when a liquid-based medium cannot be used, for example in screening of non-responders and in low-resource settings. Self-sampling by brush, transferred to the FTA cartridge requires further investigation specifically in these settings.

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chapter 3

Dry storage and transport of a cervicovaginal self-sample by use of the Evalyn brush, providing reliable human papillomavirus detection combined with comfort for women

Romy van Baars, Remko P. Bosgraaf, Bram W.A. ter Harmsel, Willem J.G. Melchers, Wim G.V. Quint, Ruud L.M. Bekkers

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ABSTRACT

Primary screening using high-risk human papillomavirus (hrHPV) detection has been suggested as a way of improving cervical cancer prevention. Women currently not attending screening (nonresponders) are more likely to participate when given the opportunity of self-sampling for hrHPV testing. The Evalyn Brush is a new cervicovaginal self-sampling device, developed specifically to meet women's demands, which is user-friendly and easy to use. The aims of this study were to investigate agreement of hrHPV detection by two PCR methods between the Evalyn Brush and physician-obtained samples and to study women's acceptance of this self-sampling device. Each of 134 women visiting the gynecology outpatient clinic collected a self-obtained sample (self-sample) and completed a questionnaire. The brush was stored dry. After self-sampling, a trained physician obtained a conventional cervical cytology specimen in ThinPrep medium. HrHPV detection was performed using the SPF_{10}-DEIA-$LiPA_{25}$ and GP5+/6+-LQ-test. The overall agreement for hrHPV detection using SPF_{10}-DEIA-$LiPA_{25}$ between the self-sample and the physician-taken sample was 85.8% (kappa value, 0.715; 95% confidence interval [CI]: 0.597 to 0.843; p=1.000). The overall agreement for hrHPV detection using GP5+/6+-LQ between the self-sample and the physician-taken sample was 86.6% (kappa value, 0.725; 95% CI: 0.607 to 0.843; p=0.815). Ninety-eight percent of the women rated their experience as good to excellent. Moreover, 95% of women preferred self-sampling to physician sampling. Self-sampling using the dry Evalyn Brush system is as good as a physician-taken sample for hrHPV detection and is highly acceptable to women. To validate this self-sampling device for clinical use, a large screening cohort should be studied.

BACKGROUND

Cervical cytology screening programs have significantly decreased the incidence and mortality of cervical cancer. Primary screening using high-risk human papillomavirus (hrHPV) detection has been found to be more sensitive than conventional cervical cytology for detecting cervical precancer. [1-4] All data argue for the implementation of hrHPV testing as a primary test in cervical cancer screening, and the Health Council in the Netherlands has advised the Minister of Health to implement primary screening with hrHPV detection as a way of improving cervical cancer prevention.[5]

Cervical cancer incidence is higher among women who do not respond (nonresponders) or have no access to cervical screening programs than in screened women. A substantial number of nonresponders participate in screening when given the opportunity of self-sampling for hrHPV testing.[6, 7] Self-sampling for hrHPV therefore has the potential to reduce cervical cancer incidence, especially among nonresponders.[8]

Cervicovaginal self-collected samples (self-samples) have proved to be as reliable as physician-obtained cervical samples for the detection of hrHPV.[9-16] Studies on HPV self-sampling have used a great variety of collection devices, such as tampons, swabs, cervicovaginal brushes, and cervicovaginal lavage. Women are more familiar and comfortable with tampons than with other self-sampling methods, and the use of tampons is an attractive self-sampling method for women.[10, 17, 18] However, tampons need more extensive processing than swabs and brushes for performance of HPV analysis.[19] Furthermore, studies that used a brush or lavage[9, 20-22] for self-collection have demonstrated a higher sensitivity for cervical intraepithelial neoplasia grade two or worse (CIN2+) than studies that used a Dacron or cotton swab.[23-26]

Although cervicovaginal lavage is the most studied self-sampling technique,[6, 9, 11, 27-29] the main disadvantage is that liquid specimens are not convenient to send by mail. This might be an obstacle in national screening programs.[30] Brushes, on the other hand, may be used for dry transport and storage.[31] Richman et al.[32] showed that the majority of women who were offered the choice between the Qiagen cervical brush, the Fournier cervical self-sampling device, and the Pantarhei cervicovaginal lavage preferred the brush. Brushes are flexible and easy to use, can be processed in the same way as physician-obtained smears, and are suitable for sending by mail.[14, 15, 30] Although self-sampling for HPV testing is very acceptable to women, they are still concerned about performing the self-sampling procedure properly.[10, 11, 33-36]

To improve women's confidence and the convenience of performing self-sampling, a new cervicovaginal self-sampling device, the Evalyn Brush, was developed. This device is more understandable and user-friendly to women, as it indicates a standard depth of insertion and the number of rotations (Fig. 1). The depth of insertion is controlled by the wings. The brush needs to be rotated five times, and at each rotation, there is an audible click indicating the number of rotations. After self-sampling, the cap can be clicked onto the case and the

Figure 1. The Evalyn Brush
The Evalyn Brush® is about 20cm in length and consists of a transparent case with wings. Within the casing is a pink stick with a pink plunger at one end and a white brush at the other. You can push the white brush out of the case by pushing the pink plunger towards the transparent casing. After self-sampling, you can pull the brush back in and a cap can be clicked onto the case before transport.

brush can be sent by mail as is. The FTA cartridge, another previously reported dry storage system,[30, 37] has the disadvantage that the DNA from the brush can be only partly transferred to the cartridge.

We conducted the present study to investigate clinical applicability of the Evalyn Brush as a dry transport system compared to concurrently physician-obtained samples for the detection of hrHPV. We also investigated the acceptability of self-sampling using this device and women's preferences for self-sampling or physician sampling.

MATERIALS AND METHODS

Clinical specimen collection

Clinical specimens were collected between September 2010 and May 2011 from 134 women aged 18 years and above visiting the gynecological outpatient clinics of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, and of the Reinier de Graaf Hospital, Voorburg, The Netherlands, for colposcopic evaluation due to an abnormal Pap smear or for

a follow-up visit after an abnormal Pap smear. Women self-collected a cervicovaginal sample with the Evalyn Brush (Rovers Medical Devices B.V., Oss, The Netherlands) after they had received verbal and written instructions with illustrations and consented to the study. After the specimen was obtained, a cap was clicked onto the case, and it was stored dry in the original state. After self-sampling, a trained physician obtained a liquid-based cytology sample using a Rovers Cervex-Brush (Rovers Medical Devices B.V., Oss, The Netherlands). The Cervex-Brush was rinsed in ThinPrep medium (Hologic, Marlborough, MA) at Radboud University Nijmegen Medical Centre and in SurePath medium (Klinipath BV, Duiven, The Netherlands) at Reinier de Graaf Hospital. Cytological examination and classification were performed at the local laboratory according to the CISOE-A (composition, inflammation, squamous epithelium, other and endometrium, endocervical columnar epithelium, and adequacy of the smear) classification, which can easily be translated into the Bethesda 2001 classification.[38] All samples were stored and transported at room temperature to DDL Diagnostic Laboratory, Voorburg, The Netherlands, for molecular testing. All samples were assigned an anonymous, unique patient code.

Questionnaires

To investigate the acceptability of using the Evalyn Brush, all women were asked to fill out a short questionnaire using a 5-point ordinal scale to record their general experience, their response to the instructions, and their assessment of the convenience of using the Evalyn Brush. Participants were also asked whether they preferred self-sampling or physician sampling.

Specimen preparation

The dry Evalyn Brush was resuspended in 1 ml of ThinPrep. The vials were vortexed for 3x15 s, stored overnight at 4°C, and again vortexed for 2x15 s. From each resuspended dry Evalyn brush specimen and from each cervical cytological specimen in liquid-based medium, 250 µl was used to obtain 100 µl of eluate with the QIAamp MinElute Virus Spin kit (Qiagen Inc., Valencia, CA) as described by the manufacturer. The mean interval between obtaining the specimen and HPV DNA isolation was 2 months, with a range of 2 weeks to 6 months. Each DNA isolation and PCR test run contained HPV-positive and -negative controls. All self-collected and physician-obtained samples were tested for HPV with both the analytically sensitive SPF_{10}-PCR system [39, 40] and the clinically validated GP5+/6+-PCR-based test.[41, 42]

HPV detection and genotyping

SPF_{10} PCR-DEIA-LiPA25 system

Broad-spectrum HPV DNA amplification was performed using a short-PCR-fragment assay (HPV SPF_{10}-$LiPA_{25}$, version 1; Labo Bio-medical Products B.V., Rijswijk, The Netherlands). This assay amplifies a 65-bp fragment of the L1 open reading frame of HPV genotypes, as described by Kleter et al.[39, 40] HPV detection of at least 54 anogenital HPV genotypes was performed

using a cocktail of 9 conservative probes in a microtiter hybridization assay, the DNA enzyme immunoassay (DEIA).[40, 43] The samples positive for HPV by DEIA were then analyzed with the line probe assay ($LiPA_{25}$) by reverse hybridization with type-specific probes for HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74.[39] The LiPA strips were visually inspected and interpreted following the standardized reference guide.

GP5+/6+-EIA-LQ HPV amplification and detection

The samples were also tested with the clinically validated hrHPV GP5+/6+ primer-mediated PCR assay (Diassay, Rijswijk, The Netherlands). With this, detection of DNA from 14 hrHPV genotypes, i.e., HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68, can be determined.[44] Briefly, 10 µl of DNA was amplified with the biotin-labeled GP5+/6+ primer set. The GP5+/6+ amplimers were subsequently genotyped by the digene HPV Genotyping LQ test using xMAP technology for high-throughput screening (Qiagen, Hilden, Germany) according to the manufacturer's instructions.[45]

For the comparison of the two collection systems, only the 14 hrHPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 were evaluated. Comparing the presence of hrHPV between the samples, results were classified as identical, concordant, or discordant. If all genotypes were the same in both samples, the results were called identical. If analyses showed at least one identical genotype in both samples, the results were called concordant. Genotype results were called discordant when the genotypes were different.

Statistical analysis

The level of agreement was determined using Cohen's kappa statistics. The two-tailed McNemar's test was used for mutual comparison of positivity rates. The level of statistical significance was set at 0.05. All analyses were performed using SPSS version 17.0 for Windows (Chicago, IL). Cytology and histology data were used to investigate clinically relevant differences in hrHPV detection.

This study was approved by the local medical ethical committees of both hospitals.

RESULTS

A self-collected sample and a subsequent conventional physician-taken cervical smear were obtained from 134 women (mean age, 40 years [standard deviation (SD), 9.5 years]; range, 21 to 66 years). For 44 of the 134 women, histology results were available. Of the 44 biopsy specimens, 8 contained normal tissue, 9 had a CIN1 lesion, 13 a CIN2 lesion, and 14 a CIN3 lesion. Cytology results were available for all women. If a histology diagnosis was available, this was used in the analyses of hrHPV detection in relation to cytohistological diagnosis. Five of the cytology results were not obtained during the same visit as that in which the sample for

HPV analysis was obtained. Of these five women, three had an earlier smear with borderline dyskaryosis and two had an earlier negative result. These earlier results were used as the diagnoses in the analyses of hrHPV detection for women without concurrent cytohistological diagnoses.

SPF_{10} PCR-DEIA-$LiPA_{25}$ system

Table 1 shows the SPF_{10} PCR-DEIA-$LiPA_{25}$ results in relation to the cytohistological diagnoses. The hrHPV positivity rate in physician-taken samples was 72/134 (54%) using the SPF_{10}-DEIA-$LiPA_{25}$ system. By comparison, 71 (53%) of the self-samples were hrHPV positive with SPF_{10}-PCR. Ten women were SPF_{10} positive in the physician-taken samples but negative in self-samples, and 9 women tested positive in self-samples only but negative on the physician-taken sample. Fifty-three women were hrHPV negative in both samples. These differences in hrHPV results were observed in all diagnostic categories. There was no difference in the percentage of HPV positivity and the number of discordant cases between the specimens that were tested after 2 weeks to 1 month and the specimens that were tested after 2 to 6 months (data not shown). There was good agreement for hrHPV detection using SPF_{10}-DEIA-$LiPA_{25}$ between the self-sample and the physician-taken sample (kappa value [κ]=0.715; 95% confidence interval [CI], 0.597 to 0.843; p=1.000) with 85.8% concordance. Of the 62 samples that were SPF_{10} positive in the physician-taken sample and the self-sample, 41 (66%) showed identical hrHPV genotypes, 18 (29%) showed concordant hrHPV genotypes, and 3 (5%) showed discordant genotypes. In the concordant cases, in 7/18 (39%) cases the self-sample detected an additional hrHPV genotype and in 5/18 (28%) cases an additional hrHPV type was detected in the physician-taken sample. In the 6 other cases, one or two genotypes were replaced by one or two other genotypes in the other sample. In 3 discordant

Table 1. Agreement in hrHPV positivity (14 hr types) in self-sampled dry Evalyn Brush samples compared to physician-obtained samples with SPF_{10}-DEIA-$LiPA_{25}$ in relation to the diagnoses

Diagnoses	n	hrHPV positivity by SPF_{10} in:				kappa value (95% CI)	P-value
		Dry Brush and physician-obtained	Physician-obtained only	Dry Brush only	None of both systems		
Negative†	70	21	7	3	39	0.695 (0.522-0.868)	0.344
BMD*#	28	15	0	5	8	0.632 (0.360-0.904)	0.063
CIN 1	9	4	1	1	3	0.550 (0.001-1.000)	1.500
CIN 2	13	11	0	0	2	1.000 (1.000-1.000)	2.000
CIN 3	14	11	2	0	1	0.440 (0-1.000)	0.500
TOTAL	134	62	10	9	53	0.715 (0.597-0.834)	1.000

† 2 of these results were not obtained on the same moment as the sample for HPV analysis was obtained

*3 of these results were not obtained on the same moment as the sample for HPV analysis was obtained

1 of these samples was a vagina top smear

BMD borderline or mild dyskaryosiss

Table 2. Comparison for hrHPV genotyping by SPF_{10}-DEIA-$LiPA_{25}$ in physician-obtained and dry Evalyn Brush samples

Genotype	hrHPV positivity by SPF_{10}-$LiPA_{25}$ in:					
	Dry Brush and physician-obtained	Physician-obtained only	Dry Brush only	None of both systems	kappa value (95% CI)	P-value
HPV16	13	1	3	117	0.850 (0.706-0.994)	0.625
HPV18	8	2	0	124	0.881 (0.719-1.000)	0.500
HPV31	8	3	6	117	0.604 (0.369-0.839)	0.508
HPV33	5	1	0	128	0.905 (0.721-1.000)	1.000
HPV35	2	1	0	131	0.796 (0.407-1.000)	1.000
HPV39	2	4	2	126	0.378 (0-0.770)	0.687
HPV45	2	0	0	132	1.000 (1.000-1.000)	2.000
HPV51	7	0	4	123	0.763 (0.540-0.985)	0.125
HPV52	5	5	5	119	0.460 (0.177-0.743)	1.000
HPV56	4	3	3	124	0.548 (0.226-0.870)	1.000
HPV58	1	1	0	132	0.663 (0.044-1.000)	1.000
HPV59	4	4	0	126	0.653 (0.339-0.967)	0.125
HPV66	9	1	4	120	0.763 (0.563-0.962)	0.375
HPV68/73	1	2	2	129	0.318 (0-0.812)	1.000
HPV39/68/73	0	2	0	132	n.c	0.500
Any type	71	30	29	1880	0.691 (0.617-0.766)	1.000

n.c. this quantity cannot be calculated

BMD borderline or mild dyskaryosis

cases, the physician-taken samples showed HPV types 52, 56, 31, and 39/68/73 ($LiPA_{25}$ cannot distinguish between these types), whereas the self-samples showed HPV types 16, 31, and 16, respectively.

The 72 physician-taken samples and 71 self-samples that were SPF_{10}-DEIA positive were genotyped by $LiPA_{25}$. Only the 14 hrHPV types were considered. Table 2 shows that the overall agreement for hrHPV genotyping between physician-taken samples and self-samples was good (κ=0.691; 95% CI: 0.617 to 0.766; p=1.000). No statistically significant differences were found. From the 72 hrHPV-positive physician-taken samples, 25 (35%) contained a multiple infection with two or more hrHPV types, compared to 20/71 (28%) in the self-samples.

GP5+/6+-LQ

Table 3 shows the GP5+/6+-LQ test results in relation to the cytohistological diagnoses. With GP5+/6+-PCR, hrHPV was detected in 58 (43%) of 134 physician-taken samples. A similar number of self-samples tested hrHPV positive (56/134 [42%]; p=0.815). Ten samples were found GP5+/6+positive in physician-taken samples but negative in self-samples. Only two of these physician-taken samples were also SPF_{10} positive. Both were negative by SPF_{10} in self-samples. With GP5+/6+-PCR, hrHPV was detected in eight self-samples that were negative in the physician-taken sample. For 68 women both samples were hrHPV negative, and for 48

Table 3. Agreement in hrHPV positivity (14 hr types) in self-sampled dry Evalyn Brush samples compared to physician-obtained samples with GP5+/6+-LQ in relation to the diagnoses

Diagnoses	n	hrHPV positivity by GP5+/6+ in:					
		Dry Brush and physician-obtained	Physician-obtained only	Dry Brush only	None of both systems	kappa value (95% CI)	P-value
Negative†	70	13	4	4	49	0.689 (0.490-0.889)	1.273
BMD*#	28	12	1	3	12	0.716 (0.460-0.971)	0.625
CIN 1	9	4	2	0	3	0.571 (0.098-1.000)	0.500
CIN 2	13	9	1	1	2	0.567 (0.032-1.000)	1.500
CIN 3	14	10	2	0	2	0.588 (0.107-1.000)	0.500
TOTAL	134	48	10	8	68	0.725 (0.607-0.843)	0.815

† 2 of these results were not obtained on the same moment as the sample for HPV analysis was obtained
*3 of these results were not obtained on the same moment as the sample for HPV analysis was obtained
#1 of these samples was a vagina top smear
BMD borderline or mild dyskaryosis

women both samples were hrHPV positive. None of the diagnostic categories showed a significant difference in hrHPV detection. The concordance for hrHPV detection using GP5+/6+-LQ between self-samples and physician-taken samples was 86.6%, with good agreement (κ= 0.725; 95% CI: 0.607 to 0.843; p=0.815).

All GP5+/6+-positive samples were genotyped by LQ. Only the 14 hrHPV types were considered. The results are shown in Table 4. The 48 samples that were GP5+/6+-LQ positive in both the physician-taken sample and the self-sample did not show discordant genotypes, 37/48 samples (77%) had identical hrHPV genotypes, and 11/48 (23%) had concordant hrHPV genotypes. We found good agreement for hrHPV genotyping between physician-taken samples and self-samples (κ=0.768; 95% CI: 0.691 to 0.846; p=0.110). A multiple infection with two or more genotypes was found in 24% (14/58) of the physician-taken samples and 25% (14/56) of the self-samples.

Detection rate of CIN2+

CIN2+ was present in 27 women (20.1%). The sensitivities for the detection of CIN2+ in physician-obtained samples with the SPF_{10} and the GP5+/6+-PCR were 88.9% and 81.5%, respectively, and in the self-samples 81.5% and 74.1%, respectively (Table 5). The specificities for the detection of CIN2+ samples in physician-taken samples with the SPF_{10} and the GP5+/6+-PCR were 55.1% and 66.4%, respectively, and in the self-samples 54.2% and 66.4%, respectively. No significant difference in the sensitivity for the detection of CIN2+ could be found between the physician-taken samples and the self-samples with both detection methods (for SPF_{10}, p=0.500; and for GP5+/6+, p=0.625).

Table 4. Comparison for hrHPV genotyping by GP5+/6+-LQ in physician-obtained and dry Evalyn Brush samples

Genotype	hrHPV positivity by GP5+/6+-LQ in:					
	Dry Brush and physician-obtained	Physician-obtained only	Dry Brush only	None of both systems	kappa value (95% CI)	P-value
HPV16	11	4	3	116	0.729 (0.539-0.920)	1.000
HPV18	7	1	1	125	0.867 (0.686-1.000)	1.000
HPV31	6	3	2	123	0.686 (0.427-0.945)	1.000
HPV33	5	1	0	128	0.905 (0.721-1.000)	1.000
HPV35	2	0	0	132	1.000 (1.000-1.000)	2.000
HPV39	2	0	1	131	0.796 (0.407-1.000)	1.000
HPV45	2	0	0	132	1.000 (1.000-1.000)	1.000
HPV51	4	3	1	126	0.651 (0.334-0.969)	0.625
HPV52	1	1	1	131	0.492 (0-1.000)	1.000
HPV56	4	4	0	126	0.653 (0.339-0.967)	0.125
HPV58	2	1	0	131	0.796 (0.407-1.000)	1.000
HPV59	3	0	1	130	0.853 (0.570-1.000)	1.000
HPV66	8	2	1	123	0.830 (0.642-1.000)	1.000
HPV68	0	1	0	133	n.c.	1.000
Any type	56	21	11	1658	0.768 (0.691-0.846)	0.110

n.c. this quantity cannot be calculated

Table 5. Sensitivity and specificity for the two collection devices with the SPF_{10} and the GP5+/6+-system for the detection of CIN2+

	Physician-obtained				Dry Brush			
	SPF_{10}		GP5+/6+		SPF_{10}		GP5+/6+	
Sensitivity	24/27	88.90%	22/27	81.50%	22/27	81.50%	20/27	74.10%
Specificity	59/107	55.10%	71/107	66.40%	58/107	54.20%	71/107	66.40%

Questionnaires

Of the 134 questionnaires, 127 (95%) were returned for analysis. The results from the questionnaires are shown in Table 6. From this group, 124 (98%) women rated their experience with the brush as good to excellent. The instructions for using the Evalyn Brush were considered good to excellent by 124 (98%) of the 127 women, and 125 (98%) women rated the convenience of using this self-sampling device as good to excellent. Most women (n=120 [95%]) preferred self-sampling to physician sampling because it was simple, easy, and less painful than a physician-collected smear. Also women that never used tampons judged their experience with the brush as very good. Women also liked the option of self-sampling because it was time saving, as no visit to the clinician was needed. The most frequent reason (6/7 [86%]) for preferring the physician-taken smear was that the women considered it more reliable. Among the women who preferred self-sampling to physician sampling, 2/120 (2%) nevertheless considered the physician-taken sample more reliable and 3/120 (3%) questioned whether they had performed the test correctly. Women commented on the appearance of the Evalyn Brush and said that they liked the color.

Table 6. Questionnaire results

Question	Excellent		Very Good		Good		Moderate		Poor	
	n	%	n	%	n	%	n	%	n	%
Experience	43	34	39	31	42	33	3	2	0	0
Instructions	46	36	35	28	43	34	3	2	0	0
Convenience	45	35	45	35	35	28	1	1	1	1
Convenience compared to physician-taken smear	56	44	30	24	34	27	5	4	2	1

DISCUSSION

The dry self-samples showed good agreement with the physician-taken samples in hrHPV detection with both the analytically sensitive SPF_{10}-PCR and the clinically validated GP5+/6+-PCR. Our results indicate that self-sampling using the dry Evalyn Brush system is as good as a physician-taken smear for hrHPV detection. Our results are in line with previous studies showing repeatedly that self-collected cervicovaginal samples are as reliable as clinician-collected specimens for hrHPV detection.[7, 9, 11-14, 46-48]

Previous HPV self-sampling studies have used a variety of collection devices and HPV DNA tests. The concordance between the dry brush system and physician sampling in this study was 85.8% with SPF_{10} and 86.6% with GP5+/6+. This is comparable with the mean concordance calculated in the meta-analysis of Petignat et al. (87%)[13] and with the more recent review of Schmeink et al. (85.2%).[14] The kappa statistic showed good agreement between self-sampling and physician sampling for hrHPV in this study (κ=0.715 and κ=0.725). This agreement was higher than the mean κ obtained by Schmeink et al. (κ=0.60)[14] and by Petignat et al. (κ= 0.66).[13] In our study, the sensitivities for CIN2+ did not differ significantly between the self-samples and the physician-taken samples. Some previous publications reported that self-sampling has a lower sensitivity than clinician sampling for HPV detection,[11, 15, 21, 23, 26, 49-51] but these results have not been consistently found.[9, 10, 47] The difference in sensitivity between studies might be due to differences in collection devices (brush, swab, tampon, or lavage), populations (screening population or women with an abnormal Pap smear), and the HPV DNA tests used. Schmeink et al. concluded that PCR-based HPV testing shows better results than studies performed with HC2. From our results, it appears that the use of an analytically sensitive test, like the SPF_{10}, results in a lower specificity than that obtained with the less sensitive GP5+/6+. Further studies are needed to determine the most suitable test in different populations.

The Evalyn Brush is a well-accepted self-sampling method for HPV detection according to 98% of women who used this device because it is easy to use, time saving, and more comfortable than collection by a physician. This self-sampling device was specifically designed to improve women's confidence in, and the convenience of, self-sampling. Indeed, 95% of

women preferred self-sampling to physician sampling. The few women in our study who preferred clinician sampling specified their main reason as fear of inadequate self-sampling. This is in line with findings of previous studies.[18, 32, 34, 36, 48] Acceptability of the self-sampling device may be important for women who ignore the invitation to attend the national cervical cancer screening program or in settings without organized cervical screening programs.[52] Use of the Evalyn Brush may help increase the participation rate for cervical screening programs.

A limitation of this study is that it was performed in a hospital setting. Self-sampling is shown to be accepted well by women with a history of an abnormal Pap smear, but this study population is not representative of the broader population of women not participating in screening. Therefore, this study cannot be generalized to such a population. Another theoretical limitation is that the self-sample was always obtained before the physician-taken smear. This was done to avoid interference with HPV detection by the lubricating gel used on the speculum. The order of sampling could influence the amount of HPV DNA sampled, but Harper et al.[19] showed in a randomized controlled trial that the order of sampling did not influence the result. Third, the number of patients included in this study is small. The response rate and performance of the Evalyn Brush are currently being investigated in nonresponders to the Netherlands national screening program.

In conclusion, although the number of women included in this study was limited, the dry-stored Evalyn Brush showed good agreement for hrHPV detection with the physician-taken smears and is a well-accepted self-sampling device. Clinical validation and evaluation of the acceptability of this self-sampling device in screening populations should be the next step.

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chapter 4

The impact of Human Papillomavirus genotype on colposcopic appearance: a cross-sectional analysis

Romy van Baars*, Jacolien van der Marel*, Wim G.V. Quint, Johannes Berkhof, Marta del Pino, Aureli Torné, Jaume Ordi, Nicolas Wentzensen, Mark Schiffman, Miekel M. van de Sandt, Jan Lindeman, David Jenkins, Theo J.M. Helmerhorst, René H.M. Verheijen, Bram W.A. ter Harmsel, Inmaculada Alonso.

*Both authors contributed equally

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ABSTRACT

Objective: To study colposcopic performance in diagnosing high-grade cervical intraepithelial neoplasia or cervical cancer (CIN2+ and CIN3+) using colposcopic characteristics and high-risk human papillomavirus (hrHPV) genotyping.

Design: Cross-sectional multicentre study.

Setting: Two colposcopy clinics in the Netherlands and Spain.

Population: Six hundred and ten women aged 17 years and older referred for colposcopy because of abnormal cytology.

Methods: A cervical smear was obtained. Colposcopists identified the worst lesion, graded their impression and scored the colposcopic characteristics of the lesions. Up to four biopsies were collected, including one biopsy from visually normal tissue.

Main outcome measures: CIN2+ and CIN3+, positive for HPV16 or other high-risk HPV types (non-16 hrHPV-positive).

Results: The mean age in HPV16-positive CIN2+ women was 35.1 years compared with 39.1 years in women with other hrHPV types (p=0.002). Sensitivity for colposcopy to detect CIN2+ was 87.9% (95% CI: 83.2–91.5), using colposcopic cut-off of 'any abnormality'. The remaining CIN2+ were found by a biopsy from visually normal tissue or endocervical curettage (ECC). Detection of CIN2+ by lesion-targeted biopsies was not different between HPV16-positive women [119/135; 88.1% (95% CI: 81.2–92.9)] and non-16 hrHPV-positive women [100/115; 87.0% (95% CI: 79.1–92.3); p=0.776]. In multivariate analysis, 'acetowhitening' [odds ratio (OR) 1.91, 95% CI: 1.56–3.17], 'time of appearance' (OR 1.95, 95% CI: 1.21–3.15) and 'lesion >25% of visible cervix' (OR 2.25, 95% CI: 1.44–3.51) were associated with CIN2+.

Conclusions: In this population following European screening practice, HPV16-related CIN2+ lesions were detected at younger age and showed similar colposcopic impression as non-16 hrHPV high-grade lesions. There was no relationship between any of the colposcopic characteristics and HPV16 status.

INTRODUCTION

Almost all invasive cervical cancer (ICC) cases are caused by persistent infections with high-risk human papillomavirus (hrHPV) through premalignant stages of cervical intraepithelial neoplasia (CIN). [1, 2] Cervical cytology screening programmes have improved the detection of precancerous lesions and, as a result, cervical cancer incidence has decreased in developed countries. [3] Women with abnormal cytology are offered colposcopy, which is the standard for identifying CIN2+ (CIN2, CIN3 and ICC) in most countries. Current biopsy procedures rely on visual features to identify the area on the cervix that most likely represents the worst lesion. [4-8] However, colposcopy has a sensitivity of 50–70% to detect CIN2+. [9-13] Gains in sensitivity can be obtained by increasing the number of biopsies. [14] Moreover, in a study performing random biopsies of regions without abnormalities, 23–37% of the overall CIN2+ lesions were detected in this biopsy only. [15]

The risk of developing ICC within the group of hrHPV genotypes varies substantially. HPV16 is found to be the genotype with the highest carcinogenic potential; it is associated with the highest risk of the development of CIN3 and ICC. [16-19] Also, HPV16-related CIN2+ has been found at young age. [20, 21] HPV genotype 16 is suggested to cause more definite visual abnormalities than other HPV types. [4, 22] Women with CIN2+ frequently present with multiple HPV genotypes in cytology. [23] In a study using laser capture microdissection (LCM) in women positive for multiple HPV types in cytology we have shown that HPV16 is the most predominant causal genotype in CIN2+ lesions. [24] This suggests that etiologically, HPV16 has an even more important role than previously thought. Although a growing interest exists for clinical use of hrHPV testing to triage women who have minor cytological changes and in primary screening, HPV genotyping is currently not implemented as standard clinical practice in most countries. [25]

The objective of this study was to evaluate the visual appearance of the cervix using colposcopic characteristics combined with HPV genotyping to predict CIN2+ in women who were referred for colposcopy.

METHODS

Study design and population

The EVAH-study (Evaluating the Visual Appearance of cervical lesions in relation its histological diagnosis, Human papillomavirus genotype and other viral parameters) is a multicentre study conducted between August 2010 and October 2012. In total, 610 women aged 17 years and older who had an abnormal Pap smear result defined as atypical cells of undetermined significance (ASC-US) or worse (disregarding HPV status), who were referred for colposcopic evaluation to Reinier de Graaf Groep, Voorburg, The Netherlands or to Hospital Clínic, Barcelona, Spain, were included.

Patients were excluded if they had had previous treatment of cervical pathology, had a confirmed diagnosis of ICC at the time of referral, had insufficient knowledge of the Dutch or Spanish language or were pregnant or breast-feeding at the date of colposcopy or 3 months before. This study has received approval from the medical ethical board at both hospitals. Informed consent was obtained from all participating women.

Cytology, colposcopy and specimen collection

Before colposcopy, a liquid-based cytology sample using a Cervex-Brush® (Rovers Medical Devices BV, Oss, The Netherlands) was obtained. The Cervex-Brush was rinsed in ThinPrep® medium (Hologic, Marlborough, MA, USA) at Hospital Clínic, and in SurePath™ medium (Klinipath BV, Duiven, The Netherlands) at Reinier de Graaf Groep. Cytological examination and classification were performed at the local laboratory according to the Bethesda 2001 classification in Spain (negative, ASC-US, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), atypical glandular cells of undetermined significance (AGUS), atypical squamous cells, cannot exclude HSIL (ASC-H), adenocarcinoma in situ (AIS) or ICC). In The Netherlands grading was done according to the CISOE-A (composition, inflammation, squamous epithelium, other and endocervical columnar epithelium, and adequacy of the smear), which was translated to the Bethesda 2001 classification as described earlier. [26]

Nine colposcopists were involved in this study, seven expert and two junior colposcopists with supervision from an expert colposcopist. Five percent acetic acid was used to elicit the acetowhite epithelial response. Grading of visual impression was done as normal (including squamous metaplasia), CIN1, CIN2, CIN3 (including AIS) or ICC and the impression was coded in the final data analysis as normal (N), low-grade (LG; CIN1) or high-grade (HG; CIN2+, including ICC). Colposcopists were asked to score the lesion margins (geographical, smooth, internal borders), colour of the lesions (absent, translucent, intermediate white, opaque white), punctation (absent, fine, coarse), mosaicism (absent, fine, coarse), the presence of atypical vessels, time of appearance (slow, fast) and size of the lesions (0, <25, 25–50, >50% of the cervix). This scoring system was based on the Reid colposcopy index designed by Reid

and Scazi. [27] In this study, multiple biopsies during colposcopy were collected. Up to four biopsies were taken from different lesions, or distinct areas within a large complex lesion were biopsied separately. If fewer than four directed biopsies were taken, a biopsy from visually normal tissue was added. Using this refined biopsy protocol, we aimed to detect more underlying CIN2+ than with the current approach, in which usually only the most severe lesion is identified and biopsied. Endocervical curettage (ECC) was performed in cases where the squamocolumnar junction (SCJ) was not completely visualized. In accordance with the national guidelines in the Netherlands and Spain, histologically confirmed high-grade lesions diagnosed as CIN2+ were treated.

Histological processing

Individual biopsy specimens, including ECC samples when taken, were processed separately, fixed in 10% neutral buffered formalin and paraffin-embedded. Haematoxylin and eosin (H&E) sections were examined by a local pathologist and classified as negative (including squamous metaplasia), CIN1, CIN2, CIN3 (including AIS) or ICC. All biopsies were independently reviewed by a second gynaecological pathologist. In case of disagreement between the original and review diagnosis, a third pathologist reviewed the discordant cases independently. Consensus diagnosis was determined by the agreement of two of three interpretations. All pathologists were blinded for HPV status. The final histological diagnosis per case was based on the worst diagnosis found in all specimens of each woman.

HrHPV genotyping

DNA extraction was performed using 250 µl of the cytology specimen to obtain 100 µl of eluate with the QIAamp MinElute Virus Spin kit (QIAgen Inc., Valencia, CA, USA). GP5+/6+ PCR-based HPV genotyping was performed at both laboratories in the Netherlands and Spain. In The Netherlands, 10 µl of isolated DNA was amplified and genotyped using the LMNX HPV GP Genotyping kit (Labo Bio-medical Products BV, Rijswijk, The Netherlands) as described by the manufacturer. [28] The LMNX test utilizes Luminex xMAP high-throughput technology to identify 18 HPV types; i.e. HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82. In Spain, 10 µl of isolated DNA was amplified by the GP5+/6+ PCR and hrHPV was detected by the EIA (Diassay, Rijswijk, The Netherlands) according to the manufacturer's instructions. With the GP5+/6+ PCR–EIA test, 14 hrHPV types can be targeted: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. Next, the EIA-positive GP5+/6+ amplimers were genotyped using the Genotyping kit HPV GP (Diassay), according to the manufacturer's instructions. The strip-based genotyping test targets the same 18 HPV types as the LMNX test. To compare the performance of the two genotyping tests, the first 60 cytology samples included in the study were analysed with both HPV genotyping methods. The agreement between both methods in hrHPV detection was 86.7% (p=0.727, kappa= 0.72, 95% CI: 0.54–0.90). Of the 32 samples

that were hrHPV-positive in both methods, 20 (63%) showed identical hrHPV genotypes, 10 (31%) concordant hrHPV genotypes and 2 (6%) discordant genotypes.

Statistical analysis

Independent sample t-tests and chi-square tests were used to compare age, age at sexual debut, cytology diagnosis and hrHPV status. Analyses were stratified by hrHPV status and HPV16 status. HPV detection and genotype analyses were restricted to 14 hrHPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). HPV16 status was defined as positive if HPV16 was detected by GP5+/6+ in the cytology specimen. Positivity for any other hrHPV type present except HPV16 was defined as non-16 hrHPV-positive.

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for colposcopic performance were calculated. As a cut-off for abnormal colposcopy, both low-grade or worse visual impression (any grade of abnormality) and high-grade or worse visual impression (HG+) were used. The association between lesion size, HPV16 status, and age in women with CIN2+ or CIN3+ was examined using linear logistic regression. Level of statistical significance was set at 0.05. Colposcopic characteristics were studied on the cervix level: when multiple lesions with different colposcopic characteristics were present, the worst colposcopic features were used for analysis. Contingency tables using the Mann-Whitney U-test and Pearson's chi-square statistics were used to analyse the association between the different colposcopic characteristics and CIN2+/CIN3+ diagnosis and the association between HPV16 status and the different features. Odds ratios (ORs) and 95% CI: were calculated. In binary logistic regression, the association between CIN2+ and colposcopic characteristics was studied, adjusted for age. Colposcopic characteristics were included in the model by forward selection using the Wald test-statistic as selection criterion. The selection was stopped when the P-value of the regression coefficient was above 0.05. Analyses were performed using SPSS version 20.0 for Windows (Chicago, IL, USA).

RESULTS

Baseline characteristics

All women with an abnormal cytology result and no exclusion criteria who were referred between August 2010 and October 2012 were included in the study (n=610). Clinical characteristics of the study group are described in Supplementary table 1. The mean age of the women was 36.5 years (SD 10.9) but the age was slightly different between the study centres (Barcelona: 36.0 years and Voorburg: 39.3 years; p=0.002). The mean age of sexual debut was 18 years (SD 2.8). Referral cytology diagnoses were: ASC-US (n=71; 11.6%), LSIL (n=200; 32.8%), HSIL (n=319; 52.3%), ASC-H (n=18; 3.0%), AGUS (n=1; 0.2%) and adenocarcinoma (n=1; 0.2%). Cytology results at enrolment are shown in Supplementary table 1. HrHPV

positivity at enrolment was not significantly different between the two sites (p=0.510); 461 women (75.6%) were positive for hrHPV. Overall, 221 (36.2%) women had a histologically negative diagnosis, 123 (20.2%) women were diagnosed with CIN1, 144 (23.6%) with CIN2 and 122 (20.0%) with CIN3+ (including six with ICC). For two women the HPV genotyping data were not available and these women were excluded from further analysis.

HPV genotyping results

The HPV genotype distribution among the 461 hrHPV-positive women was as follows; HPV16 (n=193; 41.9%), HPV31 (n=73; 15.8%), HPV66 (n=43; 9.3%), HPV18 (n=42; 9.1%), HPV56 (n=39; 8.5%), HPV51 (n=37; 8.0%), HPV33 (n=29; 6.3%), HPV52 (n=29; 6.3%), HPV58 (n=26; 5.6%), HPV39 (n=22; 4.8%), HPV45 (n=19; 4.1%), HPV59 (n=16; 3.5%), HPV35 (n=15; 3.3%), HPV68 (n=11; 2.4%). Additionally, the following HPV types were detected; HPV73 (n=10; 2.2%), HPV53 (n=6; 1.3%), HPV82 (n=5; 1.1%), HPV26 (n=1; 0.2%). HrHPV was detected in 211/343 (61.5%) women with a histological diagnosis of negative or CIN1 and in 250/265 (94.3%) of women with CIN2+ (p<0.001). Both single and multiple infections were detected and HPV genotype distribution was calculated per individual HPV type. Multiple hrHPV infections were detected in 116/461 (25.2%) of the hrHPV-positive women. Fifty-seven of 211 (27.0%) hrHPV-positive women with a diagnosis of negative or CIN1 versus 59/250 (23.6%) of women with CIN2+ had a multiple hrHPV-type infection (p=0.45).

Age and HPV16 status

The mean age in the HPV16-positive women with CIN2+ and CIN3+ was significantly lower compared with the non-16 hrHPV-positive cases; the mean age for HPV16-positive women with CIN2+ was 35.1 years and for non-16 hrHPV-positive women 39.1 years (p=0.002). The mean age for HPV16-positive CIN3+ cases was 36.4 years, versus 40.5 years in the non-16 hrHPV-positive group (p=0.048). Also, there was a significant shorter sexual activity span between HVP16-positive women (17.2 years) with CIN2+ lesions compared with non-16 hrHPV-positive women (21.3 years; p=0.001). An equal trend was found for CIN3+ cases, although not significant (18.6 years versus 22.2 years; p=0.068). No difference in number of lifetime partners was found between the two groups (n=2.7; p=0.701). In linear regression, age in women with CIN2+ was associated with HPV16 status (HPV16-positive women; estimate 3.7 years younger, 95% CI: -6.2 to -1.3, p=0.003). The association remained significant after adjusting for lesion size categorized as <25, 25–50% or >50% of the cervix (HPV16-positive women; estimate 3.7 years younger, 95% CI: -6.1 to -1.3, p=0.003). For women with CIN3+ only, the univariate association between age and HPV16 was also significant (HPV16-positive women; estimate 4.1 years younger, 95% CI: -8.0 to -0.2, p=0.040), and the association adjusted for lesion size was nearly significant (HPV16-positive women; estimate 3.7 years younger, 95% CI: -7.7 to 0.2, p=0.065).

Colposcopic performance and HPV16 status

To evaluate the colposcopic performance we studied the relationship between colposcopic impression and histological outcome per HPV group (total hrHPV-positive, HVP16-positive and non-16 hrHPV-positive) (Tables 1 and Supplementary table 2). In total, 233/265 (87.9%) CIN2+ cases and 108/122 (88.5%) CIN3+ cases had an abnormal colposcopic impression (any grade of abnormality). There was an impression of HG or ICC (HG+) in 163/265 (61.5%) CIN2+ cases and in 87/122 (71.3%) CIN3+ cases (Table 1). Five of six ICC were recognized as ICC by the colposcopists. In one woman with adenocarcinoma as the cytological referral diagnosis, the SCJ was not visualized and an ECC was performed which showed ICC. In HPV16-positive women, 119/135 (88.1%) CIN2+ cases had a colposcopic impression of any grade of abnormality compared with 100/115 (87.0%) CIN2+ cases in non-16 hrHPV-positive women (p=0.776). Sensitivity for detection of CIN3+ in HPV16+ versus non-16 hrHPV-positive cases was 91.8 versus 82.2%, respectively (p=0.119) (Table 1). When HG+ visual impression was used as cut-off for abnormal colposcopy, sensitivity was not significantly different either (p=0.279 for CIN2+ and p=0.332 for CIN3+). Previous studies have shown that multiple HPV infections often show complex and confluent multiple lesions. [24, 29, 30] Therefore calculations were limited to cases with single HPV genotype infections only (single HPV16+; n=131 versus single non-16 hrHPV-positive; n=215) and revealed no differences in colposcopic detectability for CIN2+ and CIN3+. The sensitivity for CIN2+ detection with any grade of abnormal impression in single HPV16-positive and single non-16 hrHPV-positive women was not significantly different: 88/100 (88.0%) versus 77/92 (83.7%); p=0.393. Also, there was no difference in sensitivity for CIN3+ detection [52/58 (89.7%) versus 28/36 (77.8%); p=0.118, respectively].

To investigate further the role of HPV16 in CIN2+ and CIN3+, the PPV of colposcopy in HPV16-positive versus non-16 hrHPV-positive women in this population was calculated (Supplementary table 3). PPV was higher in HPV16-positive women with CIN2+ than in non-16 hrHPV-positive women, for both any grade of abnormality and HG+ impression (75.8 versus 54.6%; p<0.001, and 88.8 versus 74.3%; p=0.011, respectively). This difference was also significant in CIN3+ cases (any grade of abnormality: 42.7 versus 20.2%; p<0.001, and HG+: 55.1 versus 33.7%; p=0.003). We observed similar results for each of the nine colposcopists involved in the study.

Colposcopic characteristics and HPV16 status

In 429/608 (70.6%) cases, the SCJ was visible during colposcopy, at least one lesion-targeted biopsy (119 women had a random biopsy only) was collected and no missing colposcopic characteristics were reported. These cases were included in the analysis of colposcopic characteristics. 'Time of appearance' data were available for 422 women and 'size of lesion' for 423 women (Table 2). CIN2+ lesions had more distinct margins (p=0.001), more distinct acetowhitening (p<0.001), coarser punctation (p<0.001), coarser mosaic (p=0.02), appeared

Table 1. Sensitivity and specificity of colposcopy for the detection of CIN2+ and CIN3+ lesions stratified by HPV status

	CIN2+						CIN3+					
	Sensitivity			Specificity			Sensitivity			Specificity		
Visual impression	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI
Overal												
Any abnormality	233/265	87.9	83.2 - 91.5	152/343	44.3	39.0 - 49.8	108/122	88.5	81.2 - 93.4	170/486	35.0	30.8 - 39.4
High-grade*	163/265	61.5	55.3 - 67.3	282/343	82.2	77.7 - 86.0	87/122	71.3	62.3 - 79.0	349/486	71.8	67.5 - 75.7
hrHPV positive												
Any abnormality	219/250	87.6	82.7 - 91.3	90/211	42.7	35.9 - 49.6	104/118	88.1	80.6 - 93.1	107/343	31.2	26.4 - 36.4
High-grade*	154/250	61.6	55.2 - 67.6	175/211	82.9	77.0 - 87.6	83/118	70.3	61.1 - 78.2	236/343	68.8	63.6 - 73.6
HPV16 positive												
Any abnormality	119/135	88.1	81.2 - 92.9	20/58	34.5	22.8 - 48.2	67/73	91.8	82.4 - 96.6	30/120	25.0	17.7 - 33.9
High-grade*	79/135	58.5	49.7 - 66.8	48/58	82.8	70.1 - 91.0	49/73	67.1	55.0 - 77.4	80/120	66.7	57.4 - 74.8
non-16 hrHPVpositive												
Any abnormality	100/115	87.0	79.1 - 92.3	70/153	45.8	37.7 - 54.0	37/45	82.2	67.4 - 91.5	77/223	34.5	28.4 - 41.2
High-grade*	75/115	65.2	55.7 - 73.7	127/153	83.0	75.9 88.4	34/45	75.6	60.1 - 86.6	156/223	70.0	63.4 - 75.8
HPV negative												
Any abnormality	14/15	93.3	66.0 - 99.7	62/132	47.0	38.3 - 55.8	4/4	100	39.6 - 100	63/143	44.1	35.8 - 52.6
High-grade*	9/15	60.0	32.9 - 82.5	107/132	81.1	73.1 - 87.1	4/4	100	39.6 - 100	113/143	79.0	71.3 - 85.2

Results are stratified for the visual impression of low-grade cervical intraepithelial neoplasia (CIN) or worse (any abnormality) versus high-grade CIN or worse (high-grade).

*Including ICC

Table 2. Different colposcopic characteristics in CIN2+ and CIN3+ cases (Table 2A) and in CIN2+ and CIN3+ cases stratified by HPV16 status (Table 2B). In total, 429 (70.6%) women with a visible transformation zone, at least one lesion targeted biopsy taken and no missing reported characteristics were studied.

Table 2A.

	Histology					
Characteristics	<CIN2	CIN2+	p-value	<CIN3	CIN3+	p-value
Margins						
Geographical	95	82		144	33	
Smooth	91	134	0.001	162	63	0.003
Internal borders	7	20		15	12	
Colour						
Absent	9	6		13	2	
Translucent	84	44	<0.001	114	14	<0.001
Intermediate	85	128		156	57	
Opaque	15	58		39	34	
Punctation						
Absent	144	135		231	48	
Fine	45	69	<0.001	77	37	<0.001
Coarse	4	32		14	22	
Mosaic						
Absent	132	139		208	63	
Fine	46	61	0.020	85	22	0.092
Coarse	15	36		29	22	
Atypical vessels						
Absent	187	222	0.168	313	96	0.001
Present	6	14		9	11	
Time of appearance*						
Slow	123	82	<0.001	177	28	<0.001
Fast	68	149		140	77	
Size lesion#						
0%	5	4		9	0	
<25%	106	65	<0.001	151	20	<0.001
25-50%	43	73		86	30	
>50%	35	92		72	55	

* N=422. Missing data n=7

N=423. Missing data n=6

Table 2B.

	hrHPV positives					
	CIN2+			CIN3+		
Characteristics	HPV16 positive	non-16 hrHPV positive	p-value	HPV16 positive	non-16 hrHPV positive	p-value
Margins						
Geographical	44	32		20	11	
Smooth	67	59	0.592	40	21	0.829
Internal borders	11	9		7	5	
Colour						
Absent	3	3		2	0	
Translucent	25	16	0.817	10	4	0.813
Intermediate	63	57		33	22	
Opaque	31	24		22	11	
Punctation						
Absent	72	53		31	15	
Fine	33	33	0.454	22	14	0.656
Coarse	17	14		14	8	
Mosaic						
Absent	65	64		36	24	
Fine	38	20	0.200	14	8	0.191
Coarse	19	16		17	5	
Atypical vessels						
Absent	114	94	0.865	59	34	0.545
Present	8	6		8	3	
Time of appearance*						
Slow	48	30	0.167	19	9	0.777
Fast	72	67		48	26	
Size lesion#						
0%	3	1		0	0	
<25%	37	21	0.180	16	4	0.932
25-50%	35	35		13	15	
>50%	46	42		37	17	

* N=422. Missing data n=7

N=423. Missing data n=6

faster (p<0.001) and had a larger lesion size (p<0.001) than negative or CIN1 lesions. In addition, women with CIN3+ lesions had atypical vessels more often (p=0.001) than did women with negative tissue, CIN1 or CIN2. CIN3+ lesions did not show coarser mosaic (p=0.092). Atypical vessels were present in four of five women diagnosed with ICC and a visible SCJ. The time of appearance was fast and the lesion size was >50% in all ICC with SCJ visible. Mosaic, punctation and colour had a variable score in these cases (Table 2A).

The associations between colposcopic characteristics and CIN2+ (and CIN3+) were expressed by Mantel-Haenszel ORs, corrected for age. For end-point CIN2+, statistically significant associations were found for most characteristics but not for 'atypical vessels' or

Table 3. Odds ratios (OR) for the different colposcopic characteristics in all women with CIN2+ and in women with CIN2+ stratified by HPV16 status (Table 3A) and ORs for women with CIN3+ and women with CIN3+ stratified by HPV16 status (Table 3B). All ORs are corrected for age.

Table 3A.

Colposcopic characteristics	Total CIN2+	HPV16 positive versus non-16 hrHPV positive in CIN2+
	OR (95% CI)	OR (95% CI)
Margins (geographical vs smooth/internal borders)	1.75 (1.18 - 2.61)	0.77 (0.43 - 1.38)
Acetowhitening (absent/translucent vs intermediate/opaque)	3.25 (2.12 - 4.99)	0.70 (0.34 - 1.44)
Punctation (absent vs present)	1.98 (1.29 - 3.03)	0.78 (0.45 - 1.37)
Punctation (absent/fine vs coarse)	6.74 (2.30 - 19.78)	1.07 (0.49 - 2.33)
Mosaic (absent vs present)	1.48 (0.98 - 2.26)	1.26 (0.70 - 2.25)
Mosaic (absent/fine vs coarse)	1.98 (1.03 - 3.81)	0.93 (0.43 - 1.97)
Atypical vessels (absent vs present)	1.50 (0.56 - 4.07)	1.64 (0.51 - 5.26)
Time of appearance (slow vs fast)	3.26 (2.17 - 4.91)	0.59 (0.32 - 1.09)
Lesion size (cut off 25%)	3.24 (2.16 - 4.88)	0.58 (0.30 - 1.10)
Lesion size (cut off 50%)	2.53 (1.62 - 3.96)	0.90 (0.51 - 1.57)

Table 3B.

Colposcopic characteristics	Total CIN3+	HPV16 positive versus non-16 hrHPV positive in CIN3+
	OR (95% CI)	OR (95% CI)
Margins (geographical vs smooth/internal borders)	1.79 (1.12 - 2.85)	1.01 (0.42 - 2.42)
Acetowhitening (absent/translucent vs intermediate/opaque)	3.51 (1.97 - 6.28)	0.79 (0.22 - 2.90)
Punctation (absent vs present)	2.93 (1.86 - 4.63)	0.97 (0.41 - 2.27)
Punctation (absent/fine vs coarse)	5.16 (2.52 - 10.55)	1.40 (0.49 - 3.99)
Mosaic (absent vs present)	1.32 (0.83 - 2.09)	1.78 (0.72 - 4.37)
Mosaic (absent/fine vs coarse)	2.69 (1.46 - 4.95)	2.31 (0.72 - 7.37)
Atypical vessels (absent vs present)	3.71 (4.47 - 9.40)	1.84 (0.46 - 7.30)
Time of appearance (slow vs fast)	3.43 (2.11 - 5.59)	1.10 (0.40 - 3.06)
Lesion size (cut off 25%)	4.02 (2.37 - 6.80)	0.64 (0.21 - 1.96)
Lesion size (cut off 50%)	3.54 (2.22 - 5.63)	1.75 (0.75 - 4.12)

'mosaicism (absent versus present)' (Table 3A). For end-point CIN3+, the presence of atypical vessels was significant and the presence of 'mosaicism (absent versus present)' was not significant (Table 3B). Colposcopic characteristics were also compared between HPV16-positive and non-16 hrHPV-positive CIN2+ and CIN3+ cases. There was no significant relationship between any of the colposcopic characteristics and HPV16 status, either for CIN2+ or for CIN3+ (Tables 2B and 3A,B). In a binary logistic regression, the association between CIN2+ and multiple colposcopy characteristics were studied, adjusted for age. Significant independent associations with CIN2+ were found for 'acetowhitening', 'time of appearance' and 'lesion size (>25% of visible cervix)'. The corresponding ORs were 1.91 (95% CI: 1.56-3.17), 1.95 (95% CI: 1.21-3.15), and 2.25 (95% CI: 1.44-3.51), respectively.

DISCUSSION

Main findings

This study in women referred for colposcopy due to abnormal cervical cytology reveals that the colposcopic visual appearance of HPV16-related CIN2+ is not different from other hrHPV-type related lesions. The women were referred according to conventional European screening practice with a mean age of over 35 years and on the basis of any abnormal cytology grade including a single ASC-US smear. HPV testing was not part of the screening or triage procedure. No difference was observed in either sensitivity or specificity of colposcopic impression or individual colposcopic criteria for CIN2+, when caused by HPV16 or other hrHPV types. However, we confirmed that HPV16-positive women had a younger age at diagnosis of CIN3. [20, 31] We observed this effect for both CIN2+ and CIN3+ separately. Moreover, we found a significantly shorter span of sexual activity among women with HPV16-related CIN2+ than other carcinogenic HPV types, although these women had a similar number of lifetime partners. A similar, but non-statistically significant, trend for CIN3+ women was found. These findings suggest that HPV16-related CIN2+ lesions appear to develop faster than those related to other hrHPV types. When stratifying for age, we found no significant association between lesion size and HPV16 positivity. This implies that CIN2+ is detected at the same size irrespective of genotype but HPV16-related CIN2+ lesions reach the detection threshold faster than lesions related to other types because they are found at younger age.

In the present study, the sensitivity for detecting CIN2+ was 87.9% for any grade of abnormal impression and 61.5% for HG+ impression. In CIN3+ the sensitivities were 88.5 and 71.3% for any grade of abnormal impression and HG+ impression, respectively. When studying performance of different colposcopic characteristics irrespective of HPV status, all characteristics except for 'presence of atypical vessels' and 'mosaicism (absent versus present)' were significantly associated with CIN2+. The highest correlations were found for 'acetowhitening', 'time of appearance' and 'lesion size of >25% of visible cervix'. In general,

colposcopy has a sensitivity of 50–70% to detect high-grade lesions and ICC, [9-12] but the sensitivity can be increased by raising the number of collected biopsies. [14] The use of an extended biopsy protocol in our study with multiple targeted biopsies from all abnormal areas and a biopsy from visually negative tissue could explain the high sensitivity we have found. Unlike measures like sensitivity and specificity, the PPV depends on the prevalence of CIN2+ in the study population. We found a PPV of 72.8% for CIN2+ using colposcopic impression of HG+, representative for this population and broadly comparable to a recent UK study.[32]

Strengths and limitations

The main strength of this study is the large well described European study population representative of women with abnormal smears referred for colposcopy. In The Netherlands there is an organized 5-yearly screening program starting at the age of 30. In Spain there is opportunistic screening. In both countries the median age of women referred for colposcopy was over 35 years. A large number of women had CIN2+ as a histological endpoint. Various colposcopists from two different centres took part. We used a refined protocol of collecting multiple biopsies. Histological diagnosis of all biopsies was done by consensus diagnosis including expert pathologists and local pathologists who were blinded to HPV status at the time of diagnosis.

A limitation of this paper is that it is a cross-sectional study and loop electrosurgical excision procedure (LEEP) outcomes were not included as a histological endpoint. Hence, we lack longitudinal HPV genotyping and histological end point data. Different test algorithms were used for HPV genotyping in the two study centres. A pilot study performed on the first samples included in the study showed good agreement between HPV positivity rates and HPV genotypes. Furthermore, previous studies demonstrated a high genotyping agreement between both test algorithms. [28, 33] As in most clinical and epidemiological studies, HPV genotyping was performed on cytology specimens. In multiple infections, it is uncertain which genotype causes the lesion. Without techniques to define the presence of different HPV types in pre-neoplastic cells they may be incorrectly associated with CIN. Therefore, LCM combined with sensitive PCR (LCM-PCR) is currently being applied to study HPV type attribution on lesion level in this study.

Interpretation

Our findings are in contrast with Jeronimo et al. [4] who suggested that HPV16 causes more definite visual abnormalities than other HPV types, regardless of eventual histological diagnosis. The mean age of the study population of Jeronimo et al. was substantially lower than that of our study population (24 years versus 36.5 years). An explanation for the difference in findings between the studies is that high-grade cervical precancer has to grow to a certain size before it becomes detectable during colposcopy and that high-grade lesions evolve more rapidly in women with HPV16 infections than many other hrHPV types. This is consistent

with evidence that ICC associated with HPV16 occurs at an earlier age than that associated with many other hrHPV types and that the development of ICC from CIN3 is associated with a large lesion size. [17, 34] Lesions driven by other hrHPV types than HPV16 in the older women in the present study have had the chance to grow over time. Our theory is in line with the findings of Wentzensen et al., [31] who observed a difference in lesion size between women with HPV16 and other types in CIN2, but not in CIN3. Zaal et al. [22] found that with conventional visual colposcopy, no difference in sensitivity for CIN2+ lesions caused by different HPV types was found. However, the sensitivity of dynamic spectral imaging colposcopy for CIN2+ was higher in HPV16-positive than in non-16 hrHPV-positive women which may be explained by the better identification of small CIN2 lesions with this technique. [22] This is in agreement with our finding that in this population of women with abnormal cervical screening results, the visual colposcopic appearance of HPV16-positive women does not differ from that in women infected with other HPV types. Studies have shown that HPV16 is the genotype with the highest oncogenic potential because it is associated with the highest risk of the development of CIN3 and cancer and has the highest worldwide attribution for CIN2+ and ICC. [16, 19]

CONCLUSIONS

In this cross-sectional study in women with abnormal cytology and referred for colposcopy according to current European screening practice, we found that the sensitivity for detecting CIN2+ during colposcopy is similar in HPV16-positive and non-16 hrHPV-positive women. Irrespective of HPV status, all studied colposcopic characteristics were associated with CIN2+, in particular acetowhitening and lesion size. Furthermore, the mean age of HPV16-positive women with CIN2+ was significantly lower than in the non-16 hrHPV-positives. These findings confirm the important etiologic role of HPV16 in the development of cervical neoplasia, although HPV16-related CIN2+ lesions are not easier to detect during colposcopy. This might be a result of more rapid development of HPV16-related CIN2+. However, the performance of colposcopy in detecting these lesions is not improved, which may limit the benefit of this knowledge during colposcopic examination. As there is debate as to the accuracy of colposcopy in women vaccinated against HPV16 and 18, this study shows that colposcopic performance is similar for any hrHPV-type-positive woman. [35] To improve our understanding of the development of CIN2+ and CIN3+ related to HPV16 and other carcinogenic types, we are currently performing lesion-based genotyping using LCM-PCR.

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SUPPLEMENTARY TABLES

Supplementary table 1. Population characteristics

	Barcelona		Voorburg		Total		
	n	%	n	%	n	%	p-value
Total patients	511	83.8	99	16.2	610	100	
Age at enrolment (mean, SD)	36.0 (11.1)		39.3 (9.2)		36.5 (10.9)		0.002*
Age at sexual debut (mean, SD)	18.2 (2.8)		17.2 (2.6)		18.0 (2.8)		0.001*
Cytology at enrolment							
Negative	119	23.3	25	25.3	144	23.6	
ASC-US	38	7.4	1	1.0	39	6.4	
LSIL	103	20.2	28	28.3	131	21.5	
HSIL	228	44.6	43	43.4	271	44.4	
ASC-H	14	2.7	0	0.0	14	2.3	0.053**
AGUS	1	0.2	0	0.0	1	0.2	
Adenocarcinoma in situ	1	0.2	1	1.0	2	0.3	
Cervical cancer	6	1.2	0	0.0	6	1.0	
Not enough material	1	0.2	1	1.0	2	0.3	
hrHPV status at enrolment (GP5+/6+)							
Positive	390	76.3	71	71.7	461	75.6	
HPV16 positive	165	42.3	28	39.4	193	41.9	
non-16 hrHPVpositive	225	57.7	43	60.6	268	58.1	0.510**
Negative	121	23.7	26	26.3	147	24.1	
Sample lost	0	0.0	2	2.0	2	0.3	

*independent-samples t-test

**chi-square test

Supplementary table 2. The relation between histological diagnosis and visual impression of the cervix stratified by HPV status

		Total		hrHPV positive						HPV negative	
				Total hrHPV positive		HPV16 positive		non-16 hrHPV positive			
Histology	Visual impression	n	%	n	%	n	%	n	%	n	%
Negative/CIN1											
	Normal	152	44.3	90	42.7	20	34.5	70	45.8	62	47.0
	Low-grade	130	37.9	85	40.3	28	48.3	57	37.3	45	34.1
	High-grade	61	17.8	36	17.1	10	17.2	26	17.0	25	18.9
	Total	343*	100	211	100	58	100	153	100	132	100
CIN2											
	Normal	18	12.6	17	12.9	10	16.1	7	10.0	1	9.1
	Low-grade	49	34.3	44	33.3	22	35.5	22	31.4	5	45.5
	High-grade	76	53.1	71	53.8	30	48.4	41	58.6	5	45.5
	Total	143*	100	132	100	62	100	70	100	11	100
CIN3+											
	Normal	14	11.5	14	11.9	6	8.2	8	17.8	0	0.0
	Low-grade	21	17.2	21	17.8	18	24.7	3	6.7	0	0.0
	High-grade	87	71.3	83	70.3	49	67.1	34	75.6	4	100
	Total	122	100	118	100	73	100	45	100	4	100
Total		608	100	461	75.8	193	41.9	268	58.1	147	24.2

*1 sample lost

Supplementary table 3. The positive predictive value (PPV) and negative predictive value (NPV) of colposcopy for the detection of CIN2+ and CIN3+ lesions stratified by HPV status

		CIN2+						CIN3+					
		PPV			NPV			PPV			NPV		
	Visual impression	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI
Overall													
	Any abnormality	233/424	55.0	50.1 - 59.7	152/184	82.6	76.2 - 87.6	108/424	25.5	21.4 - 29.9	170/184	92.4	87.3 - 95.6
	High-grade*	163/224	72.8	66.4 - 78.4	282/384	73.4	68.7 - 77.7	87/224	38.8	32.5 - 45.6	349/384	90.9	87.4 - 93.5
hrHPV positive													
	Any abnormality	219/340	64.4	59.0 - 69.5	90/121	74.4	65.5 - 81.7	104/340	30.6	25.8 - 35.8	107/121	88.4	81.0 - 93.3
	High-grade*	154/190	81.1	74.6 - 86.2	175/271	64.6	58.5 - 70.2	83/190	43.7	36.6 - 51.1	236/271	87.1	82.4 - 90.7
HPV16 positive													
	Any abnormality	119/157	75.8	68.2 -82.1	20/36	55.6	38.3 - 71.7	67/157	42.7	34.9 - 50.8	30/36	83.3	66.5 - 93.0
	High-grade*	79/89	88.8	79.9 - 94.2	48/104	46.2	36.4 - 56.2	49/89	55.1	44.2 - 65.5	80/104	76.9	67.4 - 84.4
non-16 hrHPVpositive													
	Any abnormality	100/183	54.6	47.1 - 62.0	70/85	82.4	72.2 - 89.5	37/183	20.2	14.8 - 26.9	77/85	90.6	81.8 - 95.6
	High-grade*	75/101	74.3	64.4 - 82.2	127/167	76.0	68.7 - 82.2	34/101	33.7	24.8 - 43.8	156/167	93.4	88.2 - 96.5
HPV negative													
	Any abnormality	14/84	16.7	9.7 - 26.7	62/63	98.4	90.3 - 99.9	4/84	4.8	1.5 - 12.4	63/63	100.0	92.8 - 100
	High-grade*	9/34	26.5	13.5 - 44.7	107/113	94.7	88.3 - 97.8	4/34	11.8	3.8 - 28.4	113/113	100.0	95.9 - 100

Results are displayed stratified for the visual impression of low-grade cervical intraepithelial neoplasia (CIN) or worse (any abnormality) versus high-grade CIN or worse (high-grade).

*Including ICC

chapter 5

The increased detection of cervical intraepithelial neoplasia when using a second biopsy at colposcopy: a cross-sectional study

Romy van Baars*, Jacolien van der Marel*, Agata Rodriguez, Wim G.V. Quint, Miekel M. van de Sandt, Johannes Berkhof, Mark Schiffman, Aureli Torné, Jaume Ordi, David Jenkins, René H.M. Verheijen, Theo J.M. Helmerhorst, Bram W.A. ter Harmsel, Nicolas Wentzensen, Marta del Pino

*Both authors contributed equally

Submitted

ABSTRACT

Objective: It has been suggested that colposcopy can miss a significant percentage of high-grade cervical intraepithelial neoplasia (CIN2+). Improved disease ascertainment was evaluated by taking multiple lesion-directed biopsies.

Design: Cross-sectional multicentre study.

Setting: Two colposcopy clinics in the Netherlands and Spain.

Population: 610 women with abnormal cytology referred for colposcopy.

Methods: Multiple directed biopsies were collected from lesions and ranked according to impression. A non-directed biopsy of normal-appearing tissue was added if fewer than four biopsies were collected. We evaluated the incremental CIN2+ yield for one and two directed biopsies and in an additional analysis, CIN2+ outcomes for second biopsies in women who had only one biopsy were imputed. Colposcopic images were reviewed for quality control.

Main outcome measures: CIN2+ and CIN3+.

Results: In women with at least two lesion-directed biopsies the yield for CIN2+ increased from 51.7% (95% CI: 45.7-57.7) for one directed biopsy to 60.4% (95% CI: 54.4-66.2) for two biopsies. The highest CIN2+ yield was observed in women who were HPV16-positive, had high-grade squamous intraepithelial lesion (HSIL) cytology, and high-grade colposcopy impression. The yield increased from 83.1% (95% CI: 71.5-90.5) with one directed biopsy to 93.2% (95% CI: 83.8-97.3) with two directed biopsies. Only 4.5% additional CIN2+ were detected in biopsies not targeting abnormal areas on the cervix.

Conclusions: A second lesion-directed biopsy is associated with a significant increase in CIN2+ detection. Performing a second lesion-directed biopsy and using a low threshold for abnormality of any acetowhitening should become the standard practice of colposcopy.

INTRODUCTION

Colposcopy with lesion-directed biopsy is currently a widely used standard for the evaluation of women referred with abnormal cervical cytology. The aims of colposcopy are the evaluation of the cervix, with particular attention for the cervical squamocolumnar junction (SCJ) and to determine which abnormal areas should be biopsied. Current biopsy procedures rely on the colposcopic identification of the area on the cervix that most likely represents the worst lesion. [1] The biopsy result determines further management. Generally, if a CIN2 or worse (CIN2+) is found the woman receives treatment. Despite the central role of colposcopy in detecting CIN2+, it has been suggested that it can miss 30-55% of high-grade lesions. [2-6] In a study investigating women with atypical cells of undetermined significance (ASC-US) cytology and CIN3 or invasive cervical cancer (ICC), it was found that gains in detecting cervical precancer can be obtained by increasing the number of lesion-directed biopsies from one to two. [6] Moreover, in studies that include biopsies of regions without colposcopic abnormality in addition to lesion-directed biopsies, 12 to 37% of the overall CIN2+ lesions were detected in this biopsy only. [5, 7, 8]

In this study, the benefit of collecting a second lesion-directed biopsy to detect CIN2+ in women with abnormal cytology from two European study sites was investigated. Furthermore, the benefit of collecting an additional biopsy of visual normal appearing tissue (non-directed biopsy) was examined. We studied CIN2+ yields taking into account the women's referral cytology grade, Human Papillomavirus (HPV) status, and colposcopic impression.

METHODS

Study population

Between August 2010 and October 2012, 610 women aged 17 years and older, visiting the gynecological outpatient clinic of the Hospital Clínic in Barcelona, Spain or the Reinier de Graaf Groep in Voorburg, The Netherlands, were enrolled in a cross-sectional study. In Spain, there is opportunistic screening and all women with abnormal cytology are referred for colposcopy. In the Netherlands, there is an organized 5-yearly screening program starting at the age of 30. Cytology grading is done according to the CISOE-A (composition, inflammation, squamous epithelium, other and endocervical columnar epithelium, and adequacy of the smear) classification. [9] Women with Pap smears graded as borderline or mild dyskaryosis (BMD) are recalled for repeat cytology after 6 and 18 months and are referred for colposcopy if the repeat cytology result is abnormal (borderline dyskaryosis or worse). Outside the national screening program, women with clinical symptoms indicative of cervical pathology (e.g. post coital bleeding) also receive cytological examination. All women in this study were referred for colposcopic evaluation because of abnormal cytology, which was detected at local health

centers between 1 and 6 months prior to the study visit. Inclusion and exclusion criteria and patient characteristics have been described previously. [10] This study was approved by the medical ethical boards of both hospitals. All women provided signed informed consent. This study is registered in the Dutch Trial register (NTR3464).

Cytology and high-risk HPV (hrHPV) detection

Before colposcopy, a liquid based cytology sample using a Cervex-Brush® (Rovers Medical Devices B.V., Oss, The Netherlands) was obtained. The Cervex-Brush was rinsed in ThinPrep® medium (Hologic, Marlborough, MA) in Spain, and in SurePath™ medium (Klinipath BV, Duiven, The Netherlands) in the Netherlands. Cytological examination and classification was performed at the local laboratory in Spain according to the Bethesda 2001 classification (negative, ASC-US, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), atypical glandular cells of undetermined significance (AGUS), atypical squamous cells- cannot exclude HSIL (ASC-H), adenocarcinoma in situ (AIS) or ICC). In the Netherlands grading was done according to the CISOE-A classification, which was translated into the Bethesda 2001 classification as described earlier. [9]

GP5+/6+ PCR based HPV genotyping was performed at both laboratories in the Netherlands and in Spain. [10] In brief, DNA extraction was performed using 250µL of the cytology specimen to obtain 100µL of eluate with the QIAamp MinElute Virus Spin kit (QIAgen Inc., Valencia, CA). In the Netherlands, 10 µl of isolated DNA was amplified and genotyped, using the LMNX HPV GP Genotyping kit (Labo Bio-medical Products BV, Rijswijk, The Netherlands). [11] In Spain, HPV detection was performed using the GP5+/6+-PCR-EIA (Diassay, Rijswijk, The Netherlands). The EIA-positive GP5+/6+ amplimers were genotyped by the Genotyping kit HPV GP (Diassay). The strip-based genotyping test targets the same 18 HPV types as the LMNX test.

Colposcopy procedure

Colposcopic examination was performed using a digital colposcopy imaging system (Boundary Marketing Tool) created by the National Cancer Institute (NCI) in collaboration with the National Library of Medicine (NLM), Bethesda, USA. [12] Nine colposcopists were involved in this study. Acetic acid 5% was applied for eliciting the acetowhite epithelial response. Up to four directed biopsies were collected from different lesions or different regions within 1 lesion. Distinct areas within a large complex lesion were biopsied separately. If fewer than 4 directed biopsies were taken, a biopsy from normal appearing epithelium of the SCJ (non-directed biopsy) was added. An endocervical curettage (ECC) was collected if the SCJ was not or only partially visible, if there was suspicion of ICC, if the visualized lesion extended in the endocervical canal and if the SCJ was visible but no or marginal abnormalities were visualized. Overall colposcopic impression was graded as normal (including acetowhitening suggestive for metaplastic changes), low-grade, high-grade or worse. [13, 14] Biopsies were

ranked by order of severity according to their colposcopic impression. When the impression was similar between two lesions, the two biopsies were ranked by order of collection. From women with an available digital colposcopic image a review of the total colposcopic impression and the impression of the location of the non-directed biopsy was conducted. In total 447/610 (73.3%) digital colposcopy images were available for review. Each colposcopist in Voorburg reviewed a subset of the Barcelona images in order to have all images reviewed and three of six colposcopists in Barcelona reviewed the Voorburg images. A low threshold for abnormality (any acetowhitening suggestive of metaplastic changes) was used in the reviewing process.

Pathological diagnosis and grading

Biopsy specimens were fixed in 10% formalin and paraffin-embedded. Hematoxylin and Eosin (H&E) sections were examined by a local pathologist and classified as normal, CIN1, CIN2, CIN3, including 2 cases with adenocarcinoma in situ (AIS), or ICC. The overall histological diagnosis per case was based on the worst diagnosis found in all specimens of each woman. All biopsies were independently reviewed by a second central gynaecological pathologist. In case of disagreement between the original and review diagnosis, a third central pathologist reviewed the discordant cases independently. Consensus diagnosis was determined by the agreement of 2 of 3 interpretations. In case of 3 different diagnoses, the 2 central pathologists came to an agreement after reviewing the discordant case together. P16 immunohistochemical staining (Clone JC8, SantaCruz Biotechnology Inc.) was performed on an adjacent section and scoring was based on the extent of the staining. The scoring of p16 included nuclear and cytoplasmic staining and was graded as negative/ patchy staining or positive staining.

Statistical analysis

Contingency tables were used to report patient characteristics of the study populations in Spain and the Netherlands. Because colposcopy is performed with knowledge of referral cytology result, the populations were stratified by referral cytology and also by colposcopic impression (normal, low-grade, high-grade or worse). Furthermore, since HPV16 is known to be the most carcinogenic HPV type, [15] populations were stratified by HPV16 status (HPV16 positive and HPV16 negative, including HPV negative and low-risk HPV).

Since only a minority (18.8%) of women received more than two directed biopsies, an unbiased evaluation of the yield of CIN2+ for three and four lesion-directed biopsies was not possible. Therefore we focused our main analyses on the yield of CIN2+ for the first and second lesion-directed biopsy only excluding women with only 1 directed biopsy from our analysis. Also, we excluded women with ECC only (n=19) from this analysis. The absolute yield of CIN2+ was used to assess the incremental benefit of collecting two instead of one directed biopsy. The absolute yield is the percentage of women with two or more directed biopsies found to have CIN2+. For each woman, only one CIN2+ was included in the calculation of

yield, even if multiple CIN2+ lesions were detected. Furthermore, CIN2+ yield was calculated for different combinations of cytologic, virologic, and colposcopic results with one and two directed biopsies. Only risk stratifications with 15 or more cases were included because data were unstable with lower numbers. We also performed a secondary analysis representing absolute CIN2+ yields for the complete study population. To achieve this, we imputed CIN2+ outcomes for second biopsies that were not performed as previously described (Unpublished observations by Wentzensen et al.) (Supplementary information 1). Comparison between CIN2+ yield percentages were calculated using McNemar's test. All analyses were performed using SPSS 20.0 (Armonk, NY) and SAS 9.1 (Cary, NC).

RESULTS

Characteristics of the study populations

Patient characteristics per histological outcome for the study sites Voorburg, The Netherlands and Barcelona, Spain are shown in Table 1. The median age of women included in the study in Voorburg (n=99) was 39.0 years (range: 21-61) and 34.0 years (range: 17-92) in Barcelona (n=511); p<0.001. Referral cytology grading and HPV status (HPV positivity, HPV16 positivity and multiple hrHPV type infections) were similar in both sites; p=0.859 for cytology grading, p=0.507, p=0.510, and p=0.961 for HPV positivity, HPV16 positivity, and multiple hrHPV infections respectively. Colposcopic impression was significantly different between both sites; more high-grade impression was found in Voorburg and more negative/acetowhite impression in Barcelona; p<0.001. The median number of biopsies in Voorburg was 3.0 and in Barcelona 2.0; p<0.001. A larger proportion of CIN2+ cases was diagnosed in Voorburg compared to Barcelona; p=0.006.

In the combined population, 172 (28.2%) women had one lesion-directed biopsy, 148 (24.3%) had two directed biopsies, 63 (10.3%) women had three directed biopsies, 52 (8.5%) had four directed biopsies and 156 (25.6%) women received only a non-directed biopsy. We found CIN2+ solely in endocervical curettage (ECC) in 19 women (7%) and this group had a median age of 43 years.

In both study sites the CIN2+ yield increased when collecting increasing numbers of lesion-directed biopsies and results for CIN3 or worse were similar although numbers were lower. When we used a biomarker enhanced endpoint for CIN2+ following recent US guidelines for grading CIN [16] and excluded p16 negative CIN2 lesions to achieve high specificity for cervical precancer, the CIN2+ yield per biopsy was slightly lower among women with low-grade and high-grade impression although not significant (data not shown).

Table 1. Patient characteristics per study site

Table 1a. Patient characteristics Voorburg

	Histology									
	Total		Negative		CIN1		CIN2		CIN3+	
Median age (range)	39.0 (21-61)		44.5 (21-61)		38.5 (23-57)		36.5 (21-61)		40.0 (25-60)	
	n	%	n	%	n	%	n	%	n	%
Referral cytology	99	100	26	100	18	100	28	100	27	100
ASC-US	13	13.1	5	19.2	4	22.2	3	10.7	1	3.7
LSIL	31	31.3	11	42.3	7	38.9	9	32.1	4	14.8
HSIL	55	55.6	10	38.5	7	38.9	16	57.1	22	81.5
ASC-H	0	0	0	0	0	0	0	0	0	0
AGUS	0	0	0	0	0	0	0	0	0	0
Adenocarcinoma	0	0	0	0	0	0	0	0	0	0
HPV status*	97	100	25	100	18	100	27	100	27	100
HPV- / Low-risk HPV+	26	26.8	13	52.0	8	44.4	4	14.8	1	3.7
High-risk HPV+	71	73.2	12	48.0	10	55.6	23	85.2	26	96.3
HPV16+	28	28.9	5	41.6	1	10.0	7	30.4	15	57.7
Multiple HPV infection	19	19.6	3	25.0	3	30.0	6	26.1	7	26.9
Colposcopic Impression	99	100	26	100	18	100	28	100	27	100
Normal	2	2.0	2	7.7	0	0.0	0	0.0	0	0.0
Low-grade lesion	35	35.4	13	50.0	9	50.0	12	42.9	1	3.7
High-grade lesion	62	62.6	11	42.3	9	50.0	16	57.1	26	96.3
Median number of biopsies	3.0 (0-4)		2.0 (0-4)		3.0 (2-4)		3.0 (1-4)		3.0 (2-4)	
P16 immunohistochemistry	99	100	26	100	18	100	28	100	27	100
Negative	34	34.3	22	84.6	10	55.6	2	7.1	0	0.0
Positive	65	65.7	4	15.4	8	44.4	26	92.9	27	100

* 2 Missing values are excluded

Detection of CIN2+ and CIN3+ using lesion-directed biopsies

In total 263/610 (43.1%) women with 2 or more lesion-directed biopsies were included in this analysis. CIN2+ and CIN3+ yields for one and two directed biopsies are shown in table 2. We first stratified the analysis of detection of CIN2+ and CIN3+ by colposcopic impression. No CIN2+ and CIN3+ was detected in women with no or marginal colposcopic abnormalities, but substantially more among women with low-grade and especially high-grade impression. Among 91 women with low-grade impression, the yield of CIN2+ increased from 30.8% for one biopsy to 38.5% for two biopsies. Among 172 women with high-grade colposcopic impression, the yield increased from 62.8% for one biopsy to 72.1% for two biopsies. In other stratified analyses, the highest CIN2+ yields for a second directed biopsy were observed for women with HPV16 positivity (83.7%) and HSIL+ (including HSIL, ASC-H, AGUS and Adenocarcinoma) referral cytology (73.0%) (Table 2). In all strata (except for women with normal/

Table 1b. Patient characteristics Barcelona

	Histology									
	Total		Negative		CIN1		CIN2		CIN3+*	
	34.0 (17-92)		34.0 (19-74)		31.0 (17-64)		34.0 (20-74)		36.0 (23-92)	
Meadian age (range)	n	%	n	%	n	%	n	%	n	%
Referral cytology	511	100	195	100	105	100	116	100	95	100
ASC-US	58	11.4	35	17.9	14	13.3	6	5.2	3	3.2
LSIL	169	33.1	103	52.8	49	46.7	15	12.9	2	2.1
HSIL	264	51.7	47	24.1	41	39.0	89	76.7	87	91.6
ASC-H	18	3.5	10	5.1	1	1.0	6	5.2	1	1.1
AGUS	1	0.2	0	0.0	0	0.0	0	0.0	1	1.1
Adenocarcinoma	1	0.2	0	0.0	0	0.0	0	0.0	1	1.1
HPV status	511	100	195	100	105	100	116	100	95	100
HPV- / Low-risk HPV+	121	23.7	90	46.2	21	20.0	7	6.0	3	3.2
High-risk HPV+	390	76.3	105	53.8	84	80.0	109	94.0	92	96.8
HPV16+	165	32.3	28	26.7	24	28.6	55	50.5	58	63.0
Multiple HPV infection	97	19.0	24	22.9	27	32.1	29	26.6	17	18.5
Colposcopic Impression	511	100	195	100	105	100	116	100	95	100
Normal	182	35.6	116	59.5	34	32.4	18	15.5	14	14.7
Low-grade lesion	165	32.3	56	28.7	52	49.5	37	31.9	20	21.1
High-grade lesion	164	32.1	23	11.8	19	18.1	61	52.6	61	64.2
Median number of biopsies	2.0 (0-5)		1.0 (0-4)		2.0 (0-4)		3.0 (0-4)		3.0 (0-4)	
P16 immunohistochemistry	511	100	195	100	105	100	116	100	95	100
Negative	220	43.1	182	93.3	27	25.7	8	6.9	3	3.2
Positive	291	56.9	13	6.7	78	74.3	108	93.1	92	96.8

*Including 6 carcinomas

acetowhite colposcopic impression), the absolute increase in CIN2+ yield from the first to the second biopsy was statistically significant and ranged from 5.5% to 10.2%. Yields for histological endpoint CIN3+ were lower, but the absolute increase in CIN3+ yield from the first to the second biopsy was statistically significant in almost all strata, except for the women with normal/acetowhite impression and ASC-US or LSIL referral cytology.

When we studied the larger population of women with at least a directed or non-directed biopsy (n=590) and imputed CIN2+ outcomes for second biopsies, we found similar relative increases in yield for second directed biopsies in all risk strata of colposcopic impression, although absolute CIN2+ yields per biopsy were lower (Supplementary table 1).

Table 2. Cumulative yield of CIN2+ and CIN3+ for 1 or 2 directed biopsies in women with ≥ 2 lesion-directed biopsies

	CIN2+ detected (n)	Cumulative CIN2+ yield (%)	95% CI	CIN3+ detected (n)	Cumulative CIN3+ yield (%)	95% CI
ALL (n=263)						
Bx1	136	51.7	45.7-57.7	63	24.0	19.2-29.5
Bx1-2	159	60.4	54.4-66.2	80	30.4	25.2-36.2
Colposcopy impression normal/acetowhitening (n=0)						
Bx1	0	0.0	n.a.	0	0.0	n.a.
Bx1-2	0	0.0	n.a.	0	0.0	n.a.
Colposcopy impression low-grade (n=91)						
Bx1	28	30.8	22.2-40.9	11	12.1	6.9-20.4
Bx1-2	35	38.5	29.1-48.7	12	13.2	7.7-21.7
Colposcopy impression high-grade (n=172)						
Bx1	108	62.8	55.4-69.7	52	30.2	23.9-37.5
Bx1-2	124	72.1	65.0-78.3	68	39.5	32.5-47.0
HPV16- (n=164)						
Bx1	63	38.4	31.3-46.0	22	13.4	9.0-19.5
Bx1-2	76	46.3	38.9-54.0	31	18.9	13.7-25.6
HPV16+ (n=98)						
Bx1	72	73.5	64.0-81.2	41	41.8	32.6-51.7
Bx1-2	82	83.7	75.1-89.7	49	50.0	40.3-59.7
Referral cytology ASC-US (n=18)						
Bx1	7	38.9	20.3-61.4	4	22.2	9.0-45.2
Bx1-2	8	44.4	24.6-66.3	4	22.2	9.0-45.2
Referral cytology LSIL (n=60)						
Bx1	12	20.0	11.8-31.8	4	6.7	2.6-15.9
Bx1-2	16	26.7	17.1-39.0	5	8.3	3.6-18.1
Referral cytology HSIL+ (n=185)						
Bx1	117	63.2	56.1-69.9	55	29.7	23.6-36.7
Bx1-2	135	73.0	66.2-78.9	71	38.4	31.7-45.6

Bx; Biopsy, Bx1; CIN2+ found with the first biopsy only, Bx 1-2; CIN2+ found with the first two biopsies

Detection of CIN2+ using non-directed biopsies targeting normal appearing areas

In 492/610 women (80.7%) a non-directed biopsy was performed exclusively or in addition to lesion-targeted biopsies. In 156 women the non-directed biopsy was the only collected biopsy. In 158 women the non-directed biopsy was the second biopsy, in 124 the third, in 53 the fourth. Twelve of 156 women with a non-directed biopsy only yielded CIN2+. Among women with one directed biopsy adding the non-directed biopsy increased the CIN2+ yield from 53 to 62/158 (33.5% (95% CI: 26.7-41.2) to 39.2% (95% CI: 32.0-47.0)). This increase in yield was statistically significant (p=0.004). Among women with two directed biopsies the CIN2+ yield increased from 67 to 71/124 (54.0% (95% CI: 45.3-62.6) to 57.3% (95% CI: 48.5-65.6) which was not statistically significant (p=0.125). Among women with three directed biopsies there was no yield of CIN2+ when adding a non-directed biopsy (Table 3).

In an analysis that considered a biopsy as non-directed only if two independent reviewers of the colposcopic images called the impression at the biopsy site normal, without any acetowhitening, only 12 (6.7%) additional CIN2+ were detected which is 4.5% in the total study population. Among women with one directed biopsy adding the consensus non-directed biopsy increased the yield of CIN2+ from 53 to 56/158 (33.5% (95% CI: 26.7-41.2) to 35.4% (95% CI: 28.4-43.2)). This increase was not statistically significant (p=0.250). Among women with two directed biopsies the yield increased from 67 to 68/124 (54.0% (95% CI: 45.3-62.6) to 54.8% (95% CI: 46.1-63.3)) which was not a statistical significant increase either (p=1.000) (Table 3).

Table 3. Detection of CIN2+ by lesion-directed biopsies and additional yield by targeting normal appearing areas, by number of biopsies targeting cervical lesions

Number of biopsies targeting lesions	Yield of CIN2+ based on lesion-directed biopsies	Additional yield of biopsy targeting normal areas	Additional yield of consensus non-directed biopsies
0	NA	12/156 (7.7%)	8/12 (6.6%)
1	53/158 (33.5%)	9/158 (5.7%)	3/96 (3.1%)
2	67/124 (54.0%)	4/124 (3.2%)	1/79 (1.3%)
3	37/53 (69.8%)	0/53 (0%)	0/25 (0%)

NA; not applicable

Yield of CIN2+ using combined risk stratifications

We calculated CIN2+ yield for different combinations of cytologic, virologic, and colposcopic results with one and two directed biopsies (Figure 1). Among women with HPV16-positive HSIL+ referral cytology and high grade colposcopic impression the yield of CIN2+ increased from 83.1% for those with one directed biopsy to 93.2% for women with two directed biopsies (Figure 1).

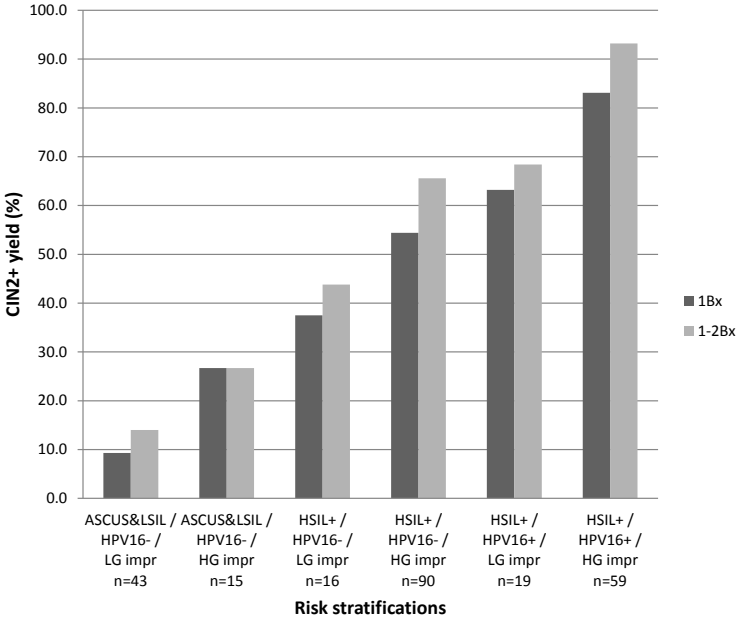

Figure 1. Cumulative CIN2+ yield for one or two directed biopsies using different risk stratifications (cytology grade, HPV16 status and colposcopic impression) in women with ≥ 2 directed biopsies.

DISCUSSION

Main findings

Colposcopy of the cervix is a key diagnostic procedure in women with abnormal cytology that aims at the detection of cervical precancer. It requires experience to acquire expertise in this procedure but even then there is only moderate correlation between colposcopic assessment and the final histological diagnosis. [3, 4] New evidence in cervical cancer prevention strategies was being translated in a supplement of the European guidelines for Quality Assurance in Cervical Cancer Screening, [1] but colposcopic practice was not addressed in detail. A recent US study used an extended biopsy protocol in a US colposcopy referral population to evaluate the incremental benefit of taking up to four biopsies (Unpublished observations by Wentzensen et al.). We applied this protocol in a European study population of women referred according to conventional European screening practice with a median age of over 35 years (compared to 27 years in the US population). We showed that the yield of CIN2+ and CIN3+ increased significantly when a second biopsy from lesion-directed tissue was added. Even when there was a high-grade colposcopic impression, not all prevalent CIN2+ was detected with one directed biopsy, highlighting the difficulty of identifying the worst lesion on the cervix. When we excluded p16 negative CIN2 lesions to achieve high specificity for cervical precancer following the recent LAST guidelines, [16] the results were similar. Dif-

ferences in number of collected biopsies and colposcopic impression for both study sites in the Netherlands and Spain with comparable referral cytology and HPV results represent the importance of the need of consensus colposcopic practice.

Strengths and limitations

To our knowledge this is one of the first studies systematically investigating colposcopic performance for the detection of high-grade cervical lesions by collecting multiple biopsies. Since this study has a cross-sectional design we did not use longitudinal histological end point data and we might underestimate the prevalence of CIN2+. Women included in the study receive 6 monthly cytological follow-up for at least 2 years after the first visit to estimate missed prevalent and incident disease. This follow-up is currently ongoing. Since only 19% of women received 3 or 4 lesion-directed biopsies in our study, we were unable to draw conclusions from the CIN2+ yield for the third and fourth directed biopsies. To study the more complete, heterogeneous population including women with less than 2 directed biopsies, we performed an analysis where we imputed biopsies that were not collected in all women with less than 4 directed biopsies (Supplementary information 1, Supplementary table 1). This analysis reflects absolute yields for the first and second directed biopsies for the complete study population. Consequently, absolute yields per biopsy were lower, but relative increases in CIN2+ yields were similar to the original yields. Furthermore, an image review was done on digital images which might not be representative for real time colposcopy. [17] We did not observe adverse events related to collection of multiple biopsies and the procedure was well tolerated.

Interpretation

Our findings are in agreement with previous studies that show that a single biopsy from the most worrisome lesion can fail to detect CIN2+. [2, 6, 7] The yield of the first directed biopsy was higher compared to a recent similar US study (Unpublished observations by Wentzensen et al.). The women in our study were referred according to conventional European screening practice with a median age of over 35 years and on the basis of any abnormal cytology grade including a single ASC-US smear. Compared to the US study the median age and the proportion HSIL referral cytology in our study were higher, and more CIN2+ cases were diagnosed. This shows that more high-grade disease was present in our study compared to the US study population and this difference determines the yield of CIN2+ in both studies. We showed that performing a second directed biopsy appeared particularly useful. For comparison, a recent large study on the benefit of ECC found a yield of only 1% for endocervical sampling, [18] which is lower compared to the 5-10% yield of a second biopsy observed in our study. In other studies on cervical biopsies, multiple non-directed biopsies were collected and it was estimated that 10-30% of CIN2+ were found by these non-directed biopsies. [5, 7, 8, 19] In our study, the total additional benefit of non-directed biopsies for detecting CIN2+ was lower

(9.4%). We regarded 'acetowhitening suggestive for metaplastic changes' as 'normal'. When considering a biopsy as non-directed only if two independent reviewers of the colposcopic images called the impression at the biopsy normal (without any acetowhitening), even less (6.7%) additional CIN2+ was found in women with non-directed biopsies. We found CIN2+ solely in an ECC in 7% of women and this group had a median age of 43 years. As the rate of ECCs showing high-grade CIN increases with age, the proportion of CIN2+ diagnosed in an ECC only in studies with younger populations will likely be less compared with our findings. [7, 20]

CONCLUSION

The results of this study confirm the importance of performing colposcopy according to standardized practice and affirm the importance of performing two instead of one lesion-directed biopsy to detect high-grade cervical disease. Our results suggest that adding an extra directed biopsy rather than collecting only one biopsy from the worst appearing site increases the yield of CIN2+ significantly. Furthermore, when a very low threshold for abnormality (any acetowhitening) is used, adding a non-directed biopsy to increase the yield in detecting CIN2+ might be redundant because prevalent high-grade lesions could be detected by directed biopsies. As a result of the increase in yield of CIN2+, management decisions for women with cervical precancer can be made earlier. However, it remains to be elucidated how relevant the earlier detection of CIN2 and also CIN3 lesions is because small lesions that are not easy to detect with one biopsy are more likely to regress spontaneously than evident lesions. [21] A growing interest exists for clinical use of hrHPV testing to triage women who have minor cervical cytological changes, in the follow-up of women who are treated for CIN2+, and in primary screening against cervical cancer. [22-25] Standardized colposcopic procedures are necessary in optimizing these new triage, follow-up and screening strategies. Our findings confirm that performing an additional lesion-directed biopsy will improve performance and efficiency of cervical cancer screening.

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DETAILS OF ETHICS APPROVAL

Medical ethical approval from the institutional ethics boards from Hospital Clinic, Barcelona, Spain and from Reinier de Graaf Groep, Voorburg, The Netherlands was received (protocol number 10-023). The study was registered in the Dutch trial registry (NTR3464).

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SUPPLEMENTARY DATA

Supplementary information 1

Imputation approach

We imputed CIN2+ outcomes for biopsies that were not performed in order to present the range of plausible assumptions. The first model imputed the unobserved yields based on an assumption that an additional biopsy would not have detected any additional CIN2+ (i.e., that yield of a second directed biopsy would have been zero), which is the implicit assumption of current clinical practice that takes only one biopsy from the worst lesion; this model minimizes the yield estimate. The second model imputed based on the assumption that an additional directed biopsy would have had the same marginal yield as among women from whom a second directed biopsy was actually collected; this model maximizes yield. As an intermediate, we estimated that a second directed biopsy had the same CIN2+ yield as an additional biopsy from normal-appearing tissue. All three reasonable assumptions gave similar answers; for data presentation, we showed the third imputation approach when estimating yield of a second biopsy in risk strata of colposcopic impression, referral cytology result and HPV16 result (Supplementary table 1).

Supplementary table 1. Imputed cumulative yield of CIN2+ for 1-2 directed biopsies

	CIN2+ detected (n)	Cumulative CIN2+ yield (%)	95% CI
All women with ≥1 biopsy (n=590)			
Bx1	195	34.9	31.1 - 38.9
Bx1-2	218	42.3	38.4 - 46.5
Colposcopy impression normal/acetowhitening (n=168)			
Bx1	3	8.3	4.8 - 13.9
Bx1-2	3	-*	-*
Colposcopy impression low-grade (n=199)			
Bx1	53	26.6	20.8 - 33.4
Bx1-2	60	32.2	25.8 - 39.2
Colposcopy impression high-grade (n=224)			
Bx1	139	62.3	56.2 - 69.2
Bx1-2	155	70.4	64.0 - 76.3
HPV16- (n=403)			
Bx1	92	24.1	20.0 - 28.6
Bx1-2	105	29.8	25.4 - 34.6
HPV16+ (n=186)			
Bx1	102	58.1	50.6 - 65.2
Bx1-2	112	68.3	61.0 - 74.8
Cytology ASC-US (n=67)			
Bx1	10	14.9	7.8 - 26.2
Bx1-2	11	19.6	11.1 - 31.2
Cytology LSIL (n=194)			
Bx1	21	11.9	7.8 - 17.5
Bx1-2	25	14.0	9.5 - 19.8
Cytology HSIL+ (n=330)			
Bx1	164	52.4	46.9 - 57.9
Bx1-2	182	62.3	56.9 - 67.6

Bx; Biopsy, Bx 1; CIN2+ found with the first biopsy only, Bx 1-2; CIN2+ found with the first two biopsies, * number of cases with ≥ one targeted biopsy is less than 15

chapter 6

Categorisation of CIN to address histological problems of CIN 1 and 2 by combining viral biomarker panHPVE4, proliferation marker MCM and transformation marker p16

Romy van Baars*, Heather Griffin*, Zhonglin Wu, Yasmina Soneji, Miekel M. van de Sandt, Rupali Arora, Jacolien van der Marel, Bram W.A. ter Harmsel, Robert Jach, Krzysztof Okon, Hubert Huras, David Jenkins, Wim G.V. Quint, John Doorbar

*Both authors contributed equally

In preparation

chapter 7

CADM1 and MAL methylation status in cervical scrapes is representative for the most severe underlying lesion in women with multiple cervical lesions

Romy van Baars, Jacolien van der Marel, Peter J.F. Snijders, Agata Rodriquez-Manfredi, Bram ter Harmsel, Henk A.M. van den Munckhof, Jaume Ordi, Marta del Pino, Miekel M. van de Sandt, N. Wentzensen, Chris J.L.M. Meijer, Wim G.V. Quint

In preparation

chapter 8

Oncogenic human papillomavirus-infected immature metaplastic cells and cervical neoplasia

Jacolien van der Marel, Romy van Baars, Inmaculada Alonso, Marta del Pino, Miekel M. van de Sandt, Jan Lindeman, Bram W.A. ter Harmsel, Mathilde Boon, Frank Smedts, Jaume Ordi, Aureli Torné, David Jenkins, Wim G.V. Quint

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ABSTRACT

Persistent cervical high-risk human papillomavirus (hrHPV) infection results in high-grade cervical intraepithelial neoplasia (CIN2/3) and cervical carcinoma. The susceptibility of the cervix to HPV carcinogenesis and the importance of HPV18 in cervical carcinoma despite relative infrequency in CIN2/3 could be linked to hrHPV infection of immature metaplasia (IM) at the squamocolumnar junction. Atypical IM (AIM) is an equivocal category used to describe changes in IM suggestive of high-grade neoplasia, which causes diagnostic and management problems. We used laser capture microscopy combined with polymerase chain reaction in 24 women with HPV18, HPV16, or other HPV infections on cytologic analysis and a cervical loop electrosurgical excision procedure to locate hrHPV in cervical tissue. HPV18-positive AIM and CIN2/3 were present in 7/12 cases with HPV18 on cytologic analysis. In 2 cases with HPV18 and other HPV types, HPV18 was only present in AIM and not in CIN2/3. HPV16-positive AIM was present in 3/7 and HPV16-positive CIN2/3 in 5/7 cases with HPV16. No cases had HPV16 AIM without CIN2/3. Other hrHPV-positive AIM and CIN2/3 cases were present, respectively, in 1/6 and 5/6 cases positive for hrHPV types other than HPV16/18. In a subset, 94% HPV18 AIM regions showed CK17 and p16 positivity, and 41% were CK7 positive. CIN2/3 and AIM with other hrHPVs showed similar patterns. AIM was a particular feature of HPV18 infection in women with CIN2/3. HrHPV infection of CK7/17-positive AIM expressing p16 was particularly seen for HPV18 with and without classical CIN2/3 and should be regarded as a high-grade precancer.

INTRODUCTION

The model of human papillomavirus (HPV) carcinogenesis is HPV infection of basal proliferative cells of squamous epithelium evolving through cervical intraepithelial neoplasia (CIN) grade 1 to high-grade CIN (HG-CIN; CIN2/3) and then cervical carcinoma (CC), but its validity at the tissue level has never been established. [1-3] Molecular and epidemiological studies confirm the association between persistent HPV infection, cervical precancer, and cancer, [4, 5] with approximately a dozen high-risk (hr)HPV types involved. [6] Studies suggest that the high risk of developing CC after exposure to hrHPV compared with other anogenital cancers relates to epithelial metaplasia at the squamocolumnar junction (SCJ) of the cervix and the specialized epithelial stem-like cells involved.[2, 7-10]

The frequency of individual hrHPV types differs between CIN2/3 and CC. [11, 12] HPV16 is the most common in both CIN2/3 and CC. [13, 14] HPV18 is the second most frequent HPV type in CC [15-17] and particularly important in cervical adenocarcinoma (CADC) but has been found less in CIN2/3. [14, 18-22] The relative infrequency of HPV18 in CIN2/3 and its importance in CADC might be explained by infection of glandular cells in the endocervical canal or immature metaplastic (IM) cells of the SCJ where lesions are less accessible for sampling and also result from the fact that HPV18 cause poorly defined morphologic abnormalities that understate the severity of the underlying disease. [23] The present study was undertaken to utilize laser capture microscopy combined with sensitive polymerase chain reaction for HPV DNA (LCM-PCR) to examine the presence of infection by HPV18, HPV16, and other hrHPV types in specific zones and cell populations of cervical epithelium including IM, atypical IM (AIM), and CIN of different grades. [24]

Epithelial remodeling at the SCJ involves expansion of reserve cells that might act as a "stem cell–like" population beneath endocervical glandular epithelial cells to produce proliferating IM cells that mature into squamous metaplastic epithelium morphologically identical to native ectocervical epithelium. [2, 25, 26] Almost all cervical (pre)cancer lesions arise in relation to this transformation zone of the cervix. [27] The reserve cells and cells of IM are positive for cytokeratin (CK)17 [28] and it has been suggested that the true progenitor cell population of the SCJ comprises cuboidal cells expressing CK7 that remodel the SCJ through a "top-down" differentiation process. [1, 2]

AIM was first described as an abnormal cytologic change [29] and has been linked to cervical lesions with immature metaplastic and dysplastic features but not showing all cell arrangement features of CIN3. AIM was described as having a uniform basal cell population, minimal nuclear crowding, variable hyperchromatism, preserved polarity, cells with enlarged nuclei confined to suprabasal areas, and no abnormal mitoses. [30] The main histologic features of IM, AIM, and CIN3 are shown in Figures 1 and 2, and an example of AIM and classical CIN3 present in the SCJ is shown in Figure 3. AIM is not part of the CIN or Bethesda classifications [31] and remains a controversial but widely used diagnosis of uncertain biological and clinical

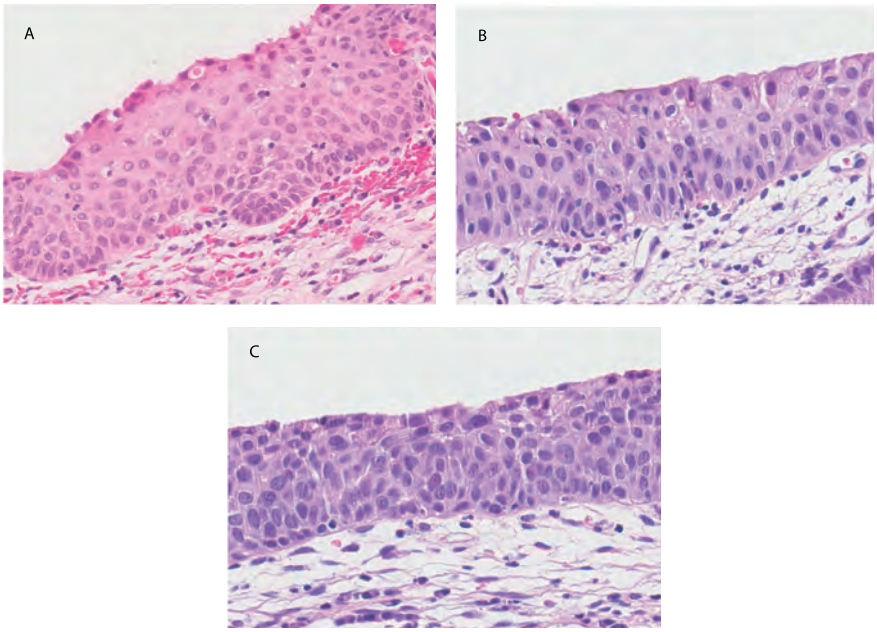

Figure 1. Characteristic H&E staining of immature metaplasia (IM) with a regular arrangement of nuclei in a multi-layered epithelium showing minimal squamous differentiation (original magnification 20x) (Fig. 1A), atypical immature metaplasia (AIM) showing an undifferentiated epithelium with more irregular nuclear profiles and nuclear enlargement and hyperchromicity (Fig. 1B) and cervical intraepithelial neoplasia grade 3 (CIN3) showing no differentiation and densely packed overlapping nuclei (Fig. 1C). AIM differs from CIN3 in showing less marked nuclear enlargement and crowding and less irregularity of arrangement of nuclei and cells.

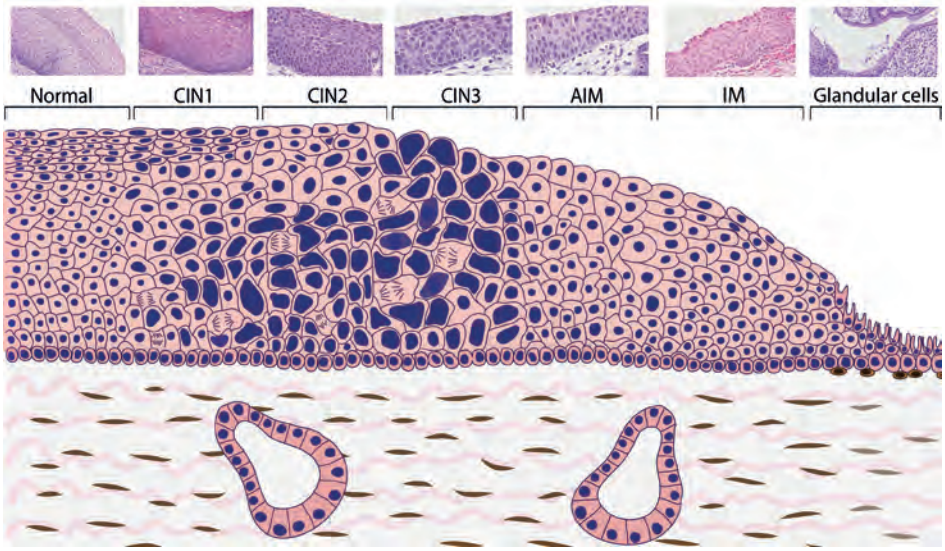

Figure 2. Schematic figure of the different histological types present on the squamo-columnar junction (SCJ) combined with characteristic H&E stainings. The images showing IM, AIM and CIN3 in this figure are presented at higher magnification (20X) in Figure 1.

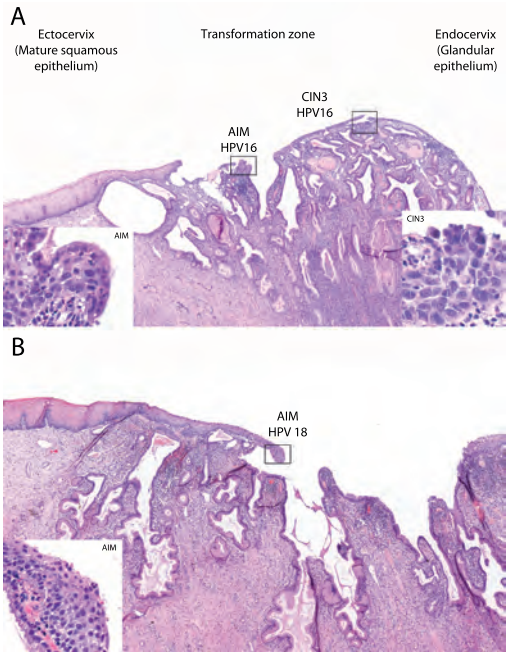

Figure 3. Low-magnification image showing the cervical transformation zone in cross-section with 40X original magnification of regions of atypical immature metaplasia (AIM) and/or CIN3 in the transformation zone. The ectocervix is lined by mature squamous epithelium that shades into the transformation zone. This is defined as the zone between the actual (squamo-columnar junction (SCJ) with endocervical glandular epithelium and the original SCJ recognized by the outer limit of endocervical glands beneath squamous epithelium. Figure 3A: Example of an HPV16 positive case. Figure 3B: Example of an HPV18 positive case.

significance, presenting a problem for deciding clinical management. A proportion of AIM lesions is associated with cervical hrHPV infection. [32] $P16^{INK4a}$ (p16) is a specific biomarker of dysplastic cervical epithelia. [33] Normal IM cells show negative or patchy positivity for p16 compared with diffuse positivity of cells in CIN2/3 or CC. [34, 35] Expression of p16 in AIM has received limited study, [36, 37] although recent US guidelines for grading CIN recommend that any p16-positive area that might be a CIN2 or worse should be managed as a high-grade precancer. [38]

The relation between keratin expression of cells at the SCJ and CIN is complex. [39] Subcolumnar reserve cells show a typical keratin expression pattern, of which CK17 is specific. [40] CK17 has also been described in CIN2/3 but is rarely seen in mature ectocervical squamous epithelium. [8] CK7 is described as a marker for a progenitor cell population at the SCJ and is expressed in CIN2/3. [1, 2] It has been recently proposed that all p16+, SCJ marker (CK7)+ "cervical lesions" should be managed as a borderline high-grade squamous intraepithelial lesion or query-squamous intraepithelial lesion group of lesions [41] even when the lesion is morphologically CIN1.[8]

We previously used LCM-PCR to demonstrate that individual areas of normal cervix and cervical precancer are associated with a single HPV type [24, 42] and that a single HPV type is

found in CC. [43] Our new LCM-PCR findings provide evidence for the view that AIM associated with hrHPV should be regarded as a HG-CIN lesion.

MATERIALS AND METHODS

Case selection

The study included 24 women from a prospective cohort study at the Reinier de Graaf Hospital, Voorburg, The Netherlands, and Hospital Clínic, Barcelona, Spain. We studied women aged 18 years and older, undergoing colposcopy for an abnormal Pap smear. The medical ethical boards of both hospitals approved this study. Written informed consent was obtained from each subject. One important aim of the study was to investigate the location of HPV18 in women with CIN2/3 and HPV18 as part of a multiple infection. Twelve women with HPV18 as a single (n=4) or multiple (n=8) infection on cytologic analysis who underwent loop electrosurgical excision procedure (LEEP) due to CIN2/3 in a biopsy were randomly selected, including 1 woman positive for both HPV16 and 18 on cytologic analysis. Six women with HPV16 as a single (n=2) or multiple infection (n=4) and 6 women with other HPV types (all multiple types) were studied as representative of other hrHPV types.

Cytologic HPV detection and genotyping

A liquid-based cytology sample using a Cervex-Brush (Rovers Medical Devices B.V., Oss, The Netherlands) was obtained. The brush was rinsed in ThinPrep medium (Hologic, Marlborough, MA) at the Hospital Clínic and in SurePath medium (Klinipath BV, Duiven, The Netherlands) at the Reinier de Graaf Hospital. DNA isolation was performed using the QIAamp MinElute Virus Spin kit (QIAgen Inc., Valencia, CA). HPV detection used the HPV-SPF$_{10}$-PCR-DNA enzyme immunoassay (DEIA) system (Labo Bio-medical Products, Rijswijk, The Netherlands), as described elsewhere. [44, 45] Briefly, 10 µL of isolated DNA was used for amplification by broad-spectrum primers targeting a 65 bp region of the L1 gene. The amplification products were detected by the HPV-SPF$_{10}$-PCR-DEIA system, and DEIA-positive SPF$_{10}$ amplimers were used to detect and identify 25 HPV genotypes (6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, and 74) by reverse hybridization on a line probe assay (SPF$_{10}$-HPV-LiPA$_{25}$ version 1; Labo Bio-medical Products). [46]

Histologic processing and diagnosis of histologic samples

A total of 181 formalin-fixed paraffin-embedded (FFPE) tissue blocks were available, 4 to 12 for each of the 24 women from routine LEEP specimens including anterior and posterior lips of the cervix. Sections of 4 µm thickness were cut, and hematoxylin-eosin (H&E) staining was used for initial pathologic diagnosis. Additional sectioning was done using a sandwich method to give one 4-µm-thick section for confirmation of diagnosis (H&E before); 2 sets of

3x8-µm-thick (24 µm) sections for whole-tissue section (WTS)-PCR analysis; 1 slide covered with a polyethylene naphthalate membrane (Zeiss Microimaging GmbH, Jena, Germany) for LCM; and finally, a 4-µm-thick section for pathologic confirmation (H&E after). From selected cases, additional slides for immunohistochemical (IHC) analysis were made. A panel of expert pathologists reviewed all sections independently without knowledge of the HPV status, and consensus diagnosis on individual regions of IM, AIM, glandular and squamous epithelium, and CIN was determined by agreement of 2 of 3 interpretations.

HPV detection and genotyping by LCM-PCR

LCM-PCR was used to locate HPV genotypes in cervical epithelium. LCM samples of different cervical regions were obtained for analysis on membrane slides. LCM-PCR was performed in cases with HPV18 on cytologic analysis and HPV18 as a single-type or multiple type infection in the WTS. For cases with HPV16 on cytologic analysis, LCM-PCR analysis was performed on samples with a multiple infection and a random selection of HPV16 single infections in the WTS. In cases positive for other types on cytologic analysis, LCM-PCR was performed if a multiple-type infection was present in the WTS. The number of LCM regions selected ranged from 5 to 82 per case and was determined by the number of HPV-positive blocks and the presence and size of the different histologic areas. Sampling of multiple regions of mature endocervical glandular tissue was stopped during the study because a large proportion of these regions showed HPV negativity. Nine regions of stroma were sampled: all were negative for HPV. All slides were scanned using digital microscopy (Aperio Technologies Inc., Vista, CA). A pathologist annotated multiple areas of interest defined by consensus review on the membrane slides using the H&E slide as a diagnostic tool. Areas were selected according to standard diagnostic criteria. [26, 30] The sample size ranged from 15,000 to 3,300,000 µm^2. Using the annotated digital image, the selected regions were extracted with the Zeiss P.A.L.M. microbeam ultraviolet laser microdissection and catapulting system and transferred to an Adhesive Cap500 opaque tube (Zeiss, Jena, Germany). LCM-PCR was performed on a negative control (human placenta) to control for potential contamination of the patient samples. No contamination was found. Total DNA from WTS and LCM specimens was isolated from FFPE material by a proteinase K procedure as described elsewhere. [24, 47] Briefly, the tissue was suspended in 100 µL proteinase K solution (1 mg/mL). DNA isolation was performed overnight at 70°C for WTS and on 56°C for LCM samples. Proteinase K was heat-inactivated at 95°C for 10 minutes, and the HPV-SPF$_{10}$-PCR system was used for HPV detection and genotyping. Each run contained negative and internal and external positive controls to monitor for efficiency of DNA isolation, PCR amplification, hybridization, and genotyping procedures. Contamination or failure of analyses was not encountered. An internal DNA control (Rnase P [48]) was used for all samples. Two LCM samples of glandular tissue were excluded due to poor DNA quality.

Immunostaining procedures and antibodies

Immunostaining was performed on additional sections of an available subset of the tissue blocks with AIM areas. Primary antibodies to p16, CK7, and CK17 were applied. For p16, the mouse anti-p16^{INK4a} (Roche diagnostics, Penzberg, Germany) was used. CK7 has been reported as staining a progenitor cell population at the SCJ of the cervix and was detected by anti-CK7 (clone OV-TL 12/30; Dako Netherlands BV, Belgium). CK17 (anti-CK17, clone E3; Novocastra, Leica Microsystems, Newcastle, UK) labels basal and myoepithelial cells of complex human epithelia [40] and, in the cervix, stains reserve cells and IM cells. Slides were stained in an automated immunostainer (Bond-max, Leica Biosystems GmbH, Nussloch, Germany). Antigen retrieval comprised a 20-minute TRIS buffer step. P16 immunostaining was evaluated using a semiquantitative score including both nuclear and cytoplasmic staining and was graded as 0 (negative), 1 (patchy), 2 (strong staining but limited to basal layers; diffuse basal staining), and 3 (strong and diffuse staining, uniform from basal layer to epithelial surface; full thickness diffuse staining). Scores were summarized as negative staining (0 and 1) or positive staining (2 and 3). Grading of CK7 and CK17 staining was done as follows: stratified epithelia (squamous epithelium, IM, AIM, and CIN) were subdivided into a basal compartment comprising half the thickness of the epithelium taken from the basement membrane and a superficial compartment from about 50% thickness to the epithelial surface (extensive staining). The staining in each compartment was scored as positive if >25% of cells were stained. For analyses, scores were summarized as negative or positive staining. Staining in endocervical columnar epithelium and reserve cells was noted separately.

RESULTS

Patient characteristics and histologic diagnoses

We studied 24 women with HPV18, HPV16, or other hrHPV types as a single or multiple infection on cervical cytologic analysis, 11 with HPV18, 6 with HPV16, and 6 positive for other types (HPV6, 53, 66, 31, 35, 51, 53, 56, 59, 68/73). One case was positive for both HPV16 and 18. AIM was present in 1 or more tissue blocks from 6 HPV18-positive cases, in 3 of HPV16 cases, and in 1 case positive for other hrHPV types. In all these cases, HPV18, HPV16, or other types were found in areas of AIM by LCM-PCR (Table 1).

LCM-PCR localizes HPV in different epithelia, and CIN is almost always hrHPV positive

We collected 596 LCM samples of which 263 (44%) were hrHPV DNA positive. The number of selected LCM regions was determined by the number of HPV positive blocks and the presence and size of AIM and CIN2/3 areas (see Supplementary table 1, which shows total sampled LCM area sizes in mm^2 for AIM and CIN2/3 in HPV18, HPV16, and other hrHPV cytology cases).

Table 1. Overview of collected LCM-PCR samples per case, their histological diagnosis and HPV genotyping. The number of sampled LCM regions per HPV type and HPV negative samples are shown between brackets.

	Histology (HPV type [n])					
Subject	**Glandular tissue**	**Squamous epithelium**	**IM**	**AIM**	**CIN1**	**CIN2/3**
			HPV 18 cytology positive cases			
1	neg(8)	neg(14)	-	-	-	-
2	-	neg(8)	neg (1)	18(6)	-	18(23)
3	neg(5)	neg(3)	-	18(1)	-	18(4) neg (9)
4	neg(2)	neg(3) 18(5)	18(1)	18(14) neg(8)	18(2)	-
5	neg(1)	neg(3) 18+68(1)	68(1)	-	-	-
6	neg(1)	18(1) neg(4)	neg(1)	18(1)	-	-
7	neg(2)	neg(3)	-	-	-	-
8	18(1) 31(4) neg(36)	neg(13)	18(1) 18+31(1) untyp(2) neg(2)	18(3)	18(2)	18(2) 31(14) neg(1)
9	-	neg(12)	-	18(3)	-	18(2)
10	neg(23) 18(2)	neg(11) 18(1)	neg(1)	-	-	18(7)
11	53(1)	53(1) neg(6)	-	-	68(1)	68(3) 51+68(1) 18+68(1)
			HPV 16 and 18 cytology positive case			
12	neg(12) 56(2) 18(1)	56(1) neg(5)	51(1) neg(2)	18(1) 56(1) neg(2)	-	18(9) 16(3) 51(2) 51+56(2) 56(1) 18+56(1) neg(2)
			HPV 16 cytology positive cases			
13	neg(3)	neg(6)	-	-	-	-
14	16(1) neg(4)	neg(11)	16(2) neg(1)	16(2)	-	16(3)
15	neg(1)	11+52+53(1) neg(8)	neg(3)	-	-	35(2)
16	16(1) 31(1) neg(4)	16(1) neg(6)	neg(2)	16(1) 31(1)	-	16(7) 31(5) 16+31(1)
17	neg(8)	39(1) neg(11)	neg(7)	16+39(1)	16(1)	16(20)
18	-	neg(5)	58(1)	-	-	16(13) 58(6) 53+58(2)
			Other hrHPV type cytology positive cases			
19	-	neg(1)	-	-	35(1)	35(20)
20	-	neg(3)	-	-	-	56(6) 53(5)
21	neg(3)	neg(9)	neg(1)	-	-	33(1) neg(1)
22	-	neg(7)	-	-	-	-
23	neg(7)	6(2) neg(12)	-	-	31(2)	35(2) 31+35(1) 31(5)
24	neg(1)	56(2) neg(4)	-	31(1)	51(1) 56(1)	31(1)

AIM; atypical immature metaplasia, CIN; cervical intraepithelial neoplasia, HPV; Human papillomavirus, hrHPV; high-risk Human Papillomavirus, IM; immature metaplasia, LEEP; loop electrosurgical excisional procedure, Untyp; untypable by SPF_{10}-PCR, -; no LCM areas present

All LCM samples except 2 regions of glandular epithelium were suitable for HPV DNA testing. All 9 regions of stroma were negative for HPV. HPV genotyping results by LCM-PCR per case have been summarized (Table 1). For CIN of any grade, 186/199 (94%) LCM-PCR samples were hrHPV positive, and HPV18, HPV16, and other types were found in all grades of CIN.

LCM-PCR shows high frequency of hrHPV located in IM and especially in AIM compared with normal glandular and squamous epithelium

Only 4% of LCM areas containing glandular tissue in the HPV18 cases were positive for HPV18 and 5% in the HPV16 cases. No areas of glandular tissue were positive for hrHPV in the other hrHPV-type cases. A similar pattern of infrequent hrHPV positivity was seen in squamous epithelium: 8/95 (8%), 1/56 (2%), and 4/40 (10%) of these areas in HPV18, HPV16, and other HPV type cases, respectively, were positive for the index HPV type. AIM was extensive in HPV18 cases, and HPV positivity rates in AIM were high for HPV18, HPV16, and other HPV-type cases: 29/40 (73%), 4/9 (44%), and 1/1 (100%), respectively (Table 2).

Table 2. Numbers of LCM samples positive for HPV18, 16 and other hrHPV types by HPV index group (HPV18, 16 or other hrHPV types in cytology) and by histological diagnosis. The proportion of HPV positive LCM samples is shown by diagnostic category.

	Histology					
Index HPV type in cytology	**Glandular tissue**	**Squamous epithelium**	**IM**	**AIM**	**CIN1**	**CIN2/3**
HPV18*						
LCM areas sampled	101	95	14	40	5	87
HPV18 positive (% of areas sampled)	4 (4)	8 (8)	3 (21)	29 (73)	4 (80)	49 (56)
HPV16*						
LCM areas sampled	38	56	19	9	1	79
HPV16 positive (% of areas sampled)	2 (5)	1 (2)	2 (11)	4 (44)	1 (100)	47 (59)
HPV other						
LCM areas sampled	11	40	1	1	5	42
Other hrHPV types positive (% of areas sampled)	0 (0)	4 (10)	0 (0)	1 (100)	5 (100)	41 (98)

* One case was positive for HPV 18 and 16 in cytology and LCM samples from this case are analyzed in the HPV18 and 16 index group. AIM; Atypical immature metaplasia, CIN; Cervical intraepithelial neoplasia, HPV; Human papillomavirus, hrHPV; High-risk Human Papillomavirus, IM; Immature metaplasia, LCM; Laser capture microdissection

AIM was not frequently found in cases positive for hrHPV types other than HPV18 and HPV16

In total, 51/100 (51%) LCM regions from cases positive for hrHPV other than HPV16 and 18 on cytologic examination were positive for hrHPV other than HPV16/18. Only 1 area of AIM was available for sampling and was positive for hrHPV. In contrast, 41/42 (98%) of CIN2/3 areas were hrHPV positive (Table 2). Distribution of other hrHPV types in relation to the histologic diagnoses of the LCM regions have been summarized (see Supplemental table 2,

which shows distribution of HPV types in LCM samples from cases positive for other hrHPV types on cytologic examination).

The presence of AIM and CIN2/3 in HPV18, HPV16, and other hrHPV-type cases

When we compared the presence of AIM and CIN2/3 on the case level, in 5 cases no AIM and CIN2/3 was available for LCM-PCR. In 9 cases, both AIM and CIN2/3 were present and sampled. In 8 cases, CIN2/3 was present but AIM was not, and in 2 cases AIM was present but CIN2/3 was not (Table 1). HPV18 cytology–positive cases had relatively more HPV18-positive AIM than HPV16 and other hrHPV cases (58% vs. 43% and 17% of cases, respectively) (Table 3). CIN2/3 was positive for the index HPV type in 58%, 71%, and 83%, respectively (Table 3).

Table 3. Comparison of HPV positivity per case in AIM and CIN2/3 for index HPV type in cytology

	Histology	
	AIM	CIN2/3
Index HPV type in cytology	n (%)	n (%)
HPV18 n=12 cases*	7 (58)	7 (58)
HPV16 n=7 cases*	3 (43)	5 (71)
Other hrHPV n=6 cases	1 (17)	5 (83)

* One case was positive for HPV 18 and 16 in cytology and analyzed in the HPV18 and 16 index group

AIM; Atypical immature metaplasia, CIN; Cervical intraepithelial neoplasia, HPV; Human papillomavirus, hrHPV; High-risk Human Papillomavirus

HrHPV-positive AIM is usually positive for p16, CK7, and CK17, similar to HG-CIN

Twenty-five FFPE tissue blocks from 10 cases were available for IHC staining: 14 with HPV18, 1 with HPV16 and 18, and 10 with HPV16. P16, CK7, and CK17 immunostaining patterns, correlated with HPV typing for each LCM region, are shown in Table 4. Sixteen of 17 (94%) HPV18-positive AIM LCM regions were positive for both p16 and CK17. Two CIN2 HPV18-positive regions were available for IHC, and both were positive for p16 and CK17. Three of 4 HPV16-positive AIM regions and 15/16 (94%) CIN2/3 regions were positive for p16 and CK17 (Table 4A). Both AIM regions with types other than HPV16 or 18 were positive for p16 and CK17. When combining p16 and CK7 positivity (Table 4B), 7 of 17 (41%) HPV18-positive AIM regions were positive for p16 and CK7 and 10/17 for p16 only. Three of 4 HPV16-positive AIM regions were both p16 and CK7 positive. All CIN2/3 regions, disregarding HPV type, showed positivity for p16 and CK7. Different epithelia with HPV genotypes and IHC patterns for p16, CK17, and CK7 are shown in Figure 4.

Table 4. Immunostaining patterns of p16, CK17 and CK7 in LCM areas of glandular tissue, squamous epithelium, IM, AIM, CIN1, 2 and 3.

Table 4A. Immunostaining patterns for p16 combined with CK17.

Histological diagnosis by hrHPV type	Immunostaining pattern			
	p16+/ CK17- (n)	P16+/ CK17+ (n)	P16-/ CK17+ (n)	Total
HPV16 +				
Glandular tissue	0	0	0	0
Squamous epithelium	0	1	0	1
IM	0	1	1	2
AIM	0	3	1	4
CIN 1	0	1	0	1
CIN 2/3	1	15	0	16
HPV18 +				
Glandular tissue	0	0	0	0
Squamous epithelium	2	3	1	6
IM	0	0	1	1
AIM	1	16	0	17
CIN 1	0	1	0	1
CIN 2/3	0	2	0	2
Other hrHPV+				
Glandular tissue	0	0	0	0
Squamous epithelium	0	1	1	2
IM	1	0	1	2
AIM	0	2	0	2
CIN 1	0	0	0	0
CIN 2/3	1	5	0	6

Table 4B. Immunostaining patterns for p16 combined with CK7.

Histological diagnosis by hrHPV type	Immunostaining pattern			
	p16+/ CK7- (n)	P16+/ CK7+ (n)	P16-/ CK7+ (n)	Total
HPV16 +				
Glandular tissue	0	0	2	2
Squamous epithelium	1	0	0	1
IM	0	1	1	2
AIM	0	3	1	4
CIN 1	0	1	0	1
CIN 2/3	0	16	0	16
HPV18 +				
Glandular tissue	0	0	0	0
Squamous epithelium	3	2	0	5
IM	0	0	1	1
AIM	10	7	0	17
CIN 1	1	0	0	1
CIN 2/3	0	2	0	2
Other hrHPV+				
Glandular tissue	0	0	5	5
Squamous epithelium	0	1	1	2
IM	0	1	1	2
AIM	0	2	0	2
CIN 1	0	0	0	0
CIN 2/3	0	6	0	6

p16-/CK17-; Negative staining for p16 (including patchy staining) and CK17, p16+/CK17+; Positive staining for p16 and CK17, p16-/CK17+; Positive staining for CK17 only, hrHPV; High-risk Human Papillomavirus, IM; Immature metaplasia, AIM; Atypical immature metaplasia, CIN; Cervical intraepithelial neoplasia

Data is shown for HPV16, HPV18, other hrHPV type positive LCM samples. Available cases: HPV18: 6 (14 blocks), HPV16&18: 1 (1 block), HPV16: 3 cases (10 blocks).

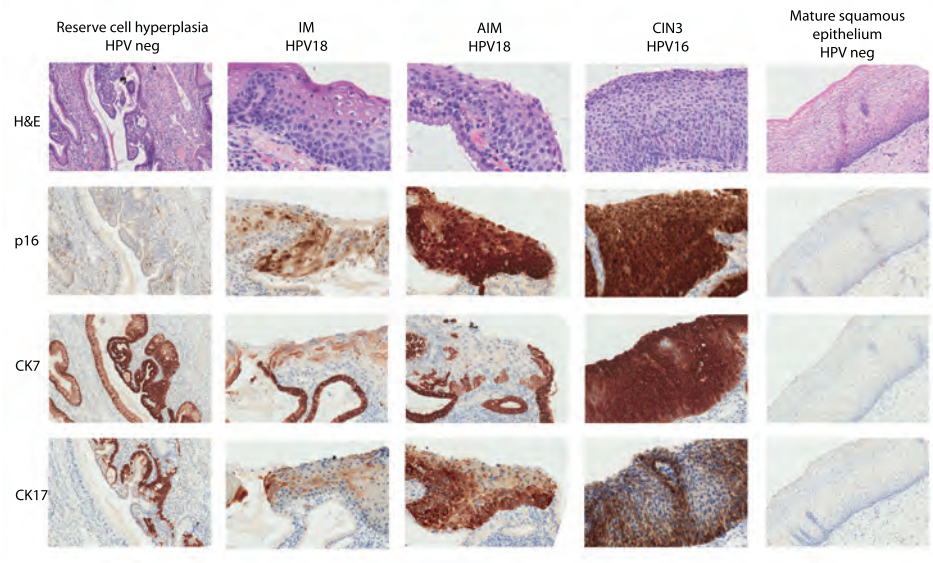

Figure 4. Glandular tissue and mature cervical squamous epithelium without HPV infection, normal IM and AIM and CIN3 associated with HPV18 or 16 infection as seen by H&E, p16, CK7 and 17 staining. Normal mature cervical squamous epithelium does not express p16 or CK7 or CK17. In normal reserve cell hyperplasia and immature metaplasia there is usually expression of CK17, some of CK7, but not of p16. In morphologically normal immature metaplasia associated with hrHPV infection, particularly HPV18, there is also expression of p16. In AIM associated with HPV18 the pattern of p16 and CK7 and CK17 expression is similar to the one seen in CIN3. The subtle differences between IM, AIM and CIN3 in H&E sections are more difficult to identify than the changes in immunohistochemical biomarkers.

DISCUSSION

This study of cervical LEEP specimens from women with CIN2/3 showed that infection of IM cells, and particularly AIM, with hrHPV was more common than infection of mature squamous or glandular epithelium. Furthermore, we observed more extensive AIM areas positive for HPV18 in cases positive for this type on cytologic analysis compared with cases positive for HPV16 or other hrHPV types.

AIM was distinguished from IM by nuclear enlargement and atypia in an undifferentiated multilayered epithelium at the inner part of the SCJ and was positive for p16, CK17, and sometimes CK7. Both cytokeratins were associated with normal cervical remodeling and metaplasia and were also seen in CIN3 and CC as in previous studies. [40] We found diffuse p16 expression in AIM similar to HG-CIN, a pattern that is regarded as marking neoplastic transformation by HPV in the cervix. This pattern has been found in CIN1-expressing markers of epithelial cell remodeling. [8] These findings suggest that infection by hrHPV (particularly HPV18) of IM cells in cervical epithelium being remodeled at the SCJ is a frequent event and in AIM is accompanied by p16 and cytokeratin patterns similar to HG-CIN. Transformation of IM cells by hrHPV provides a potential pathway for the development of CIN and CC in which AIM is an important step, rather than dedifferentiation of mature squamous cervical

epithelium by hrHPV producing CC through CIN1, 2, and 3. The frequency of AIM with HPV18 may be a key to explaining the important, unique role of HPV18 in cervical carcinogenesis.

Using LCM-PCR, we provide new evidence that hrHPV infection of specific CK7+ and/or CK17+ cells at the SCJ could be potentially important in the pathway to CC. One previous study showed a discrete population of CK7+ cuboidal cells at the SCJ of the embryonal cervix persisting into adult life [1] and suggested that this might be the key population susceptible to neoplastic transformation by hrHPV. Interestingly, in our study 7/17 (41%) cases of HPV18-associated AIM and most cases of other hrHPV associated AIM expressed CK7, and its presence was associated with complete absence of any squamous differentiation. Overall, CK7 staining was similar to that described previously but with wider endocervical expression. CK7 staining was generally diffuse in CIN2/3 and AIM. We used CK17 as described previously to mark reserve and IM cells. [28] In the present study, CK17 and CK7 identified related but not identical cell populations. Diffuse p16 expression in hrHPV-associated AIM was identical to that seen in CIN2/3. AIM might be an unrecognized form of CIN3 or an immediate precursor. [49, 50]

The cyto-histologic features of AIM have been known for many years, although the diagnosis remains controversial. We used 3 independent pathologists to ensure consistent diagnosis. AIM is recognized as showing features of neoplastic transformation but is distinguished from usual CIN3 and "reactive" changes by showing less marked nuclear enlargement and crowding, no abnormal mitoses, and less irregular, densely packed arrangement of nuclei and cells than CIN3 (Figs. 1–3). Moreover, AIM may appear with more classical CIN2/3 lesions. In our study, 9/11 cases with AIM also had CIN2/3, and in nearly all cases this was associated with the same HPV type. Although accurate localization of hrHPV infection to AIM has not previously been possible, it has been reported that 19% of AIM showed p16 immunostaining identical to CIN2/3. [36] Another study found hrHPV in whole biopsy specimens from 67% of AIM cases. [32] A concurrent or subsequent diagnosis of CIN2/3 was made in 80% of their hrHPV-positive AIM cases. Our study provides more conclusive evidence that AIM can contain hrHPV, and when it does it is a form of HG-CIN.

Identifying HPV18-associated AIM as a precancerous lesion could explain why HPV18 has a specific role in CC, different from other types. [51, 52] It is not frequently found in CIN, although it is important in CC and especially CADC. [14] CK7/CK17 positivity of IM and AIM cells identifies them as multipotential cells capable of differentiating to both squamous and glandular cells [26] and thus as possible precursor cells for CADC and squamous CC. The demonstration of hrHPV infection, diffuse p16 expression, and CK7/CK17 positivity in routine practice could give a more objective assessment of the difficult morphologic diagnoses posed by atypia in IM cells, by identifying a subset of AIM cases as a form of HG-CIN with a metaplastic phenotype. [49] That approach is consistent with the strategy currently recommended for using p16 in grading CIN in the United States. [38]

This is a cross-sectional study of selected cases of HPV18, HPV16, and other hrHPV-associated cervical abnormalities and did not investigate HPV-associated AIM in women without CIN2/3. More clinical studies are needed to support routine use of cell markers such as CK7 and CK17 in combination with p16 to identify more accurately true HG-CIN and predict progression. Nonetheless, the uncertain histologic changes of AIM when associated with hrHPV or AIM positive for p16, CK17, and/or CK7 should be regarded as potentially HG-CIN and managed accordingly. The association of AIM particularly with HPV18 may explain the relative infrequency of CIN2/3 associated with HPV18 compared with its important role in cervical cancer.

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SUPPLEMENTARY TABLES

Supplementary table 1. Total sampled LCM area sizes of AIM and CIN2/3 in cases positive for HPV18, 16 and other hrHPV types in cytology

	Region size (mm²)		
	HPV18	HPV16	Other hrHPV
AIM	5.0	0.3	0.04
CIN2/3	5.9	3.2	1.8

AIM; atypical immature metaplasia
CIN; cervical intraepithelial neoplasia, HPV; Human Papillomavirus

Supplementary table 2. Frequency of HPV DNA positivity of LCM samples in cases positive for other hrHPV types than HPV16 or 18 in cytology.

	Histologic diagnoses LCM regions						
HPV type	Glandular epithelium (n)	Squamous epithelium (n)	IM (n)	AIM (n)	CIN1 (n)	CIN2/3 (n)	Total
6		2					2
31				1	2	6	9
31+35						1	1
31+56							0
33						1	1
35					1	22	23
51					1		1
53						5	5
56		2			1	6	9
Total	0	4	0	1	5	41	51

AIM; atypical immature metaplasia, CIN; cervical intraepithelial neoplasia, HPV; Human Papillomavirus,
IM; immature metaplasia, LCM; Laser capture microdissection

chapter 9

Discussion

DISCUSSION

The introduction of cervical cancer screening programs has significantly reduced cervical cancer incidence and mortality in developed countries. The conventional screening algorithm consists of cytomorphological assessment of cervical scrapes and if indisputably abnormal or repeatedly borderline, colposcopy with confirmatory biopsy follows. Histological assessment by the pathologist confirms absence or presence of clinically relevant lesions, and women with HG-CIN or worse receive treatment. However, all steps in this screening system have their limitations. In this thesis, we focused on three topics aiming at improving current cervical cancer prevention practice and clinical management: 1. performance and acceptability of self-sampling devices, and corresponding transport modes in relation to various HPV detection methods to improve screening coverage, 2. the improvement of colposcopic detection of HG-CIN through using HPV typing data and by performing multiple biopsies, and 3. the use of additional immunohistochemical and DNA methylation biomarkers to understand more and improve classification of precancer. In the following section the clinical implications of our findings and future research directions will be discussed.

Cytology-based screening

Despites its effectiveness in population-based screening programs, cytology-based screening has several limitations. The main problem is its moderate sensitivity (50-70%), resulting in false-negative results. [1] This limited sensitivity is compensated for by relatively frequent screening (many countries use screening intervals of 1 to 3 years), [2] which is a burden for the vast majority of women not at risk of cervical disease. Moreover, women with repeated equivocal cytology results receive further examination, resulting in over-investigation, overdiagnosis and overtreatment of a substantial number of women with insignificant lesions that will regress spontaneously. [3] Furthermore, interpretation of cytology is subjective and has a low reproducibility. [4, 5] Thirdly, only about 65% of women respond to the invitation to participate in the Dutch screening program. [6] Finally, the detection of adenocarcinoma and its precursor lesions is restricted. [7, 8] Thus, there is an opportunity for improvements.

HPV-based screening

Several randomised-controlled trials have shown substantially higher sensitivity of hrHPV testing compared to cytology testing for the detection of CIN3+. [9-14] Therefore, the implementation of hrHPV testing as a primary screening test is expected to improve further cervical cancer prevention. Consequently, in 2016 hrHPV testing will be implemented as the primary screening test in the Netherlands. Because of the lower specificity of hrHPV testing, triage testing will be needed to identify hrHPV positive women, who need further investigation (i.e. colposcopy) to prevent too many women with transient hrHPV infections being referred for colposcopy with resulting higher health care costs and overtreatment. At present, cytology

at baseline and repeat cytology at 6-12 months is the most feasible and logical option and will be introduced in the Netherlands. [15] However, it is expected that because cytology is subjective, it will face growing competition from more objective biomarkers, some of which are also applicable to self-sampled specimens.

Conclusions on HPV-based screening:

- HrHPV testing is more sensitive compared to cytology for the detection of CIN3 and cervical cancer.
- Like the Health Counsil in the Netherlands has advised, hrHPV-based screening should become the primary screening method.
- Women positive for hrHPV need to be triaged to prevent over-investigation, over-diagnosis and over-treatment.

Impact of HPV vaccination on screening

In 2009, the prophylactic bivalent HPV vaccine was introduced in the Dutch National Immunisation Program for girls of 12 years old with a catch-up for 13-16-year old girls. [16] The HPV vaccines have shown to be highly effective against persistent and incident infections with the vaccine HPV types and high-grade cervical lesions that can develop from such infections. [17-21] However, in the Dutch population the uptake of the HPV vaccine during the catch-up campaign was around 55%. A lower vaccine uptake was observed in girls from conservative religious communities and with lower socioeconomic status. [22] The bivalent HPV vaccination covers the most important HPV types HPV16 and 18, which together account for approximately 70% of cervical cancer cases. Also some degree of cross-protection against phylogenetically related HPV types 31, 33 and 45 has been reported. [23] Until HPV vaccine uptake is increased, an immune cohort has been created, and all HPV types causing cervical cancer have been covered, there is still a need for screening for cervical cancer prevention. Hence, HPV-based screening and new developments to improve the specificity of detecting cervical (pre)cancer will remain necessary for many upcoming years.

Conclusion on HPV vaccination:
HPV 16/18 vaccination has become available. However, until HPV vaccine uptake and HPV type coverage is complete, screening will remain important to prevent cervical cancer for many years, even for vaccinated women

Improved detection of women at risk with hrHPV self-sampling

In the Netherlands, about 35% of women do not respond to the invitation to the cervical screening program (non-responders). [6] Over half of the cervical cancer cases are detected in

this group of non-responders, [24-26] and increasing the participation rate is therefore desirable. Offering the option of hrHPV self-sampling has been shown to increase attendance rate for screening. [27, 28] Cervicovaginal self-collected samples have proved to be as reliable and accurate as physician-obtained cervical samples for the detection of hrHPV. [29, 30]

Furthermore, self-sampling has shown to facilitate access to cervical screening in developing countries with a lack of medical staff and without a screening program. [31, 32] Introducing hrHPV self-sampling in such countries may help to increase worldwide screening coverage and reduce the global impact of cervical cancer which is mainly seen in developing countries. However, in low-resource settings there is limited access to refrigeration and special attention should be paid to storage and transportation conditions at room temperature. Here, brush sampling combined with solid state sample carriers might constitute a suitable self-collection and dry storage and transport system of cervicovaginal specimens for hrHPV testing. [33, 34] In **Chapter 2** we described that hrHPV detection on self-samples using the Viba-brush combined with application of the brush sample to the Indicating FTA-elute cartridge for storage and transport in combination with both the SPF_{10} and the GP5+/6+ HPV assays was significantly lower compared to hrHPV detection on physician-obtained samples. Remarkably however, the sensitivity and specificity for CIN2+ of the FTA cartridge in combination with the highly sensitive SPF_{10}-PCR approached that of physician-obtained samples combined with the clinical validated GP5+/6+-PCR. This underlines the fact that the clinical performance is defined by all the different steps of the diagnostic chain, e.g. the sample collection system as well as test method.

Previous studies have shown that hrHPV self-sampling is highly acceptable to women. [35-39] A pilot study showed that self-sampling is also acceptable to women of non-Caucasian ethnical groups and no cultural or religious barriers were foreseen.[40] Nonetheless, a subset of women is insecure about performing self-sampling properly. [29-33] The Evalyn brush was developed to increase women's confidence and convenience of performing self-sampling and was studied in **Chapter 3**. We found similar hrHPV detection with the Evalyn brush compared to physician-taken smears. Furthermore, the Evalyn brush was highly acceptable to the women who participated in the study. Most women preferred self-sampling to physician sampling because it was simple, time saving by obviating the need for a visit to a physician, easy to use, and less painful than a physician-obtained smear. Interestingly, it appeared that with the dry stored Evalyn brush sufficient material of good quality for molecular testing was obtained, obviating the need for a cartridge for dry storage and transport. Therefore, the Evalyn brush is a good self-sampling device that might be suitable for cervical screening not only in developed countries but also in the developing world. However, our studies were in women referred for colposcopy and before introducing hrHPV self-sampling into the screening program clinical accuracy of hrHPV self-sampling versus hrHPV physician sampling for detecting CIN2+ should be determined in a screening population. [41]

Also, further triage testing of hrHPV positive women needs to be determined before the introduction of hrHPV self-sampling. Cytological evaluation is not feasible on self-sampled material. [42] As a result, hrHPV positive women have to be referred to the gynaecologist for a cervical smear, causing loss to follow-up. Triage testing directly applicable to self-samplers by a non-morphological biomarker would be a fitting alternative. It has been shown that the detection of methylation of tumour suppressor genes is feasible on self-samples. [43, 44] Moreover, recently Verhoef et al. showed that direct triage on self-samples by methylation analysis of MAL and miR124 is non-inferior to cytology triage on an additional physician-taken smear for the detection of CIN2+. [45] This opens the way to full non-morphological screening.

Conclusions on hrHPV self-sampling:

- Offering self-sampling for hrHPV testing is a promising method to improve screening participation rates.
- Cervicovaginal self-collected samples obtained with the Evalyn brush are as reliable and accurate as physician-obtained cervical samples for the detection of hrHPV.
- The Evalyn Brush is a well-accepted self-sampling method for women because it is easy to use, time saving, and more comfortable than collection by a physician.
- Molecular triage on self-sampled material makes screening without interference of a physician possible.

Improved colposcopic detection of lesions

Women with abnormal cytology are offered colposcopy, which is the current standard for identifying high-grade disease and targeting the location of biopsies. Current biopsy procedures rely on visual features of the lesion (i.e. mosaic and punctation patterns, vessels, acetowhitening, etc.) to identify the area on the cervix that most likely represents the worst lesion. [46-50] Further management of the woman depends on the biopsy result and thus on the biopsy placement. Generally, if CIN2 or worse is found the woman receives treatment. Despite the central role of colposcopy in detecting CIN2+, it has been found that it can miss 30-55% of high-grade lesions. [51-55]

We investigated which colposcopic characteristics are associated with the presence of CIN2+ and CIN3+ and if HPV16 positive lesions have a colposcopic appearance different from lesions positive for other hrHPV types (**Chapter 4**). In multivariate analysis, acetowhitening, time of appearance and a lesion size of more than 25% of the visible cervix were the colposcopic characteristics associated with CIN2+. Thus these criteria, which the colposcopist already uses to get an impression of the severity of the lesion and to locate the biopsy, certainly helps identifying women with CIN2+ lesions. In contrast to a previous study [46] we found that the sensitivity for detecting CIN2+ during colposcopy was similar for HPV16 positive and non-16 hrHPV positive CIN2+ lesions. The difference in results between our study and the previous one might be due to the substantially higher age of our study population. HPV16

related high-grade lesions are known to evolve more rapidly than lesions caused by other hrHPV types.[56, 57] This might result in differences in detectability by colposcopy in a younger population, in whom lesions caused by non-HPV16 types might be too small for visualization by colposcopy. Conversely, in an older population high-grade lesions driven by non-HPV16 types have had the chance to grow over time, increasing their detectability.

In conclusion, the performance of colposcopy in detecting CIN2+ lesions is not improved by HPV genotyping in the age category of most European screening programs as studied here, which limits the benefit of this information during colposcopic examination. Moreover, since colposcopic performance seems similar for any hrHPV-type-positive woman, it is likely that the implementation of HPV16 and 18 vaccination should not influence the accuracy of colposcopy in the future.

A previous study in women with ASCUS or LSIL cytology showed that a gain in sensitivity can be obtained by increasing the number of biopsies. [58] Moreover, in a study performing biopsies of colposcopically normal appearing areas, 37% of detected CIN2+ lesions were found in this biopsy only. [59] The results of our study confirmed the importance of collecting two instead of one lesion-directed biopsy to detect CIN2+ and CIN3+ (**Chapter 5**). However, when using a very low threshold for abnormality (any acetowhitening), adding a non-directed biopsy showed only a minimal increase in CIN2+ yield. Therefore, a non-directed biopsy might be redundant if the threshold for abnormality is set low. However, it still remains to be elucidated how relevant the earlier detection of CIN2 and also CIN3 lesions is because small lesions that are not easy to detect with one biopsy are more likely to regress spontaneously than evident lesions. [60] Nevertheless, standardized colposcopic procedures are necessary for optimising new triage, follow-up and screening strategies. Our findings confirm that performing an additional lesion-directed biopsy will improve performance and efficiency of cervical cancer screening. Based on these findings, we advocate implementing a second lesion-directed biopsy and using a low threshold for abnormality in colposcopy guidelines to detect cervical precancer.

Conclusion on colposcopy:

- The colposcopic appearance of HPV16-positive CIN2+ lesion is not different from CIN2+ lesions positive for other hrHPV types.
- Knowledge of the HPV type present does not have additional clinical value during colposcopy.
- Performing a second lesion-directed biopsy improves CIN2+ detection significantly, and should be implemented in colposcopy guidelines.

Improved reproducibility of pathology grading by using immunohistochemical markers

Histology is the gold standard for the grading of cervical disease, but the gold standard itself is hampered by a high inter- and intraobserver variability among pathologists. [61-64] Previous studies have shown that p16 improves the interobserver agreement on histological diagnosis. [65, 66] An impediment to the use of p16 staining in CIN grading is that up to 70% of classical koilocytotic CIN1 lesions are p16 positive (**Chapter 6**). [67, 68] Recent guidelines have recommended treatment of p16 positive lesions that histologically might be CIN2. [69] Given the fact that CIN1 and 2 are the least reproducible histological grades, [64, 70] this has been predicted to lead to overdiagnosis of CIN2 and consequently overtreatment of CIN1 lesions that are p16 positive. [71]

In **Chapter 6** we showed that adding panHPVE4 to p16 staining helps to provide a simple, reliable approach to diagnosing and categorising CIN lesions. We identified with this combination of panHPVE4 and p16 4 distinct staining patterns indicating:

1. Absence of lesions;
2. Lesions with features of a productive infection;
3. Intermediate lesions (both productive and transforming features);
4. Lesions representing transforming infections.

Our data indicate that the CIN2 category is a heterogeneous category that can represent both intermediate (productive and transforming) lesions and transforming infections. This is in line with the paper from Castle et al. who suggest CIN2 to be a mixture of transient HPV infections and true cancer precursors. [72] This, together with a relatively high regression rate of CIN2 [73, 74] suggests that the use of panHPVE4 combined with p16 could provide a reliable approach for grouping CIN2 into two subcategories: CIN2 lesions that are p16 and E4 positive might indicate lesions that are likely to regress, while CIN2 lesions that express p16 but not E4 are more likely to progress to cervical cancer. Therefore, the combination of p16 and E4 might be promising to diagnose CIN more objectively and as a result prevent overtreatment of CIN2 lesions that would have regressed. However, before panHPVE4 staining can be introduced into clinical practice, immunofluorescence staining has to be transformed into a DAB staining and the feasibility of E4 staining on cytology preparations has to be studied. Furthermore, follow-up studies are needed to investigate the frequency and natural history of the subcategories in relation to age, incident HPV infection, regression rate and treatment response to define the best approach to patient management.

Proliferation markers such as MCM and ki-67 are not specific markers for cervical dysplasia as they are also upregulated by inflammation, tissue repair or metaplasia (**Chapter 6**). Therefore, a proliferation marker seemed less clinically useful in grading CIN.

Conclusions on CIN grading:
- p16 staining improves diagnostic accuracy of CIN grading and reduces interobser variability.
- With the aid of HPV-E4 and p16 staining 4 distinct staining patterns can be identified.
- CIN2 is a mixture of productive infections and true premalignant lesions.
- Combined HPV-E4 and p16 staining provides an objective approach to categorising CIN2 into these two groups.

Improved risk assessment by using methylation markers

In order to prevent over-referral rates for colposcopy of women with transient HPV infections triage testing of hrHPV positive women is needed. Host genome DNA methylation testing of the combination of CADM1 and MAL has shown to be a promising triage tool. [75, 76] CADM1 and MAL promotor methylation levels in cervical smears of hrHPV positive women are proportionally related to persistence of hrHPV infections of more than 5 years and increasing severity of underlying cervical (pre)malignant disease. [77] These findings suggest that CADM1 and MAL methylation testing can distinguish women with persistent infections from those with transient hrHPV infections and early lesions with a high likelihood of regression.

However, multiple lesions or complex lesions consisting of different histological grades and duration can be present on the cervix [78] and these can even contain distinct HPV types. [79] It is currently unknown to what extent the methylation status of the cervical scrape reflects methylation of the respective lesions in case of multiple lesions. To explore the possible variation in epigenetic alterations between different CIN lesions present in the same women, we examined the MAL and CADM1 methylation status in multiple biopsies of varying histological grade present on the cervix. In addition, we correlated the expression of methylation markers CADM1 and MAL on corresponding cervical scrapes to that of the different biopsies of these women (**Chapter 7**). The methylation status varied between the individual biopsies representing different degrees of disease. Most biopsies representing CIN2/3 lesions were hypermethylated, while almost all histologically negative or CIN1 biopsies were methylation negative. Moreover, all carcinomas were methylation positive. In some women hypermethylated HG-CIN biopsies were present in coexistence with hypermethylation negative HG-CIN biopsies. This indicates that hypermethylation is not a homogeneous process on the cervix reflecting a kind of field effect, but is clearly restricted to particular high-grade lesion areas. A good agreement in methylation status between biopsies and scrapes was found. This agreement increased with lesion grade and a perfect agreement was found in women with cervical carcinoma. Lack of concordance between scrapes and biopsies, as observed in some women, may reflect inadequacy of either or both sample types, incorrect sampling at colposcopy, and/or differences in cell composition between scrape and biopsy, resulting in different levels of background methylation (**Chapter 7**). [76, 80] Accordingly, assay thresholds for positivity need to be adjusted to the type of sample. Other sample types may even need adjustment

of the marker panel. For example, whereas CADM1 and MAL have been shown to be informative for cervical scrapes [75, 76] and histological specimens (**Chapter 7**), CADM1 was less suited to self-sampled specimens and needed to be replaced by another marker (i.e. miR124) for cervicovaginal self-samples. [44, 45] Also, other human DNA methylation markers and viral DNA methylation have been suggested as alternative triage markers. [81-85] Nevertheless, our work has shown that despite the epigenetic heterogeneity of even high-grade cervical lesions, methylation analysis of a cervical scrape as a triage test is representative of the most severe underlying lesion.

However, as in previous studies, [75, 76] we found that only 65% of cervical scrapes of women with CIN3+ lesions were hypermethylated. Therefore, hrHPV positive, methylation negative women cannot be dismissed from further follow-up and need a repeat HPV test after 12 to 18 months to determine viral clearance or persistence and decide for further follow-up steps.

Conclusions on methylation:

- CADM1 and MAL methylation is heterogeneous on the cervix and lesion-specific.
- Despite this heterogeneity and possible presence of alternating CIN lesions, the overall methylation status determined on the cervical scrape is representative for the methylation status of the underlying cervical lesion, particularly in women with CIN3+.
- This supports the suitability of CADM1/MAL methylation analysis as a triage test for HPV positive women.

HrHPV positive atypical immature metaplasia shows strong evidence for being a cancer precursor lesion

Although atypical immature metaplasia (AIM) was already described in 1943 by Traut and Papanicolaou [86] as a cytological abnormality and in 1983 by Crum et al. [87] as a histological entity, it remained an equivocal diagnosis and was not included in the Bethesda or CIN classification. [88] As a result clinical management of these lesions has been uncertain.

Infection with hrHPV is regarded to be an important initiating factor of cervical carcinogenesis. [89, 90] We studied the presence of HPV18, HPV16 and other hrHPV types using laser capture microdissection in combination with SPF_{10}-PCR [79] in specific regions and cell populations of the cervical epithelium, including immature metaplasia (IM), AIM and CIN (**Chapter 8**). HPV18 was particularly found in AIM areas in women with CIN2/3. The prevalence of HPV18 in normal glandular tissue and normal squamous epithelium was 4% and 8%, respectively, in women with HPV18 in cervical scrapes. By contrast, 73% of AIM regions were HPV18 positive in HPV18 positive cases, comparable to whole tissue results from Geng et al., who found 67% of AIM to be hrHPV positive. [91] HrHPV-positive AIMs are more likely to have concurrent or subsequent CIN3 than HPV-negative AIMs. [91] Furthermore, in a subset of AIM, we found p16 and cytokeratin (CK) 7 and 17 expression patterns similar to those of HG-CIN. P16 is consid-

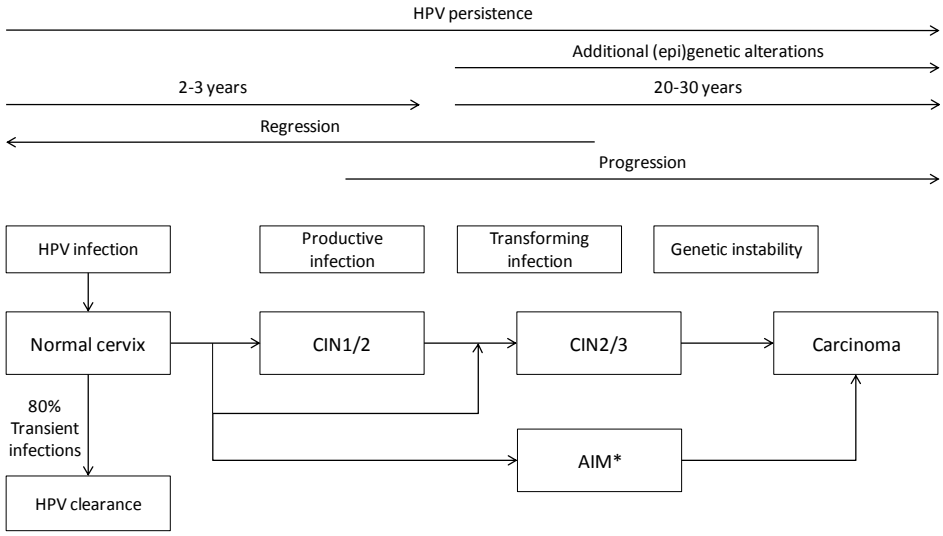

Figure 1. Progression model of cervical cancer [Adapted from Snijders *et al*. 2006] [90]
* HrHPV infected, p16 positive, CK7 and/or CK17 positive AIM might be a high-grade precursor lesion of cervical carcinoma
HPV: human papillomavirus, CIN: cervical intraepithelial neoplasia, IM: immature metaplasia, AIM: atypical immature metaplasia, CK: cytokeratin

ered a specific biomarker for transforming hrHPV infections [92] and previous studies described that p16 positive AIM may represent a spectrum of HG-CIN. [93, 94] Herfs et al. recently proposed that CK7 positive cuboidal cells at the SCJ might represent the cell population that is most susceptible to neoplastic transformation by hrHPV. [95] Consequently, they suggested that all p16 positive, CK7 positive cervical lesions should be managed as a borderline HSIL, or query (Q)-SIL group of lesions. [71] With progression of CIN, increased expression of CK17 is found. [96] Given these findings, hrHPV positive AIM with p16 and CK7 and/or CK17 overexpression can be considered a precancerous entity, similar to HG-CIN (Figure 1). The preferential association of AIM with HPV18 may explain the relative infrequency of HPV18 in CIN2/3, compared with its important role in cervical cancer.[97-99] The demonstration of hrHPV infection, diffuse p16 expression, and CK7/CK17 positivity in routine practice could provide an objective tool to identify the precancerous AIM lesions as counterparts of HG-CIN.[100] Therefore, hrHPV positive AIM showing strong p16, and CK7 and/or CK17 immunostaining should be regarded as potentially precancerous and treated as such.

Future studies are needed to determine whether combined CK7/CK17 and E4/p16 marker testing on cervical smears can be used as triage marker of hrHPV positive women. Moreover, it would be highly interesting to explore further the molecular changes of AIM, by testing the methylation status of these lesions.

Conclusion on atypical immature metaplasia:
Atypical immature metaplasia positive for hrHPV, p16, CK7 and/or CK17 should be regarded as a premalignant lesion and treated as such.

Conclusion

The studies presented in this thesis demonstrated several ways of improving cervical cancer prevention. These new insights might help to improve existing screening algorithms as described in this chapter. We found that improvements can be expected by increasing the participation rate of the screening program through introducing suitable hrHPV self-sampling methods. Self-collected cervicovaginal samples obtained with the Evalyn brush are as reliable and accurate as physician-obtained cervical samples for the detection of hrHPV. Moreover, the Evalyn brush is a well-accepted self-sampling method for women because it is easy to use, time saving, and more comfortable than collection by a physician. Providing hrHPV positive women the option of molecular triage on self-sampled material makes screening without interference of a physician possible. Furthermore, CIN2+ detection during colposcopy can be increased by performing a second lesion-directed biopsy and should therefore be implemented in colposcopy guidelines. Finally, improvements can be made by identification of true precancerous lesions by the use of biomarkers in triage and histological assessment. Although cytology has shown to be an effective triage method in hrHPV positive women, cytology is subjective and unfeasible in low-resource countries. Combined CADM1 and MAL methylation analysis on cervical samples is a suitable triage test to identify women with true precancerous lesions. Histological or cytological assessment with combined CK7/CK17 and E4/p16 staining are also biomarkers that can be used to identify women with true precancerous lesions. Therefore, it is likely that biomarker panels such as mentioned above will play a more prominent role in secondary prevention of cervical cancer in the near future.

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chapter 10

Summary
Samenvatting

SUMMARY

Worldwide, cervical cancer is the third most common cancer in women with the highest incidence in developing countries. In countries with cervical cancer screening programs, the incidence and mortality of cervical cancer have significantly decreased. However, all different steps in current screening algorithms also have their drawbacks. In this thesis, we have explored alternative methodologies aiming at improvements at various steps, including sampling method for the primary screening test, the diagnostic work-up step (colposcopy), and the use of biomarkers.

Chapter 1 is a general introduction describing cervical cancer incidence, human papillomavirus (HPV) infections, cervical cancer screening and treatment and the use of additional biomarkers.

In the new screening program, self-sampling for high risk (hr)HPV detection will be offered to non-responders for primary screening in the Netherlands. In **Chapter 2** we evaluated the clinical sensitivity and specificity for high grade cervical intraepithelial neoplasia (HG-CIN) of self-sampling using the Viba-brush combined with application of the brush sample to the Indicating FTA-elute cartridge for dry storage and transport for hrHPV testing using a clinical validated (GP5+/6+) and highly sensitive (SPF_{10}) HPV test. We found that hrHPV detection with both the SPF_{10} and the GP5+/6+ HPV assays was significantly lower in the self-samples stored on the FTA cartridge compared to physician-obtained samples. However, self-collection by FTA-based self-sampling combined with SPF_{10} hrHPV testing showed a clinical performance close to that of GP5+/6+ on physician-taken samples in this cohort of women referred because of an abnormal smear. This combined collection and test algorithm might therefore be valuable when a liquid-based medium cannot be used, for example in screening of non-responders and in low-resource settings. Our results showed that the clinical performance of hrHPV detection is determined by both the sample collection system and the test method.

In **Chapter 3**, we investigated the clinical feasibility of the Evalyn Brush as a dry transport system compared to concurrently physician-obtained samples for the detection of hrHPV. We also investigated the acceptability of self-sampling using this device and women's preferences for self-sampling or physician sampling. In this study, the dry stored Evalyn Brush showed good agreement for hrHPV detection with the physician-taken smears with both the GP5+/6+ HPV test and the SPF_{10} test. Moreover, the Evalyn Brush was a highly acceptable self-sampling device to 98% of the women in this study.

In **Chapter 4** we evaluated the visual appearance of the cervix using colposcopic characteristics combined with HPV genotyping to predict CIN2+ and CIN3+ in women who were

referred for colposcopy. The outcome of our study revealed that HPV16 related CIN2+ is detected at younger age than lesions positive for other types, but does not show worse colposcopic characteristics. Furthermore, colposcopic sensitivity for HPV16 positive lesions is not improved compared to that for other types in this population of women with an average age over 35 years, following European screening practice.

Because colposcopy can miss 30-50% of HG-CIN lesions, we systematically studied the incremental benefit of one and two lesion-directed biopsies in a study designed for this purpose in **Chapter 5**. A second lesion-directed biopsy was associated with a significant increase in CIN2+ detection. In women with at least two lesion-directed biopsies the yield for CIN2+ increased from 51.7% (95% CI: 45.7-57.7%) for one directed biopsy to 60.4% (95% CI: 54.4-66.2%) for two biopsies. The highest CIN2+ yield was observed in women who were HPV16-positive, had high-grade squamous intraepithelial lesion (HSIL) cytology, and high-grade colposcopy impression. The yield increased from 83.1% (95% CI: 71.5-90.5%) with one directed biopsy to 93.2% (95% CI: 83.8-97.3%) with two directed biopsies. The total additional benefit of non-directed biopsies for detecting CIN2+ was 9.4%. When biopsies were only considered non-directed in case two reviewers called the impression at the biopsy site normal (i.e. without any acetowhitening), only 4.5% additional CIN2+ was detected in non-directed biopsies. These results support that performing a second lesion-directed using a low threshold for abnormality of any acetowhitening should become the standard practice of colposcopy.

In **Chapter 6** we examined the immunohistochemical expression patterns of E4, $p16^{INK4a}$ (p16) and MCM in different histological grades of cervical lesions and we investigated whether E4 gave additional information beyond $p16^{INK4a}$ and MCM in grading CIN objectively. Distinct immunostaining patterns were found in different grades of CIN. All agreed histological negative regions were E4 negative; 82% were p16 negative and had only basal MCM staining. Extensive p16 and MCM staining identified missed CIN3 lesions. Agreed CIN1 was typically E4 positive with limited p16 staining below MCM positive layers, indicating productive hrHPV infection. Some CIN1 resembled CIN2 and was positive for E4 and p16. CIN2 was divided into lesions expressing E4 and p16 (intermediate lesions) and lesions that expressed p16 but not E4 (transforming lesions). CIN3 showed consistent high levels of MCM and p16 with no or minimal superficial E4 staining, indicating transforming lesions. The combination of E4 and $p16^{INK4a}$ provided a simple approach to diagnosing CIN and might therefore provide a basis for refining management decisions about CIN1 and CIN2.

To gain knowledge about possible epigenetic heterogeneity of CIN lesions, in **Chapter 7** we studied CADM1 and MAL methylation in women of whom multiple biopsies representing different CIN grades were taken. We found that methylation positivity increased proportionally

with lesion grade and, importantly, all carcinomas were methylation positive. However, the methylation status varied between the various biopsies representing different degrees of disease. Most HG-CIN biopsies were hypermethylated, while almost all histologically negative or CIN1 biopsies were methylation negative. In some women hypermethylated HG-CIN biopsies were present in coexistence with hypermethylation negative HG-CIN biopsies. Despite the presence of alternating lesions, methylation results of the cervical scrapes were in good agreement with those of the biopsies of the most severe lesion. Hence, methylation status measured in cervical scrapes is representative for the worst underlying lesions, further supporting the use of methylation analysis as disease biomarker.

In **Chapter 8** we studied the presence of HPV18, HPV16 and other hrHPV types using laser capture microdissection combined with SPF_{10}-PCR (LCM-PCR) in specific areas of the cervical epithelium, including immature metaplasia (IM), atypical IM (AIM) and CIN. HPV18 was particularly found in AIM areas in women with CIN2/3. Furthermore, immunostaining with p16, cytokeratin (CK) 7 and 17 was performed in an available subset of the tissue blocks with AIM areas. In those hrHPV positive AIM, we found p16 and CK7 and 17 expression patterns similar to those of HG-CIN, suggesting that these lesions represent cancer precursor lesions.

Finally, in **Chapter 9** a general discussion is provided of the results presented in this thesis, and discusses possible future developments, prospects and clinical consequences.

SAMENVATTING

Baarmoederhalskanker is wereldwijd de op twee na meest voorkomende vorm van kanker onder vrouwen en komt met name voor in ontwikkelingslanden. In landen met een bevolkingsonderzoek voor het opsporen van baarmoederhalskanker zijn de incidentie en sterfte aan deze vorm van kanker aanzienlijk afgenomen. Niettemin hebben de verschillende aspecten van het huidige bevolkingsonderzoek ook hun nadelen. In dit proefschrift hebben we alternatieve methoden onderzocht gericht op de verbetering van deze verschillende facetten, waaronder de afnamemethode van de screeningstest, de diagnostische vervolgstap (colposcopie) en het gebruik van biomarkers.

Hoofdstuk 1 is een algemene introductie over de incidentie van baarmoederhalskanker, humaan papillomavirus (HPV) infecties, de ontstaanswijze van baarmoederhalskanker, de preventie en de behandeling van baarmoederhalskanker en het gebruik van aanvullende biomarkers.

In Nederland zal in het nieuwe bevolkingsonderzoek een zelftest voor de detectie van hoog risico (hr)HPV worden aangeboden aan vrouwen die niet deelnemen aan het bevolkingszonderzoek als primaire screenings methode. In **Hoofdstuk 2** hebben we de klinische betrouwbaarheid van hrHPV-detectie en typering voor het detecteren van hooggradige cervicale intraepitheliale neoplasia (HG-CIN) afwijkingen onderzocht van zelfafnames die aangebracht zijn op een filterpapier dat verkleurt na het aanbrengen van het materiaal, de 'indicating FTA cartridge'. Hierbij hebben we gebruik gemaakt van een klinisch gevalideerde (GP5+/6+) en een zeer gevoelige (SPF_{10}) HPV-test. Hoog risico HPV-detectie op de zelfafgenomen FTA cartridge was lager in vergelijking met het conventionele, door een gynaecoloog afgenomen, in vloeistof bewaard uitstrijkje. In dit cohort van vrouwen die waren verwezen vanwege een afwijkend uitstrijkje, gaf de zelfafname gecombineerd met de SPF_{10} hrHPV-test een klinische sensitiviteit en specificiteit vergelijkbaar met die van de GP5+/6+ hrHPV-test op een door de gynaecoloog afgenomen uitstrijkje. De combinatie van zelfafname met de GP5+/6+-test kan bruikbaar zijn wanneer een vloeistof bewaarmedium niet gebruikt kan worden, bijvoorbeeld bij het screenen van vrouwen die niet reageren op de uitnodiging van het bevolkingsonderzoek (non-responders) en in ontwikkelingslanden. Uit onze resultaten blijkt tevens dat de klinische betrouwbaarheid van hrHPV-detectie wordt bepaald door zowel de afnamemethode als de gebruikte HPV test.

In **Hoofdstuk 3** hebben we de detectie van hrHPV op een nieuw ontworpen borstel voor zelfafname, de Evalyn Brush, vergeleken met een door de gynaecoloog afgenomen uitstrijk. De Evalyn brushes werden droog, zonder bewaarmedium, verzonden. Daarnaast hebben we onderzocht wat de vrouwen van het gebruiksgemak van de nieuwe borstel vonden en

gekeken naar de voorkeur van de vrouwen voor zelfafname of een door een arts afgenomen uitstrijkje. In deze studie kwam de hrHPV positiviteit op de droog bewaarde Evalyn Brushes overeen met die op het door de gynaecoloog afgenomen uitstrijkje en werd het gebruiksgemak door 98% van de vrouwen hoog gewaardeerd.

In **Hoofdstuk 4** beschrijven we een studie in een Europese populatie van vrouwen verwezen voor colposcopie vanwege een afwijkend uitstrijkje. We onderzochten de colposcopische impressie en karakteristieken van HPV16-positieve vrouwen in vergelijking met vrouwen positief voor andere hrHPV types (niet-16 hrHPV-positief). De uitkomst van deze studie was dat HPV16-positieve CIN2+ afwijkingen op jongere leeftijd werden gedetecteerd dan niet-16 hrHPV-positieve CIN2+ afwijkingen. Alle bekende colposcopische karakteristieken waren voorspellend voor CIN2+, maar er was geen verschil in zowel de impressie alsook de colposcopische karakteristieken tussen HPV16-positieve vrouwen en niet-16 hrHPV-positieve vrouwen.

Omdat met het colposcopisch onderzoek 30-50% van de HG-CIN afwijkingen gemist kan worden, hebben we in **Hoofdstuk 5** de opbrengst van CIN2+ voor één en twee laesiegerichte biopten systematisch bestudeerd in een studie ontworpen voor dit doeleinde. Een tweede laesiegericht biopt bleek geassocieerd met een significante toename van het opsporen van CIN2+. In vrouwen met tenminste twee laesiegerichte biopten nam de detectie van CIN2+ toe van 51,7% (95% CI: 45,7-57,7%) met één gericht biopt tot 60,4% (95% CI: 54,4-66,2%) met twee biopten. De hoogste CIN2+ opbrengst werd gevonden in de groep vrouwen die HPV16 positief waren, een ernstige dysplasie (HSIL) in de uitstrijk hadden en een hooggradige colposcopische impressie vertoonden. In deze groep vrouwen nam de opbrengst toe van 83,1% (95% CI: 71,5-90,5%) met één gericht biopt tot 93,2% (95% CI: 83,8-97,3%) met twee gerichte biopten. De toegevoegde waarde van een niet-gericht biopt voor het detecteren van CIN2+ was 9,4%. Wanneer een biopt alleen als niet-gericht werd beschouwd wanneer twee recensenten een normale (d.w.z. zonder enige azijnzuurwitheid) impressie hadden van de afnameplaats van het biopt, werden er maar 4,5% additionele CIN2+ laesies in een niet-gericht biopt gedetecteerd. Deze resultaten ondersteunen dat het afnemen van een tweede laesiegericht biopt de standaard zou moeten worden bij colposcopie en een laag afkappunt voor wat als afwijkend wordt beschouwd moet worden gebruikt.

In **Hoofdstuk 6** hebben we de kleuringspatronen van het HPV E4 eiwit, de transformatiemarker p16 en de proliferatiemarker MCM in verschillende stadia van afwijkingen van de baarmoedermond bestudeerd en gekeken of E4-kleuring toegevoegde waarde had ten opzichte van p16- en MCM-kleuring in het objectief graderen van CIN. We vonden duidelijke immunohistochemische kleuringspatronen in relatie tot de CIN graad. Alle histologisch negatieve regio's waren E4-negatief; 82% was p16-negatief en had alleen basale MCM-kleuring. Door middel van uitgebreide p16- en MCM-kleuring werden gemiste CIN3 laesies geïdentificeerd. Door drie pathologen overeengekomen CIN1 laesies waren typisch E4-positief met gelimiteerde

p16-kleuring beneden MCM- positieve lagen, indicatief voor productieve hrHPV infecties. Sommige CIN1 laesies leken op CIN2 laesies en waren positief voor E4 en p16. CIN2 was verdeeld in afwijkingen die E4 en p16 tot expressie brachten (intermediaire laesies) en afwijkingen die p16 tot expressie brachten, maar geen E4 (transformerende laesies). CIN3 toonde consistente MCM- en p16-kleuring van de volledige dikte van het weefsel, zonder of met minimale oppervlakkige E4 kleuring, indicatief voor transformerende laesies. De combinatie van E4 en p16 leverde een simpele benadering voor het diagnostiseren van CIN en kan mogelijk een basis zijn voor het optimaliseren van het beleid van vrouwen met CIN1 of CIN2 laesies.

Epigenetica bestudeert omkeerbare erfelijke veranderingen in de genfunctie die optreden zonder wijzigingen in de volgorde van de basenparen van het DNA. DNA methylatie is een epigenetisch proces, waarbij een methylgroep (CH_3) aan een cytosine wordt gekoppeld, waardoor de structuur van het DNA verandert. Dit kan leiden tot inactivatie van het gen. Als tumorsupressorgenen geïnactiveerd worden door methylatie, kan dit leiden tot de verandering van een normale cel in een kankercel. CIN laesies kunnen bestaan uit verschillende delen van uiteenlopende duur die elk veroorzaakt kunnen worden door diverse HPV types (met andere woorden; CIN laesies zijn heterogeen). Om de kennis over de mogelijke epigenetische heterogeniteit te vergroten, hebben we in **Hoofdstuk 7** CADM1- en MALmethylatie in verschillende CIN-afwijkingen bij vrouwen met meerdere biopten onderzocht. Methylatiepositiviteit nam proportioneel toe met de ernst van de afwijking. Alle carcinomen waren methylatie positief. De methylatiestatus varieerde echter wel tussen de diverse biopten met een verschillende laesiegraad in dezelfde patient. De meeste HG-CIN biopten waren gehypermethyleerd, terwijl bijna alle histologisch negatieve of CIN1 biopten methylatienegatief waren. Sommige vrouwen hadden gehypermethyleerde HG-CIN biopten naast hypermethylatienegatieve HG-CIN biopten. Ondanks de aanwezigheid van verschillende laesies kwam het methylatieresultaat van het uitstrijkje overeen met het resultaat van het biopt met de meest ernstige afwijking. Methylatie gemeten in uitstrijkjes is dus representatief voor de onderliggende meest ernstige afwijking en kan worden gebruikt voor moleculaire analyses.

In **Hoofdstuk 8** hebben we met behulp van laser-capture microdissection (waarmee je door middel van een laser microscopisch kleine stukjes weefsel kunt bemachtigen) in combinatie met SPF_{10}-PCR (LCM-PCR) de aanwezigheid van HPV18, HPV16 en andere hrHPV types bestudeerd in specifieke regio's van het cervicale epitheel, waaronder immature metaplasie (IM), atypische IM (AIM) en CIN. HPV18 werd voornamelijk gevonden in AIM-regio's in vrouwen met CIN2/3. Daarnaast hebben we een beschikbaar gedeelte van de paraffineblokken met AIM regio's immunohistochemisch gekleurd met p16, cytokeratine (CK) 7 en 17. De p16, CK7 en 17 kleuringspatronen van AIM waren vergelijkbaar met de kleuringspatronen van hooggradige CIN.

Tenslotte bevat **Hoofdstuk 9** een algemene discussie over de resultaten van dit proefschrift, de mogelijke toekomstige ontwikkelingen en de klinische implicaties.

Addendum

List of abbreviations
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LIST OF ABBREVIATIONS

AGUS	Atypical glandular cells of undetermined significance
AIM	Atypical immature metaplasia
AIS	Adenocarcinoma in situ
ASC-H	Atypical squamous cells- cannot exclude HSIL
ASC-US	Atypical squamous cells of undetermined significance
CADC	Cervical adenocarcinoma
CADM1	Cell adhesion molecule 1
CC	Cervical carcinoma
CI	Confidence Interval
CIN	Cervical intraepithelial neoplasia
CIN2+	CIN2, CIN3 or cancer
CIN3+	CIN3 or cancer
CISOE-A	Composition, Inflammation, Squamous epithelium, Other and endometrium, Endocervical columnar epithelium and Adequacy of the smear
CK	Cytokeratin
Cq	Quantifiquation cycle
DEIA	DNA enzyme immuno assay
DNA	Deoxyribonucleic acid
ECC	Endocervical curettage
FFPE	Formalin fixed paraffin embedded
H&E	Hematoxylin and Eosin
HG	High-grade
HPV	Human papillomavirus
HrHPV	High-risk human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
ICC	Invasive cervical carcinoma
IHC	Immunohistochemistry
IM	Immature metaplasia
κ	Kappa-value
LCM	Laser capture microdissection
LEEP	Loop electrosurgical excision procedure
LG	Low-grade
LiPA	Line probe assay
LSIL	Low-grade squamous intraepithelial lesion
N	Normal
NPV	Negative predictive value
MAL	T-lymphocyte maturation associated protein

1.	MCM	Minichromosome maintenance protein
2.	MiR124-a2	MicroRNA124-a2
3.	PCR	Polymerase Chain Reaction
4.	qMSP	Quantitative methylation specific PCR
5.	QSIL	Questionable squamous intraepithelial lesion
6.	SCC	Squamous cell carcinoma
7.	SCJ	Squamocolumnar junction
8.	SPF	Short PCR fragment
9.	OR	Odds ratio
10.	PCR	Polymerase chain reaction
11.	PPV	Positive predictive value
12.	SCJ	Squamocolumnar junction
13.	SD	Standard deviation
14.	TZ	Transformation zone
15.	WTS	Whole tissue section
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AFFILIATIONS OF CO-AUTHORS

Alonso I. (Inmaculada) MD PhD *Department of Obstetrics and Gynaecology* Hospital Clínic, Barcelona, Spain

Arora R. (Rupali) MD *Department of Cellular Pathology* University College Hospital, London, United Kingdom

Bekkers R.L.M. (Ruud) MD PhD *Department of Obstetrics and Gynaecology* Sint Radboud University Medical Centre, Nijmegen, The Netherlands

Berkhof J. (Hans) PhD *Department of Epidemiology and Biostatistics* VU University Medical Center, Amsterdam, The Netherlands

Boon M.E. (Mathilde) MD PhD Leids Cytologisch Pathologisch Laboratorium, Leiden, The Netherlands

Bosgraaf R.P. (Remko) MD *Department of Obstetrics and Gynaecology* Sint Radboud University Medical Centre, Nijmegen, The Netherlands

del Pino M. (Marta) MD PhD *Department of Obstetrics and Gynaecology* Hospital Clínic, Barcelona, Spain

Doorbar J. (John) PhD *Department of Pathology* University of Cambridge, Cambridge, United Kingdom

Geraets D.T. (Daan), MSc DDL Diagnostic Laboratory, Rijswijk, The Netherlands

Griffin H. (Heather) *Department of Pathology* University of Cambridge, Cambridge, United Kingdom

Helmerhorst Th.J.M. (Theo) MD PhD *Department of Obstetrics and Gynaecology* ErasmusMC University Medical Center, Rotterdam, The Netherlands

Huras H. (Hubert) MD PhD *Department of Obstetrics and Gynaecology* Jagiellonian University Medical College, Krakow, Poland

Jach R. (Robert) MD PhD *Department of Obstetrics and Gynaecology* Jagiellonian University Medical College, Krakow, Poland

Jenkins D. (David) MD PhD DDL Diagnostic Laboratory, Rijswijk, The Netherlands

Lindeman J. (Jan) MD PhD DDL Diagnostic Laboratory, Rijswijk, The Netherlands

Meijer C.J.L.M. (Chris) MD PhD *Department of Pathology* VU University Medical Center, Amsterdam, The Netherlands

Melchers W.J.G. (Willem) MD PhD *Department of Medical Microbiology* Sint Radboud University Medical Centre, Nijmegen, The Netherlands

Okon K. (Krzysztof) MD PhD *Department of Pathomorphology* Jagiellonian University Medical College, Krakow, Poland

Ordi J. (Jaume) MD PhD *Department of Pathology* Hospital Clínic, Barcelona, Spain

Quint W.G.V. (Wim) PhD DDL Diagnostic Laboratory, Rijswijk, The Netherlands

Rodriquez A. (Agata), MD *Department of Obstetrics and Gynaecology* Hospital Clínic, Barcelona, Spain

Schiffman M. (Mark) MD PhD *Division of Cancer Epidemiology and Genetics* National Cancer Institute, Bethesda, MD, USA

Smedts F. (Frank) MD PhD *Department of Pathology* Reinier de Graaf Hospital, Delft, The Netherlands

Snijders P.J.F. (Peter), PhD *Department of Pathology* VU University Medical Center, Amsterdam, The Netherlands

Soneji Y. (Yasmina) National Institute for Medical Research, London, United Kingdom

ter Harmsel W.A. (Bram) MD PhD Roosevelt Kliniek, Leiden, The Netherlands

Torné A. (Aureli) MD PhD *Department of Obstetrics and Gynaecology* Hospital Clínic, Barcelona, Spain

van der Marel J. (Jacolien) MD DDL Diagnostic Laboratory, Rijswijk, The Netherlands

van der Munckhof H.A.M. (Henk) DDL Diagnostic Laboratory, Rijswijk, The Netherlands

van de Sandt M.M. (Miekel) MD PhD DDL Diagnostic Laboratory, Rijswijk, The Netherlands

Verheijen R.H.M. (René) MD PhD *Division of Woman and Baby, Gynaecological Oncology* University Medical Center Utrecht, Utrecht, The Netherlands

Wentzensen N. (Nicolas) MD PhD *Division of Cancer Epidemiology and Genetics* National Cancer Institute, Bethesda, MD, USA

Wu Z. (Zhonglin) National Institute for Medical Research, London, United Kingdom

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PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: Romy van Baars	PhD period: 2010-2014
Erasmus MC Department: Obstetrics & Gynaecology	Promotor(s): Prof. Th.J.M. Helmerhorst,
Research School: Molecular Medicine	Prof. P.J.F. Snijders
	Supervisor: W.A. ter Harmsel, W.G.V. Quint

PhD training

	Year	ECTS
General courses		
- Good Clinical Practice, Postgrade, Zeist	2010	0.3
- Biomedical English Writing and Communication, VUmc, Amsterdam	2011	2.3
- Basic Methods and Reasoning in Biostatistics, Boerhaave, Leiden	2011	1.8
- Access database course	2011	1.8
In-depth courses		
- Colposcopy course, Stichting O.O.G.	2010	1.7
- Vulvar pathology, Stichting O.O.G.	2011	1.0
- MGC Special Course Epigenetic Regulation in Health and Disease, LUMC, Leiden	2014	0.6
Presentations		
- Journal Club HumaVac	2010	1.5
- Study presentation for Department of Gynaecology & Obstetrics, Reinier de Graaf Groep, Delft	2010	1.0
- Scientific meeting at DDL Diagnostic Laboratory, Rijswijk	2011	1.0
- Refereeravond Rotterdams Cluster Obstetrie en Gynaecologie	2012	1.0
- Scientific meeting at DDL Diagnostic Laboratory, Rijswijk	2013	1.0
(Inter)national conferences		
- Human papillomavirus (HPV) congres, Amsterdam	2010	0.6
- 27th International papillomavirus Conference, Berlin, Germany (oral poster)	2011	2.0
- 28th International papillomavirus Conference, Puerto Rico (poster)	2012	2.0
- 29th International papillomavirus Conference, Seattle, USA (oral)	2014	2.0
- IFCPC 15th World Congress for Cervical Pathology and Colposcopy, London, UK (poster)	2014	2.0

Teaching activities

	Year	ECTS
- Supervising 3th year internship of MLO students: Fiona Hartgring en Jordy Krijgsman	2012-2013	2.0
- Supervising MLO master's thesis, Charissa Strikwerda, ROC Leiden	2013-2014	2.0

ABOUT THE AUTHOR

Romy van Baars was born on the 9th of August, 1984 in Rotterdam, The Netherlands. In 2002 she completed her secondary school (Gymnasium) at the Comenius College in Capelle aan den IJssel. From 2002 to 2008 she studied medicine at the Erasmus University in Rotterdam. In 2005 she did an internship at the Tribhuvan University Teaching Hospital in Kathmandu, Nepal. Under supervision of prof. dr. Th.J.M. Helmerhorst and dr. ir. L.J. Blok she performed her graduation thesis on "Evaluation of immunohistochemical markers for different vulvar disorders" at the department of Obstetrics and Gynaecology (division Oncological Gynaecology) at the Erasmus University.

After her graduation she worked as a junior resident at the department of Obstetrics and Gynaecology of the Reinier de Graaf Gasthuis in Delft (under supervision of dr. W.A. ter Harmsel and dr. H.A. Bremer). In April 2010 she started her PhD project under supervision of prof. dr. Th.J.M. Helmerhorst, prof. dr. P.J.F. Snijders, dr. W.G.V. Quint and dr. W.A. ter Harmsel. During her PhD period she ran the marathon of Rotterdam.

After the defense of her thesis she will start as a resident in Obstetrics and Gynaecology at the Sint Franciscus Gasthuis (under supervision of dr. R.M.F. van der Weiden) in Rotterdam.

Romy lives together with Berend de Graaf in Rotterdam.

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