RISK ASSESSMENT OF CERVICAL DISEASE BY hrHPV TESTING AND CYTOLOGY

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Risk assessment of cervical disease by hrHPV testing and cytology

Thesis, Erasmus University Rotterdam, The Netherlands

The research described in this thesis has been performed at the Department of Obstetrics & Gynaecology of the Erasmus MC University Medical Center, Rotterdam, The Netherlands, and at the Departments of Obstetrics & Gynaecology, Epidemiology & Biostatistics and Pathology of the VU University medical center, Amsterdam, The Netherlands.

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Inschatting van de ernst van een premaligne cervixafwijking met behulp van cytologie en het testen op hrHPV

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
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door

Mariëlle Kocken
geboren te Rotterdam

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Slechts wie zichzelf blijft, heeft iets te bieden dat nergens anders te vinden is.

Godfried Bomans

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INTRODUCTION

1. EPIDEMIOLOGY OF CERVICAL CANCER

With 529.512 new patients in 2008, cervical cancer accounts for 8.8% of all cancers in women worldwide. Globally, cervical cancer is the third most common cancer in women, with an age-standardised incidence rate (ASIR) of 15.2 per 100,000 women.[1-2]

Approximately 85% of all cases occur in developing countries, where this disease accounts for 13.1% of all female cancers. The cumulative risk of being diagnosed with cervical cancer before the age of 75 years in these countries is 1.9%. In more developed countries cervical cancer is responsible for 3% of all female cancers, and the cumulative risk at the age of 75 years is 0.9% (Figure 1.1).[1-2]

The mortality rates are substantially lower. The global age-standardised mortality rate (ASMR) is 7.8 per 100,000 women, with a total of 274,967 women who died of the consequences of cervical cancer in 2008. These rates vary between 9.7 per 100,000 in developing countries, being approximately 55% of the women diagnosed, and 3.2 per 100,000 in developed countries, representing roughly 36% of the diagnosed group.[1-2] Globally, cervical cancer is the fourth most common cause of cancer-related death in women, however, for women aged between 15 and 44 years it ranks second.[1]

The most important factor that influences the observed trends in incidence and mortality rates is structural cervical cancer screening, which results in earlier detection of cervical cancer and consequently to detection of this disease in lower stages with better survival.[3]

The Netherlands is a country with an effective screening programme [4-5] *(see paragraph 2.2*) and had a declining mortality rate over the last 20 years. The incidence initially decreased as well, but seems to have reached its lowest point in 2003 (Figure 1.2).[6] Since then the number stabilised, and in 2009 707 new cases were diagnosed with an ASIR of 6.0 new cases per 100,000 women. The ASMR has been estimated at 1.3 deaths per 100,000 women with a

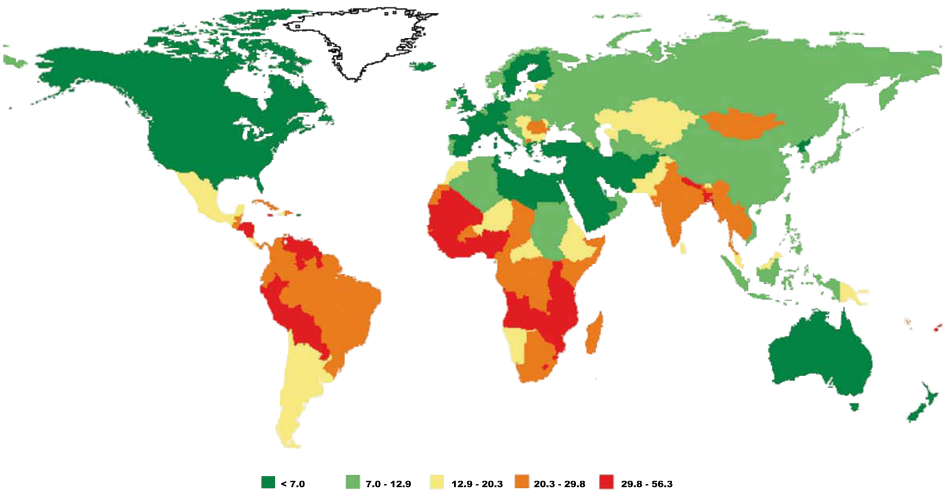

Figure 1.1 World Standard cervical cancer incidences by country (rate per 100,000) [Source: GLOBOCAN 2008].[2]

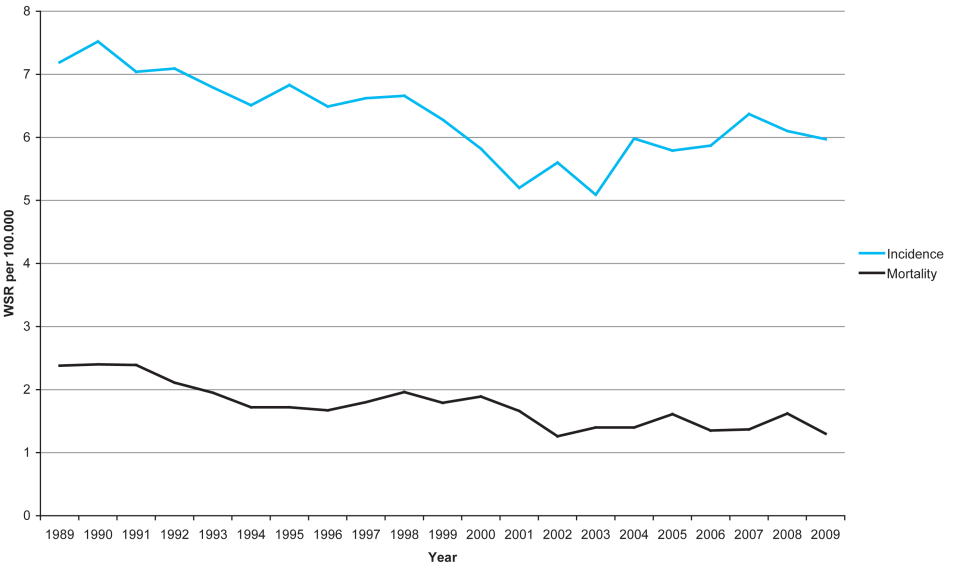

Figure 1.2 World Standardised Incidence and mortality rate of cervical cancer in the Netherlands [Source: Netherlands Cancer Registry].[7]
WSR, World Standardised Rate.

total of 209 women who died in 2009.[7-8] In this year, cervical cancer accounted for 2.1% of all newly diagnosed malignant tumours in Dutch women and for 1.3% of all cancer-related female deaths.[7] The 5-year overall survival in the Netherlands is 67%.[7]

2. PATHOLOGY AND DETECTION OF CERVICAL CANCER

2.1 Cervical cancer and the transformation zone of the cervix

The uterine cervix is the lower part of the uterus and is positioned at the edge between the uterus and the vagina. It consists of two parts; the inner part (the cervical canal, i.e. endocervix) covered by a single layer of mucus-secreting columnar epithelium and the outer part (ectocervix) which is lined by stratified non-keratinizing squamous epithelium. The boundary between these two types of epithelium is called the squamo-columnar junction (SCJ). The original SCJ is the site at which the columnar epithelium of the cervical canal touches the squamous epithelium that covers the ectocervix and vagina at time of birth. Due to hormonal changes the border between columnar and squamous epithelium shifts towards the cervix, forming the new SCJ. The area between the original and the new SCJ is called the transformation zone (TZ). This zone is easily visible on the outer part of the cervix in the majority of women between 20 and 40 years of age. In post-menopausal women it is often withdrawn into the endocervical canal and no longer visible during examination. At the TZ the metaplastic transformation of columnar into squamous epithelium occurs. This is a physiological process and arises from the

sub-columnar reserve cells. These CK17-positive cells mount up and are able to differentiate in both columnar and squamous epithelium.[9] As long as the squamous metaplastic process in the TZ has not completed, the TZ is assumed to be vulnerable for oncogenic influences, such as an infection with high-risk types of the human papillomavirus (hrHPV).[10-11] It is assumed that especially the reserve cells are susceptible for hrHPV infection. Most of these infections are cleared spontaneously, but in about 20% of the cases chromosomal instability with activation of oncogenes and inactivation of tumour suppressor genes occurs, leading to the development of cervical cancer precursor lesions (cervical intraepithelial neoplasia) and if no intervention is performed, finally to cervical cancer in the TZ. These lesions can be identified by colposcopic examination and diagnosed by histology.

2.2 Detection of cervical cancer

Cervical cancer is classified into different histological types of which squamous cell carcinomas (SCCs), accounting for 80% of all cervical cancers, adeno-squamous and adenocarcinomas (ACs), comprising approximately 15%, are the most important. The remaining 5% include rare tumours, among which neuro-endocrine carcinomas and clear-cell carcinomas.

SCCs, which derive from squamous cells, are preceded by dysplastic precursor lesions characterised by a disturbed epithelial architecture and cellular atypia. In the late 1960s the concept of cervical intraepithelial neoplasia (CIN) was introduced, assuming that cervical cancer develops from non-invasive premalignant stages.[12] CIN lesions were categorised into three groups of which CIN1 shows dysplasia in less than one third of the epithelium, CIN2 in two third of the epithelium (moderate dysplasia) and CIN3 in more than two third of the epithelium (severe dysplasia or carcinoma in situ). Invasive SCC is present when the basal membrane has been invaded (Figure 1.3).

Even without treatment most CIN lesions will regress, but the higher the CIN grade, the less often regression occurs. Approximately two third of CIN1 lesions will regress, but only one third of CIN3 lesions reverts.[13-14] To reflect their relative risk to progress to cervical cancer, CIN2 and CIN3 are also called high-grade CIN, whereas CIN1 lesions are called low-grade CIN.[14-15]

Less is known about the precursor lesions of ACs. These tumours arise from the glandular cells in de endocervix and are often preceded by adenocarcinoma *in situ* (AIS).[16] Due to the lack of criteria to define reproducible precursor lesions which can be distinguished from AIS [16], a classification similar to the squamous precursor lesions has been suggested,[17] but not established.

The recognition that cervical cancer develops through different premalignant stages (precursor lesions) which can be detected years before cervical cancer appears [10, 15], has resulted in the organisation of population-based screening programmes. The ultimate goal of population-based screening is to decrease the mortality of cervical cancer. This is achieved by early detection and treatment of precursor lesions and early stages of cervical cancer by which the development of advanced-stage cervical cancer can be prevented.[5]

Because the introduction of cervical screening programmes preceded the development of randomised controlled trials, screening effectiveness has not been investigated with what nowadays is considered the gold standard of scientific evidence.[18] Instead the effect of screening has been evaluated by tracking incidence and mortality rates over a certain time

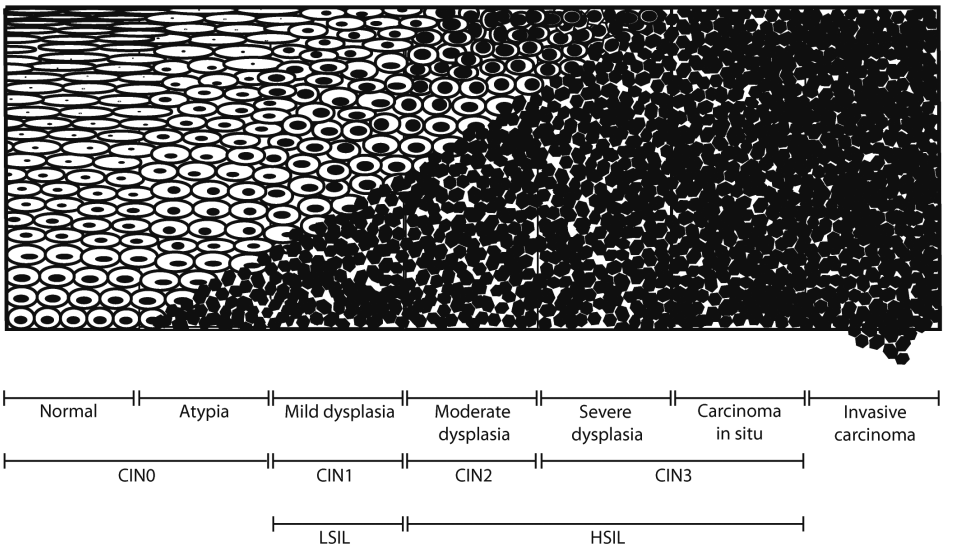

Figure 1.3 Schematic representation of histological classification during the development of cervical cancer [Adapted from: Snijders et al 2006].[15]
CIN, Cervical Intraepithelial Neoplasia; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

period. Most of these data showed a positive effect of screening.[4-5, 19-22] For all that, in both the Netherlands and the United Kingdom (UK) a decreasing cervical cancer incidence was already visible before the introduction of screening programmes, possibly due to improved sexual hygiene.[6, 20] Prerequisites for an effective programme are a high level of participation, a sensitive and specific test, availability of an adequate intervention, and adequate follow-up examinations after initial detection of an abnormality.[10] As can be seen from the decreasing incidence and mortality rates in relation to the costs, the Netherlands has, compared to other countries, an effective screening programme.[4-5]

2.2.1 Cytological screening

Already in the 1940s Dr. Papanicalaou described the relationship between exfoliated cells of the vagina and the presence of (precursor lesions of) cervical cancer.[23] This finding eventually evolved into the cytological smear, also known as PAP-smear, and resulted in the worldwide implementation of cytology-based screening as a diagnostic tool to identify cervical disease. By cytomorphologic examination of the obtained squamous and columnar epithelial cells from the TZ, cervical lesions may be detected.[24] The original PAP-classification ranges from PAP0 to PAP5 and reads as follows: PAP0 inadequate specimen, PAP1 normal cytomorphology, PAP2 borderline dyskaryosis, PAP3 mild-severe dyskaryosis, (in the Netherlands divided in PAP3a1 mild dyskaryosis, PAP3a2 moderate dyskaryosis and PAP3b severe dyskaryosis), PAP4 suspect of carcinoma *in situ* and PAP5 suspect of at least micro-invasive cancer. Women with an abnormal test result (i.e. ≥PAP2) are followed in the screening programme more closely by either repeat cytology or referral for colpscopy than women with normal cytology as they have an increased

risk of developing cervical cancer. This PAP-classification has been improved in the past years and has resulted in the Bethesda 2001 classification that is currently used in most countries.[25] In the Netherlands the CISOE-A classification (in Dutch KOPAC-B) is in use, which has resulted in a high quality and reproducibility of cytological results *(see also paragraph 3.1 and Table 1.1).*[26]

Although effective, cytological screening has several shortcomings. The first weakness is the low sensitivity to detect cervical lesions. Reasons for this limited sensitivity are sampling errors in which abnormal cells are not obtained from the cervix, and reading errors in which the few abnormal cells are not identified between the magnitudes of normal cells.[24, 27-28] Cytology has a sensitivity of approximately 65%[29] (range 30-87%).[27-28] To limit the high number of false negative test results, repeated testing over a (relatively) short interval is required.[28] This enables the detection of abnormalities during the relatively long interval between the first cytological abnormalities and the development of cervical cancer *(see paragraph 2.3)*. The second weakness is the moderate specificity of approximately 95%[29] (range 86-100%).[27-28] This results in a substantial number of women with minor abnormalities who do not harbour underlying high-grade disease. The third weakness is that although cytological screening has resulted in a decline in incidence and mortality of the most common type of cervical cancer, SCCs, the incidence of ACs has been stable or has even increased[3, 10, 16], suggesting that cytology fails to detect ACs and its precursors in an efficient manner. Finally, the interpretation of cytology is subjective and therefore has a moderate reproducibility.[27]

While cytological screening has proven its efficiency, the search for improvement is a continuing story. Previous enhancements in cytological screening have been the introduction of the endocervical brush, which has improved sampling and has lead to a better identification of premalignant lesions[30], and liquid-based cytology, which did not result in a higher sensitivity, but did lower the number of inadequate smears[31-32] and has the possibility to use excess material for additional molecular testing.[33]

2.2.2 Human papillomavirus testing

2.2.2.1 Classification of HPV

Human papillomaviruses (HPVs) are non-enveloped, double–stranded DNA viruses of approximately 8000 base pairs and belong to the family of Papillomaviridae.[34] The viral genome can be subdivided in an early (E) encoding region, containing 6 genes (E1, E2, E4, E5, E6, and E7) and a late (L) encoding region, containing 2 (L1 and L2) genes. Of the early genes, two regulatory proteins (E1 and E2) modulate replication and transcription, while oncogenic proteins E5, E6, and E7, alter the transformation process which results in the malignant alteration of cervical cells.[34] The E6 and E7 proteins interfere with the hosts tumour-suppressor genes p53 and Rb, deregulate cell-cycle and apoptosis control and thereby induce genetic instability *(see paragraph 2.3.1)*. The two late genes encode two structural proteins composing the major (L1) and minor (L2) capsid proteins.[34] Nowadays over 120 different HPV types are identified. Since the L1 gene is the most conserved gene within the genome, it has (together with E6 and E7) been used for classification of new HPV types, subtypes and variants.[34] A new type has a maximum overlap of 90% with a known HPV type, a subtype has a similarity between 90 and 98%, and if more than 98% is shared, it is considered a variant.[34]

HPVs can be divided in cutaneous and mucosal types based on their privileged site of infection. While cutaneous HPV types primarily infect the skin, the approximately 40 different mucosal types infect the mucosal epithelium of the ano-genital, respiratory and upper-digestive tract.[34] The latter comprise low-risk HPV types (lrHPV), associated with benign conditions as genital warts (condylomata accuminata), and high-risk, or oncogenic, types (hrHPV), associated with (pre)malignant lesions.[35] According to the World Health Organization the following HPV types are oncogenic: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66.[36] In addition HPV type 26, 53, 68, 73 and 82 are considered probably carcinogenic.[35] Not all hrHPV types have the same risk of inducing cervical lesions. HPV16 is most frequently found in both AC and SCC, and accounts for over half of all cases of cervical cancer. HPV18 is more associated with AC, and responsible for approximately 20% of all cervical cancer cases.[10, 37] HrHPV does not only play a causal role in the development of cervical cancer; but also in the majority of vaginal and anal cancers. In addition, a fraction of cancers of the vulva, penis, and SCCs of head and neck, including oral cavity, larynx and pharynx are caused by hrHPV.[35-37]

2.2.2.2 HPV detection methods

Because HPVs cannot be cultured efficiently and the clinical performance of serological assays is poor, alternative methods have been developed to detect and type these viruses.[38-39] Nowadays, diagnosis relies on the molecular detection of viral nucleic acids, particularly DNA and is based on either liquid hybridization or polymerase chain reactions (PCR).[39]

The first method involves hybridization of the target HPV DNA to labelled RNA probes *in situ*.[39-40] The commercially available Hybrid Capture 2 system (HC2, Digene Corporation, USA) is based on this principle. This test, approved by the Food and Drug Administration (FDA), uses a cocktail of probes and can only identify samples as positive or negative for 13 hrHPV types, without genotyping. The detected signal is expressed in relative light units and is proportional to the amount of HPV DNA present in the specimen (viral load). The cut-off can therefore be used to inform about viral load on a semi-quantative basis.

The second method is based on PCR amplification of the target HPV DNA directed by primers that bind to the highly conserved regions within the L1 gene of all mucosal HPV types.[39] As several different genotypes may be the cause of cancer, so-called consensus or general primer PCR systems have been developed that test up to 37 different types concurrently.[41] Examples of these PCR-based consensus primers are GP5+/6+[42-43], PGMY09/11 [44], and SPF10 [38], of which only the GP5+/6+ test has been clinically validated. The read-out systems for detecting the PCR product comprise enzyme immuno-assays, which are most often used for the detection of hrHPV types in general[43] and reverse line blot assays[45] or line probe assays[46] which identify individual genotypes.[39]

The sensitivity of detecting HPV is higher in PCR methods than in liquid hybridization tests as the HC2 test, however not in all situations the test with the highest sensitivity should be used.[41] Considering analytical purposes, needed to identify the epidemiological burden of HPV infections in the monitoring of vaccination studies, the sensitivity should be as high as possible. Analytical sensitivity refers to the proportion of HPV-positive women who are correctly identified by a positive test result. The SPF10 test has a very high analytical sensitivity.[47] In general, very low levels of HPV do not reflect a clinically meaningful infection, i.e. associated

with a high-grade CIN lesion or cervical cancer, but rather a transient or latent infection and a distinction between relevant and irrelevant infections should be made. Clinical sensitivity refers to the proportion of women with (pre)malignant disease who are correctly identified by a positive test result. Testing with a too sensitive test will result in a low positive predictive value and in the diagnosis of more irrelevant infections, which could result in an excess of follow-up tests, referrals and treatment. It is evident that a HPV test in a clinical setting should correctly identify all women at risk of developing pre-existent (pre)malignant cervical disease and preferably gives a negative test result for women not at risk. In order to be of clinical value, a HPV test should balance between a high clinical sensitivity as well as a high clinical specificity. In this, the HC2 and GP5+/6+ have the best results.[41, 47]

2.2.2.3 HPV infection

Since the nineteenth century it has already been recognised that cervical cancer is associated with sexual activity. In the 1970s, Harald zur Hausen identified HPV as the causal factor in cervical cancer, because the epidemiological pattern was similar to that of condylomata accuminata, which were known to be HPV related.[48] Since the late 1990s, epidemiological and biological data have demonstrated without a doubt that HPV is the main causative agent for cervical cancer, being necessary for the development, maintenance and progression of (precursor lesions of) cervical cancer.[10, 37, 49] Therefore, HPV can be detected in almost all cervical SCCs [49] and in 94% to 100% of all ACs [37, 50], and this resulted in the presumption that incorporation of HPV testing in screening programmes might better identify women at risk of developing cervical disease.[37]

hrHPV is a common sexually transmitted virus and the majority of both men and women are infected shortly after starting sexual intercourse.[51-52] Its prevalence in young women in Western countries aged 20-25 years with normal cytology is approximately 20%, and diminishes with increasing age to approximately 5% in women aged 30 years and above.[54] This decrease could be attributed to the acquisition of type-specific immunity during life.[53] In women with CIN the prevalence of hrHPV is even higher, and varies between roughly 40% in women with CIN1 to 80% in women with CIN3 and almost 100% in women diagnosed with invasive carcinoma.[49] It is assumed that the life-time risk to acquire a genital HPV infection is at least 80%.[55] An increasing number of sexual partners, increasing promiscuity of male partners[51 53], lack of condom use[56], a younger age at first intercourse, and smoking[51 53] are associated with an augmented risk of infection. At the transformation zone viral particles gain access to the epithelial basal layer via tiny tears to the mucosa and enter the basal cells.[11, 35] The attachment of the virus to the host cell can be prevented by neutralizing antibodies against HPV, which can be elicited by prophylactic HPV vaccination.[57]

2.3 *Cervical carcinogenesis*

2.3.1 hrHPV related carcinogenesis

Cervical cancer is a multistep process in which hrHPV persistence reflects the first step, but many other steps are required to result in invasive cancer (Figure 1.4).[10, 15]

Despite the fact that the human immune system is not easily alerted by the virus, approximately 80% of hrHPV infections will be cleared by the hosts' immune system within 1-2 years after exposure.[57-61] HPV evades the immune system through several mechanisms. One of

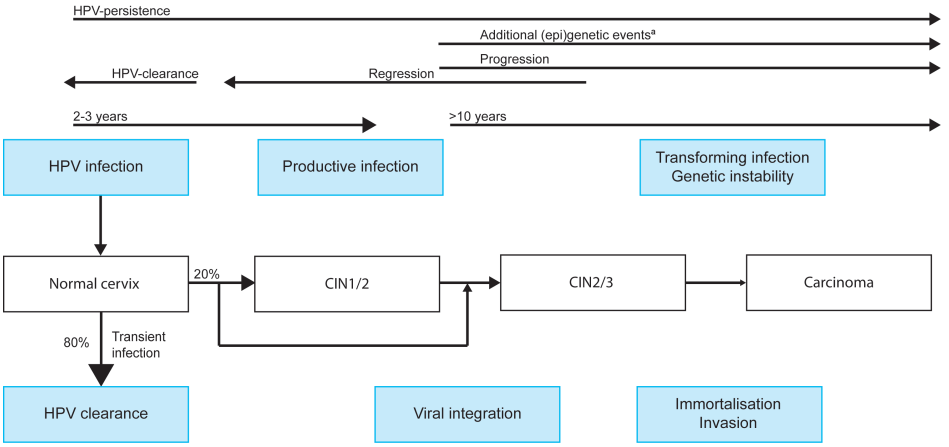

Figure 1.4 Progression model of cervical cancer [Adapted from: Snijders et al 2006].[15]
CIN, Cervical Intraepithelial Neoplasia; HPV, Human papillomavirus.
[a] Activation of oncogenes, loss of tumour suppressor gene function.

these is that HPV replication and release takes place in cells (keratinocytes) that are already programmed to die. By delaying nuclear condensation in the differentiating keratinocytes, koilocytes are formed, and viral replication is permitted. As basal cells further differentiate to produce the protective barrier normally provided by the epithelial cells, and the cells disintegrate, new and infectious viral particles are formed and released in the environment.[15, 57] This productive infection does not necessarily reflect a premalignant stage.[11] Another evasion mechanism is that Langerhans cells, the antigen presenting cells of squamous epithelium, are not activated by uptake of HPV capsids. As a result the Langerhans cells fail to migrate to the draining lymph node to process and present the HPV antigens to naive T-cells, and thereby delaying the priming of the cellular immune response.[57]

As a second step, the persistent infection has to induce the development of a premalignant lesion. Although the process between first infection and evidence of a premalignant lesion usually requires many years, time can be as short as 2 to 3 years.[15, 61-62] Of all HPV-infected women only 1-2% will have a transforming infection[37], which could ultimately result in invasive cancer.[13, 15] An infection changes from a productive into a transforming infection when the viral DNA integrates into the cellular genome of the host, characterised by methylation of E2 binding sites and expression of viral oncoproteins E6 and E7 in dividing basal cells. This results in genetic instability leading to additional genetic changes with an increased expression of E6 and E7 proteins.[15 37] These oncogenes suppress the hosts tumour suppressor genes p53 and Rb[11, 15, 37] which are responsible for cell-cycle control and apoptosis by methylation of their promoter regions.[63-64]

Ultimately this constant over-expression of viral proteins E6 and E7 will, together with additional genetic alterations lead to immortalisation and invasive growth: invasive cancer.[15] This final step takes on average 10-12 years.[15]

Only just a minority of precursor lesions will, if left untreated, eventually develop into invasive disease. While HPV infections are frequently found in sexually active women, cervical

cancers are relatively rare, and for that reason should be considered as an uncommon complication of a HPV infection.[65]

2.3.2 Non HPV-related carcinogenesis

As stated previously, hrHPV can be detected in almost all cases of cervical cancer[37, 49], however in a few rarely occurring subgroups of ACs the causal relationship between HPV and invasive cancer has not (yet) been established.[66-67] In for instance minimal deviation carcinomas and clear-cell adenocarcinomas (CCACs) other factors may be involved. Approximately 60% of all CCACs are associated with prenatal exposure to diethylstilbestrol (DES).[68] In the remaining 40% repression of the INK4a-ARF locus, mutations, or deletions in tumour suppressor genes p53 or Rb may be involved, but the exact aetiology is still unknown.

3 PREVENTION OF CERVICAL CANCER

3.1 Pre-treatment

3.1.1 Screening programme in the Netherlands

In the 1970s cytology-based screening in the Netherlands started with a pilot study in three regions (Rotterdam, Nijmegen and Utrecht) covering 24% of the Dutch female population. However, prior to disclosure of the results of this pilot study, further screening projects were developed. In 1989 a nationwide 3-yearly screening programme started for women aged 35-54 years. In the 1990s evidence pointed towards a suboptimal performance of the screening programme, in terms of both organisation and efficiency.[69] Therefore, the screening programme was revised in 1996: the screening interval was lengthened to 5 years and the age range broadened to 30-60 years.[70] This resulted in increased coverage and efficiency and a decrease in the number of smears taken outside the screening programme (opportunistic screening).[4] Also a descriptive extension was added to the PAP classification, CISOE-A (in Dutch KOPAC-B).[26, 70] This increased the reproducibility of different diagnoses and lead to more strict criteria for a PAP2 diagnosis, which resulted in a decrease of PAP2 diagnoses from 10% in 1990 to 2% in 2000.[26] In the CISOE-A classification 5 items are scored: C for Composition, I for Inflammation, S for Squamous epithelium, O for Other abnormalities and endometrium, and E for Endo-cervical columnar epithelium. The A stands for adequacy of the smear. The S, O, and E are the only parameters specifying the smear classification as used in other nomenclatures concerning cytological pathology. The CISOE-A classification can be easily translated in classifications used in other countries (Table 1.1).[26]

Nowadays approximately 800,000 Dutch women are annually invited for the population-based screening programme.[33] The attendance rate is 65% and the coverage, which is the proportion of women who had a smear taken in the preceding 5 years, is 77%.[4] To increase the coverage of the screening programme, self-sampling seems a good procedure.[33, 71-72] Over half of all invasive cancers arise in women who are not adequately screened, and increase of the participation rate is therefore desirable.[73] Women with normal cytology (PAP1), comprising 96.5% of all screened women, are recalled at the subsequent screening round after 5 years.

Table 1.1 Terminologies in use for classifying cervical cytology. [Adapted from: Bulk et al 2006 and Bulkmans et al 2004] [26, 78]

CISOE-A	C0	S1,E1-2, O1-2		S2-3, O3, E3	S4, E4-5	S5, O4-5	S6,O6,E6	S7,E7	S8-9, O7-8, E9
PAP	PAP0	PAP1		PAP2	PAP3a1	PAP3a2	PAP3b	PAP4	PAP5
Description	Inadequate	normal		Borderline	Mild	Moderate	Severe	Carcinoma in situ	Carcinoma
BETHESDA 2001	Unsatisfactory for evaluation	negative	Atrophy	ASC-H		HSIL			SCC
				ASC-US	LSIL				
				AGC	AGC favour neoplastic			AIS	AC

CISOE-A, C composition, I inflammation, S squamous epithelium, O Other abnormalities and endometrium, and E endo-cervical columnar epithelium; ASC-H, atypical squamous cells cannot exclude HSIL; ASCUS, atypical squamous cells of undetermined significance; AGC, atypical glandular cells; LSIL, low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion; AIS, endocervical adenocarcinoma in situ; SCC, squamous cell carcinoma; AC, adenocarcinoma

3.1.2 Women with abnormal cytology

In the Dutch screening programme approximately 3.5% of the women have an abnormal cytological test result (≥PAP2, in CISOE-A: ≥S2, ≥O3, and/or ≥E3).[33, 74]

In 2.5% (n=12.500) of screened women these results are borderline or mild dysplasia (BMD, similar to PAP2 and PAP3a1, CISOE-A: S2-4, O3, E3-5).[33, 75] As the vast majority of women with BMD results have no underlying high-grade cervical lesion, but will regress over time, they are not directly referred to a gynaecologist as this would result in substantial over-diagnosis and overtreatment.[13, 59, 74, 76] However, the risk of developing invasive cancer in women with BMD results is not insignificant: approximately 0.2% of them will develop cervical cancer within 24 months.[77] Therefore repeat cytology after 6 and 18 months is recommended.[26, 74, 78] Approximately one-third of the women with BMD are referred for colposcopic examination, because the abnormality persists or progresses at either of these repeat visits.[26] When the test result is normal at 6 and 18 months, women are not recalled until the next screening round.[26, 79]

Besides triaging women with BMD by cytology, triaging by hrHPV is also possible.[58, 74, 80-85] Since 2006, the Netherlands Society of Pathology (NVVP) recommends an additional HPV test in the six month follow-up visit of women with BMD.[86] Women with a repeat smear of BMD and testing positive for hrHPV are referred to a gynaecologist, while those with a negative hrHPV test will get a repeat smear 12 months after the first repeat test (Figure 1.5).

Approximately 1% of the women in the screening programme (n=3500) has a test result of moderate dyskaryosis or worse (>BMD, similar to ≥PAP3a2, CISOE-A: ≥S5, ≥O4, and/or ≥E6).[4] These women have an increased risk of (pre)malignant disease and are referred to the gynaecologist for a colposcopic examination.[78-79]

3.1.3 Colposcopy

Colposcopy was first described in 1925 by Dr. Hinselmann, correlating visual findings at colposcopy with histopathology.[87] Nowadays, colposcopy is, in combination with cytology and histology, well established for the detection of premalignant lesions. A colposcope has

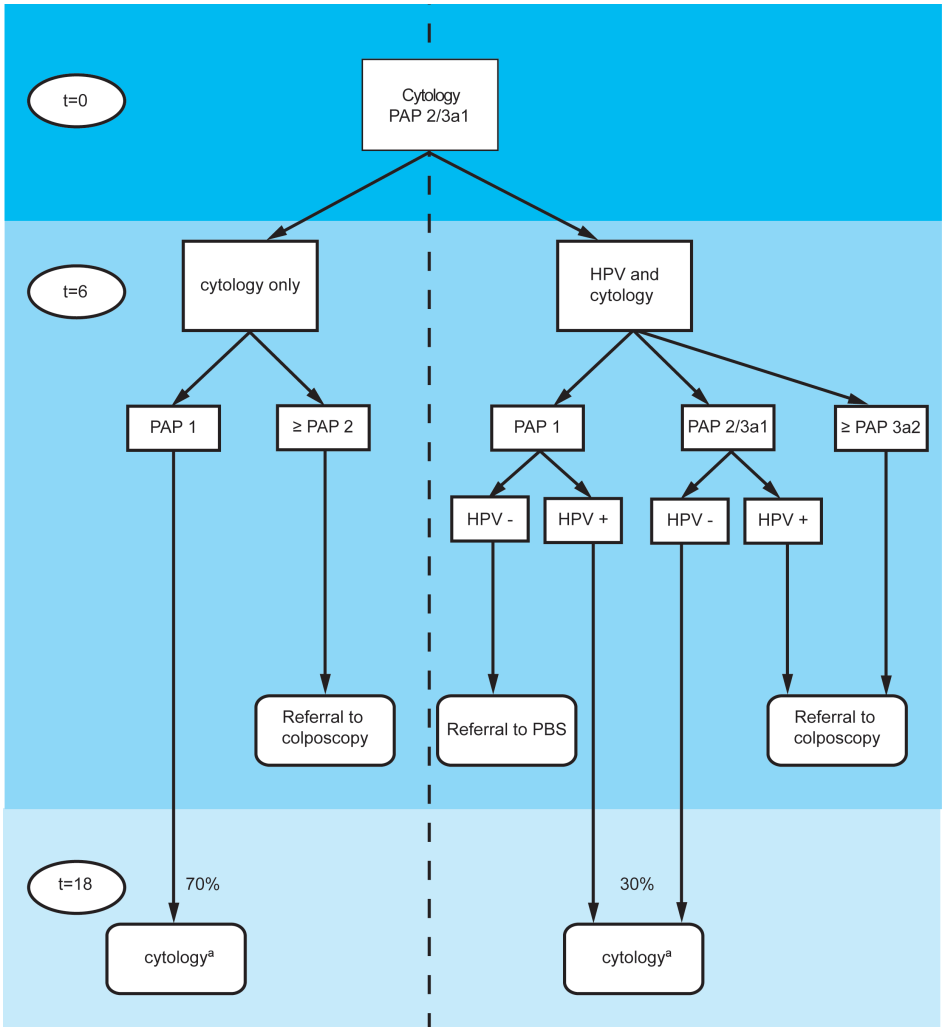

Figure 1.5 Flowchart for the follow-up of women with BMD cytology in the Netherlands. [Adapted from: Van Kemenade et al 2007].[85]
HPV, high-risk type of human papillomavirus; PBS, population-based screening.
[a] If cytology result PAP1 return to PBS, all other results (≥PAP2) referral for colposcopic examination.

6-40 fold stereoscopic magnification and allows visualisation of the microscopic changes of the cervix after application of an acetic acid solution (3-5%). This solution causes a reversible coagulation of the nuclear proteins in cells, resulting in a white discoloration and swelling of columnar and abnormal epithelium. The colposcopist determines whether the entire transformation zone can be visualised (satisfactory) or not (unsatisfactory), and also whether lesions are present. The most abnormal area is identified to perform a directed biopsy for histo-pathological diagnosis.

Colposcopy has several limitations. The first restriction is that the choice of biopsy site remains subjective and is hampered by the suboptimal correlation between visual changes and disease severity.[88-89] A second restriction is that the prediction of disease is related to the experience and skill of the colposcopist.[90] Inter-observer variation is moderate, but levels of agreement increase as cervical lesions become more severe.[90] These two restrictions result in a modest sensitivity of only 55% to distinguish high-grade from low-grade lesions.[88] As a consequence of this inaccuracy, all detected acetowhite lesions should be assessed with biopsy.[91] A final restriction is that a diagnostic procedure, such as endocervical curettage, is required when the colposcopy is unsatisfactory.[90] This is done to avoid unnecessary treatment as well as to rule out endocervical carcinoma. Therefore, trained colposcopists are of the utmost importance in an effective screening programme.

3.2 Treatment

Depending on the histology results of the biopsies taken at colposcopy treatment is performed. A conservative approach is recommended for women diagnosed with CIN1, as the majority of these lesions will regress. Follow-up of these women consists of a cytological smear every year, until there have been at least two consecutive normal smears.

Each year approximately 5000 women in the Netherlands are diagnosed with high-grade cervical disease (CIN2, CIN3, or AIS) and treated according to national guidelines.[79] The presence of a high-grade lesion indicates that the entire transformation zone (TZ) is at risk and therefore, the total TZ is treated.[10] The type of treatment needed depends on the severity and extension of the lesion, also taking into consideration the patients age and potential child wish. Nowadays, the most popular procedure is the large loop excision of the transformation zone (LLETZ). Advantages of this method are the possibility to perform this procedure under local anaesthesia at the outpatient clinic, and to examine the removed tissue histologically. When more extended tissue has to be removed (e.g. AIS, or suspected micro-invasive carcinoma) a cold-knife or laser conisation is most often performed. Other procedures include cryotherapy, which has the disadvantage that the tissue is destroyed and may not be examined, and more extensive operations such as trachelectomy and hysterectomy.

3.3 Post-treatment

Despite close surveillance, women treated for premalignant cervical disease have a risk of approximately 10%[82] to be diagnosed residual or recurrent high-grade disease.[82, 92-93] Treated women are therefore subject to close surveillance. Between countries, the guidelines vary greatly in length and intensity of follow-up.[79, 94-95]

3.3.1 Current post-treatment guidelines in the Netherlands

After treatment for a high-grade cervical lesion, women in the Netherlands are followed-up by cervical cytology 6, 12 and 24 months after treatment. Once three consecutive negative smears are found, women return to the regular screening programme.[79] Women diagnosed with AIS have a higher risk of multifocal disease and are more difficult to follow-up as their lesion is located on the endocervix. Therefore these women remain are under closer surveillance for at least 5 years.[79]

3.3.2 Alternative post-treatment algorithms

Studies have shown that successful treatment of high-grade CIN is associated with hrHPV elimination.[96-97] Because hrHPV persistence is necessary in the development of post-treatment high-grade disease, incorporating hrHPV testing in post-treatment surveillance could enhance the identification of women at risk of being diagnosed with residual and/or recurrent disease.[82, 92, 97-99] Combined testing of hrHPV and cytology has a very high negative predictive value (>99%), so women who test negative for both tests during follow-up may be monitored less intensively.[92, 97-99]

4 AIM AND OUTLINE OF THIS THESIS

The search for the perfect instrument to prevent cervical cancer is a continuing process. Although the causal link between hrHPV and most types of cervical cancer has been proven indisputably, this association has not (yet) been established in some rare subgroups of cervical adenocarcinoma. In **Chapter 2** we summarize the published literature and add new information on the association between the relatively rare clear-cell adenocarcinoma (CCAC) and hrHPV.

The second part of this thesis focuses on the risk-assessment of women with abnormal cytological test results and women treated for high-grade cervical lesions. Many studies describe the follow-up of women with abnormal cytological test results up to 5 years after diagnosis, studies concerning the long-term follow-up of these women are rarely found. In **Chapter 3** a cohort of women with abnormal cytological test results is described with a follow-up of up to 20 years. The risk of developing CIN2+ lesions in these women is assessed by comparing different test result combinations of cytology and hrHPV testing.

Women treated for high-grade cervical lesions are closely monitored as their risk of developing residual and/or recurrent disease is increased. As the risk of (unnecessary) treatment and thereby cervical damage, increases by more frequent monitoring due to false positive test results, it is of the utmost importance to determine the most optimal surveillance strategy for these women. For this reason we have followed a cohort of women treated for high-grade cervical disease for up to 20 years and compared different test result combinations of cytology and hrHPV-testing in assessing the risk of developing post-treatment disease (**Chapter 4.1**). In addition we systematically reviewed all literature published between 2003 and 2011 to determine whether hrHPV testing should be incorporated in post-treatment testing (**Chapter 4.2**).

The general discussion **(Chapter 5)** summarizes the findings of this thesis and discusses possible future prospects and clinical consequences.

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ROLE OF HRHPV TESTING IN RARE CERVICAL CARCINOMA

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HIGH-RISK HUMAN PAPILLOMAVIRUS SEEMS NOT INVOLVED IN DES-RELATED AND OF LIMITED IMPORTANCE IN NON DES-RELATED CLEAR-CELL CARCINOMA OF THE CERVIX

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SUMMARY

Introduction

Over 90% of all cervical adenocarcinoma are caused by a transforming infection with a high-risk type of the human papillomavirus (hrHPV). Previous studies demonstrated that the association between hrHPV positivity and cervical clear-cell adenocarcinoma (CCAC) varies between 0% and 100%. As approximately 60% of all CCAC are associated with intra-uterine diethylstilbestrol (DES) exposure, we determined in a cohort of both DES-exposed and DES-unexposed women the prevalence of hrHPV infections, and the potential etiological role of hrHPV by additional analysis of $p16^{INK4a}$ and p53 expression.

Methods

Representative slides of 28 women diagnosed with CCAC were tested for hrHPV by two PCR methods (the clinically validated GP5+/6+ PCR and the very sensitive SPF_{10}PCR/$LiPA_{25}$). Fifteen women were DES-exposed, 10 unexposed and of 3 women DES-exposure was unknown. Twenty-one cases with sufficient material were immuno-histochemically stained for $p16^{INK4a}$ and p53.

Results

Seven tumours, of which four DES-exposed and two DES-unexposed, tested positive for hrHPV with GP5+/6+ PCR. Thirteen tumours, of which five DES-exposed and seven DES-unexposed, tested positive with SPF_{10}PCR/$LiPA_{25}$. In one woman with unknown exposure, a CCAC tested positive in both assays. Only three cases, none in DES-exposed women, and all positive with both hrHPV assays, revealed diffuse $p16^{INK4a}$ immuno-staining and weak p53 staining as well, supporting indisputable hrHPV involvement.

Discussion

Although the prevalence of hrHPV was high, only two DES-unrelated CCAC (25%) and one tumour in a woman with unknown exposure could be attributed to hrHPV.

INTRODUCTION

Clear cell adenocarcinomas of the cervix (CCAC) are relatively rare tumours of the lower genital tract and are characterized by abundant clear cytoplasm and hobnail cells.[1-2] CCAC have a bimodal age distribution, with one peak in the early twenties and another after menopause.[3-4] In 1971, intrauterine exposure to the non-steroid oestrogen diethylstilbestrol (DES), used between 1938 and 1978 to prevent miscarriage and other pregnancy-related problems [5], was found to be associated with CCAC.[6] DES-exposed women have a 40-fold increased risk of developing CCAC, resulting in a cumulative incidence of 0.1-0.2%.[7-8] As this tumour is still very rare in DES-exposed women, DES is suggested to be an incomplete carcinogen.[7] Most CCAC are found at a relatively low stage and therefore have a good prognosis with a 5-year survival rate of 90%.[3-4, 9] Although 60% of CCAC are detected in DES-exposed women, 40% develop in DES-unexposed women, indicating the involvement of alternative etiological factors.[2, 4, 7, 10]

A factor of interest might be a transforming infection with a high-risk human papillomavirus (hrHPV) type, the key causative factor in almost all cervical squamous cell- and adenocarcinomas.[11-13] Transformation is provoked by inactivation of tumour-suppressor proteins by viral oncoproteins E6 and E7.[14-15] The E6-oncoprotein degrades p53 and can thereby block p53-mediated apoptosis. The E7-oncoprotein interferes with cell-cycle control by blocking retinoblastoma (Rb) (Figure 2.1), ultimately leading to immortalization and invasive growth.[14-15] As a consequence, hrHPV-induced cancers are generally characterized by absence of p53 whereas cancers without hrHPV often display an increase in p53 protein reflecting stabilization caused by mutations in this gene.[13-16] In addition, hrHPV-induced cancers are characterized by over-expression of $p16^{INK4a}$ [13-15, 17] most likely reflecting an oncogenes senescence-like response triggered by E7[18], but functionally ineffective because Rb is blocked downstream in the pathway (Figure 2.1). HrHPV-positive tumours without these characteristics reflect transient,

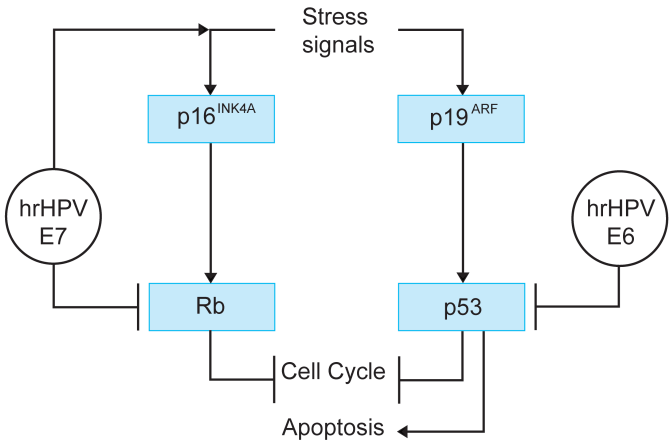

Figure 2.1 Simplified scheme of hrHPV-mediated carcinogenesis effecting Rb and p53 activity.
HrHPV-E7 degrades Rb, which results in inhibiting the cell cycle arrest, and triggers over-expression of $p16^{INK4a}$.[14,15, 18] HrHPV E6 degrades p53 leading to a block of p53-mediated apoptosis and cell cycle arrest. [14,15]

sometimes productive, infections which are commonly found in the general population. Only few studies have explored the association between hrHPV and CCAC (Table 2.1). In these small studies hrHPV positivity varied between 0% and 100%, thereby hampering any conclusion to be made about its potential causal role.[1, 16, 19-28] Only two studies provided information about immuno-histochemical staining. In one study the inverse relation between hrHPV presence and p53 presence was displayed in 11 CCAC[16], the other showed that extensive $p16^{INK4a}$ staining was absent in 3 CCAC.[24]

The aim of this study was to determine in both DES-exposed and DES-unexposed women whether hrHPV infections present in CCAC could be etiologically involved, or rather represent non-transforming infections. Therefore, we studied tissue specimens for the presence of hrHPV DNA and for the expression of $p16^{INK4a}$ and p53.

MATERIALS AND METHODS

Tumour specimens

Twenty-eight paraffin-embedded CCAC samples registered in the Central Netherlands Registry (CNR) for CCAC were collected from four university medical centres and reviewed by an expert in gynaecologic pathology (JB). Of these samples, diagnosed between 1975 and 2005, fifteen were FIGO stage 1, 12 stage 2, and one stage 3. Follow-up varied between 14 and 405 months

Table 2.1 Summary of hrHPV detection in CCAC.

					β-globin	hrHPV				
Study	**Year**	**Country**	**Primer**	**n**	**present**	**present**	**16**	**18**	**Other hrHPV types**	**DES**
Milde–Langosch[25]	1993	Germany	MY09/MY11	1	1	0				ns
Waggoner[16]	1994	USA	L1-concensus	14	11	3	0	0	HPV31 (3x)	9
Duggan[21]	1995	Canada	DBH, L1	1	1	1	1	0		ns
Tenti[28]	1996	Italy	PCR	3	3	2	0	2		ns
Pirog[1]	2000	USA	SPF10	4	4	0				ns
Ding[20]	2004	Taiwan	ns	1	1	0				0
Stewart[27]	2006	USA	ISH	1	1	0				0
Hadzisejdic[23]	2007	Croatia	E6/E7 consensus	5	5	5	0	2	HPV33, HPVX, HPV16/18/33	ns
Chen[19]	2007	Taiwan	ns	1	1	1	0	1		0
Guo[22]	2009	China	Nested PCR	1	1	1	0	1		ns
Nofech-Mozes[26]	2010	Canada	L1	3	3	2	ns	ns		0
Houghton[24]	2010	Ireland	PCR	4	3	0				ns
Total				39	35	15	1	6		
This study	2011	Netherlands	SPF10, GP5+/6+	28	28	13	7	2	HPV31, HPV45, HPV 51, HPVX	15

hrHPV, high-risk type of the human papillomavirus; DBH, dot-blot-hybridization; PCR, polymerase chain reaction; ISH, in situ hybridization; DES, diethylstilbestrol exposition; ns, not stated.

Table 2.2 Baseline characteristics.

	Study cohort (n=28)		CNR cohort (n=144)[29]	
Characteristics	**median**	**range**	**median**	**range**
Age at diagnosis *(years)*	29.0	17-54	25.0	8 -54
Follow-up *(months)*	151	14-405	161	
	n	**%**	**n**	**%**
Recurrence	5/28	17.9	34/123	27.6
Deceased	6/28	21.4	32/127	25.2
DES-exposure	15/25[a]	60.0	76/122	62.3
Tumour FIGO stage 1	15/28	53.6	55/123	44.7
10-years survival (*95%CI*)		81.2 (66.2-96.2)		77.6 (69.8-85.0)

CNR, Central Netherlands Registry for clear-cell adenocarcinoma; DES, diethylstilbestrol.
[a] Exclusion of three women with unknown DES-exposure

and depended on date of diagnosis and date of death, or last known visit to the outpatient clinic. The total number of women years in our study was 350. During follow-up, five women developed recurrent disease, four of them died of progression within 32 months. None of these women had a history of DES exposure. Another two women died of unrelated disease, respectively 81 and 220 months after diagnosis. Considering the similarities between the total CNR cohort and our sample, we believe the latter was representative (Table 2.2).[29]

Series of 4-µm sections were cut using a new blade for each tissue sample to prevent contamination. Outer sections were used for histological confirmation and immuno-histochemical assays, while inner sections were collected for DNA extraction and hrHPV analyses. Ethical approval was waived, since study material was anonymized according to Dutch regulation.[30]

DES-exposure

Previously, DES exposure was not specified uniformly and varied between a statement concerning exposure by mother, daughter, or physician, and confirmation of exposure by hospital birth records.[3, 8-10, 29] We collected information regarding intra-uterine DES exposure from CNR patient files.[29] Three categories were distinguished: (1) exposed (confirmation: (a) in medical record; or (b) by mother/daughter *and* clinical signs), (2) unknown (no data available), and (3) unexposed ((a) stated in medical record; or (b) DES denial by mother/daughter).

HrHPV testing

To ensure adequate DNA preparation, all samples were subjected to β-globin PCR. We used the primer combination PCO3 and PCO5 to generate a 209 bp product.[31] Detection of hrHPV was performed by two PCR-based assays; GP5+/6+-PCR and the ultrasensitive SPF_{10}PCR/$LiPA_{25}$.[32] The clinically validated GP5+/6+-PCR with enzyme-immuno assay read out uses a cocktail probe for 14 HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), according to established protocols.[33-34] The PCR products of hrHPV-positive women were subsequently genotyped by

reverse line blot hybridization. The SPF_{10}PCR/$LiPA_{25}$ (version 1) was performed according to specifications of the manufacturer (Labo Bio-Medical Products, Rijswijk, Netherlands) to detect and genotype 25 HPV genotypes.[35] For both HPV detection assays, samples that were positive in the enzyme-immunoassay format, but negative for any specific probe in the genotyping format were considered positive for uncharacterized HPV (sub) types or variants (HPVX).

Immuno-histochemistry (IHC)

Immuno-histochemical staining was performed according to manufacturers' instructions: $p16^{Ink4a}$ (E6H4, MTM-Laboratories, Heidelberg, Germany) and p53 (BP53-12, BioGenex-Laboratories, San Ramon, USA).

Sections were deparaffinised and incubated with the primary mouse monoclonal antibodies against $p16^{INK4a}$ or p53 after which they were incubated with a secondary biotinylated rabbit-anti-mouse bridging antibody followed by incubation with streptavidin-biotinylated peroxidase coupled with horse radish peroxidase conjugate. The peroxidase activity was detected with DAB (diaminobenzidine; Fluka, Sigma Aldrich, Buchs SG, Switzerland). They were then counterstained, washed, dehydrated and coverslipped. For positive controls sections from a breast carcinoma were used for p53 and sections from a CIN3 lesion for $p16^{INK4a}$. The negative controls were provided by performing the standard procedure replacing the primary antibody with BSA (1% bovine serum albumen).

The immuno-reactivity of $p16^{INK4a}$ and p53 was scored according to the percentage of tumour cells that stained positive as follows: no (≤10% cells), weak (>10% but ≤25% cells), moderate (>25% but ≤50% cells) and extensive (>50% cells) staining. Intensity of staining was not taken into account. All light-microscopic evaluations were scored blinded by two pathologists (CM, FS). In cases of discrepancy, slides were reviewed until consensus was reached.

Statistical analysis

The main outcome of this retrospective cohort study was the number of hrHPV-positive CCAC and the number of tumours staining positive for $p16^{INK4a}$, and/or p53. The relationships between various parameters and the outcomes in women with and without intra-uterine DES-exposure were evaluated with 2x2 tables, Fisher-Exact, Cox-regression and Mann-Whitney analysis. All calculations were performed using SPSS Version 17.0 (SPSS Inc, Chicago Illinois, USA). For all tests, the level of significance was set at 0.05.

RESULTS

DES-exposure

Fifteen women were DES-exposed *in utero*, 10 were unexposed and of three women DES-exposition was unknown (Table 2.3). Exposed women developed CCAC at a younger median age than unexposed women (21 versus 35 years, $p<0.001$). Although no difference in tumour stage (p=0.23), growth pattern (p=0.09), nuclear atypia (p=0.83), or lymph-vascular invasion (p=0.67) could be demonstrated, DES-unexposed women had a worse overall survival (p=0.04, Hazard Ratio 0.10, 95%CI 0.01-0.86).

Table 2.3 Characteristics of study population: age at diagnosis and status of human papillomavirus, p16^{INK4a} and p53.

Nr	Age[a] (years)	Year[a]	DES	hrHPV		Immuno-histochemistry[b]		Putative aetiology
				GP5+/6+ (type)	SPF_{10} (type)	p16^{INK4a}	p53	
1	20	1975	+	+ (16)	+ (16)	n.m.	n.m.	DES
2	21	1981	+	-	-	n.m.	n.m.	DES
3	21	1982	+	-	+ (18)	n.m.	n.m.	DES
4	19	1983	+	+ (16)	+ (16)	n.m.	n.m.	DES
5	19	1983	+	-	-	25	10	DES
6	17	1984	+	+ (16)	+ (16)	n.m.	n.m.	DES
7	20	1989	+	+ (16)	+ (16)	<5	<1	DES
8	21	1989	+	-	-	25	0	DES
9	19	1990	+	-	-	50	0	DES
10	27	1990	+	-	-	10	<5	DES
11	24	1991	+	-	-	0	<5	DES
12	37	1993	+	-	-	50	0	DES
13	27	1993	+	-	-	45	60	DES
14	38	1995	+	-	-	60	35	DES
15	29	2001	+	-	-	20	40	DES
16	33	1985	U	+ (45)	+ (45)	100	10	hrHPV
17	44	1997	U	-	-	90	5	unknown
18	41	1997	U	-	-	90	5	unknown
19	29	1982	-	-	+ (51)	30	0	unknown
20	41	1997	-	-	+ (X)	10	10	unknown
21	36	2000	-	-	+ (16)	n.m.	n.m.	unknown
22	54	2002	-	-	+ (31)	50	60	unknown
23	28	2003	-	-	+ (16)	30	60	unknown
24	34	2004	-	+ (16)	+ (16)	100	5	hrHPV
25	30	2005	-	+ (18)	+ (18)	100	5	hrHPV
26	33	2000	-	-	-	50	50	unknown
27	44	2000	-	-	-	n.m.	n.m.	unknown
28	48	2001	-	-	-	5	60	unknown

DES, diethylstilbestrol exposure; hrHPV, high-risk type of the human papillomavirus; SPF_{10}, SPF_{10}PCR/$LiPA_{25}$; n.m, no material available; U, DES-exposition unknown; X, HPV infection, unable to type.
[a] at diagnosis;
[b] indicated are the percentages of immuno-positive tumour cells.

HrHPV presence

DNA quality was sufficient for all samples. With GP5+/6+ PCR testing seven specimens tested hrHPV-positive. Six more tested positive by SPF_{10}PCR/$LiPA_{25}$, resulting in 13 (46.4%) positive tumours for either or both assays. Amongst hrHPV-positives, HPV16 was the most prevalent type

(7/13, 53.8%), followed by HPV18 (2/13, 15.4%). The remaining four tumours all contained a different hrHPV type: HPV31, HPV45, HPV51 and HPVX (Table 2.3). Multiple infections were not found.

Immuno-histochemistry

Of 21 tumours sufficient material remained for additional IHC. These included specimens of 10/15 DES-exposed women, of 8/10 unexposed women, and of 3/3 women with unknown DES-exposure (Figure 2.2).

Two CCAC of DES-unexposed women (Table 2.3, nr 24, 25) and one CCAC of a woman with unknown exposure (Table 2.3, nr 16) displayed characteristics supporting a causal hrHPV involvement, i.e. extensive diffuse $p16^{INK4a}$ immuno-staining in all tumour cells, and only weak, focal p53 staining. All these cases were positive by GP5+/6+ PCR and $SPF_{10}PCR/LiPA_{25}$. These three tumours all had a high nuclear mitotic activity and a mainly solid growth pattern (data not shown). All other hrHPV-positive cases (Table 2.3, nr 7, 19, 20, 22, 23) displayed a wide variation in p53 expression (0%-60% of tumour cells) and at maximum moderate $p16^{INK4a}$ staining (<50% of tumour cells). None of the tumours found in DES-exposed women that were analyzed with all parameters, showed both hrHPV presence and extensive, diffuse $p16^{INK4a}$ immuno-staining in combination with no, or weak, focal p53 staining.

In three other tumours, one of a DES-exposed woman (Table 2.3, nr 14) and 2 of women with unknown exposure (Table 2.3, nr 17, 18) extensive $p16^{INK4a}$ staining in more than 50% of all tumour cells was found. However, none of these CCAC tested positive for hrHPV.

None of the hrHPV assays or immuno-histochemical profiles was significantly associated with tumour stage, age at diagnosis, or survival rate. Table 2.3 lists the putative aetiology for each tumour.

DISCUSSION

In a relatively large group of CCAC we showed that hrHPV has a limited role in the carcinogenesis of CCAC. Taking into account that in hrHPV-positive women diffuse $p16^{Ink4a}$ staining and absence or weak p53 immuno-staining can be seen as a cellular correlate to E6/E7 mRNA expression of hrHPV and thus as functional involvement of hrHPV[13-14, 16], only three of 28 tumours could be attributed to a transforming hrHPV infection. None of these were found in DES-exposed women. Interestingly, all these three tumours tested positive in both hrHPV assays (Table 2.3). The fact that 3 out of 4 fully analyzable GP5+/6+-PCR positive tumours versus 3 out of 8 fully analyzable $SPF_{10}PCR/LiPA_{25}$ positives fulfilled the criterion of a clinically meaningful infection is in line with the higher specificity of a clinically validated PCR (i.e. GP5+/6+-PCR) for relevant disease caused by hrHPV.[32, 36] Hence, hrHPV positivity detected solely by $SPF_{10}PCR/LiPA_{25}$ most likely reflects non-transforming, transient hrHPV infections, which are also characterized by the presence of more diverse hrHPV types.

Overall, 60% (15/25) of all analyzed CCAC developed in DES-exposed women.[4, 7, 16] The estrogenic effects of DES interfere with foetal development resulting in adenosis. This tissue is thought to be more susceptible to malignant transformation.[4] In DES-exposed women CCAC were diagnosed at a younger age than in unexposed women.[3-4] Furthermore, these women had a better five-year survival.[4, 10]

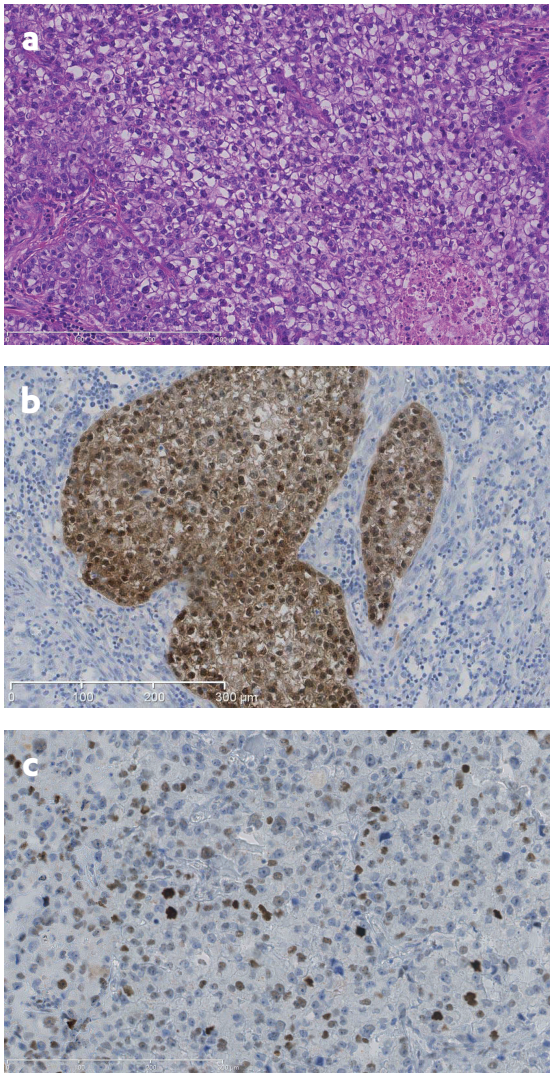

Figure 2.2 Expression of $p16^{INK4a}$ and p53 in cervical clear-cell adenocarcinoma. **a** shows the typical features of a clear cell adenocarcinoma, composed of polygonal cells with distinct cell membranes and clear cytoplasm (H-E staining). **b** clear-cell adenocarcinoma after staining with $p16^{INK4a}$, note both nuclear and cytoplasmatic staining. **c** p53 staining showing distinct nuclear staining in approximately 60% of the nuclei.

In our study hrHPV was detected in 46.4% (13/28) of all CCAC, similar to the overall percentage of 43% (15/35) found in literature (Table 2.1). When limited to DES-unexposed women the prevalence increased to 70% (7/10). Although similar to other reported frequencies in CCAC [26, 28], this is lower than the prevalence found in common cervical adenocarcinoma.[1, 12] It is unlikely that this reflects deletion of sequences targeted by our PCR assays because of viral integration in the host DNA, since in most tumours no sign of viral activity reflected by diffuse $p16^{INK4a}$ immuno-staining was found. $P16^{INK4a}$ immuno-staining is now widely considered a cellular correlate of the oncogenic expression of E6/E7 mRNA.[14, 17, 24, 37] Extensive $p16^{INK4a}$ immuno-staining was only found in three hrHPV-negative tumours. This may also reflect

undetectable hrHPV with L1-based PCR assays applied [37], however, it is more likely to reflect an hrHPV independent mechanism triggering $p16^{INK4a}$.[24]

As can be seen from Table 2.1, most previously described CCAC were positive for HPV18[19, 22-23, 28], followed by HPV31.[16] In contrast, HPV16 was most commonly found (7/13) in our cohort. HPV18 was only found in two CCAC, which was surprising as in most cervical adenocarcinoma HPV18 is more[13] or equally [1, 11] often found as HPV16. However, 14/28 women in our cohort were younger than 30 years at time of diagnosis. Because HPV DNA testing is not very specific under the age of 30 [38], the frequency of hrHPV-types might have been distorted due to the detection of transient hrHPV infections. Indeed, when we considered only the three CCAC with likely hrHPV aetiology, HPV16 and HPV18 both occurred in one tumour.

A limitation of our study is that only 21/28 samples enclosed enough material to perform immuno-histochemical assays, hampering to draw conclusions about 7 tumours remaining. Five of these tumours were positive for hrHPV of which three in both assays (Table 2.3, nr 1, 4, 6). As IHC could not be performed in these three DES-exposed CCAC, a causal role of hrHPV in DES-exposed tumours might have been missed.

A second limitation is the young median age in our cohort. Although consistent with previously published data [3, 8, 29] we can only comment on hrHPV-related carcinogenesis concerning the first peak in the bimodal age distribution.[3-4]

Conclusions

In summary, we limited our conclusions to the 21 of 28 fully analyzed CCAC. In none of the 10 DES-related tumours a causal role of hrHPV could be identified. Overall, three tumours were likely caused by a transforming hrHPV infection. Two were found in DES-unexposed women (2/8) and one in a women of whom the DES-exposition was unknown (1/3). In the remaining 8 tumours (6 in DES-unexposed women and 2 in women with an unknown exposure) the aetiology remains unclear, leaving room for other, unexplored factors in its carcinogenesis.

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3

RISK ASSESSMENT IN WOMEN WITH ABNORMAL CYTOLOGY

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3.1

LONG-TERM CIN3+ RISK IN WOMEN WITH ABNORMAL CYTOLOGY; ROLE OF HRHPV TESTING

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SUMMARY

Introduction

Many studies have examined the short-term value of high-risk human papillomavirus (hrHPV) testing in predicting cumulative risk of cervical intraepithelial neoplasia grade 3 or cancer (CIN3+). This study focuses on long-term CIN3+ risk after initial wait and see policy.

Methods

A total of 342 women with abnormal cytology of borderline/mild dyskaryosis (BMD) or worse (>BMD), included between 1990 and 1992, were followed-up by cytology and hrHPV-testing until 1996 and monitored by cytology thereafter. Primary endpoint was cumulative CIN3+ risk by December 2009.

Results

Women with BMD had a 5-year CIN3+ risk of 22.5% (95%CI 17.0-29.1) and of 0.7% (0.1-4.5) in the subsequent 5 years. HrHPV-negative women with BMD had a 5-year risk of <0.01% (95%CI 0.0-5.1) and of <0.01% (0.0-5.7) in the following 5 years, while for hrHPV-positive women these risks were 37.5% (29.0-46.9) and 1.6% (0.2-9.5), respectively. Women with >BMD had a 5-year risk of 45.1% (36.4-54.1) and of 3.5% (0.9-12.2) in the subsequent 5 years. HrHPV-negative women with >BMD had a 5-year risk of 7.3% (2.0-23.6) and hrHPV-positive women of 56.6% (46.4-66.3).

Discussion

Women with BMD have an elevated CIN3+ risk for 5 years only; afterwards their risk is similar to the general population. HrHPV-negative women with BMD may return to regular screening directly. All other women with ≥BMD should be referred for additional testing and/or colposcopy.

INTRODUCTION

The incidence of cervical cancer has been lowered by the implementation of population-based screening programs in which women are screened by cytological testing.[1-3] However, the sensitivity of cytological testing for cervical intraepithelial neoplasia grade 3 or cervical cancer (CIN3+) is moderate and compensated for by repetitive screening.[4-5] In The Netherlands, an abnormal cytological test result is detected in approximately 2-4% of all screened women.[3, 6-7] In most developed countries, women with minor cellular abnormalities of borderline and mild dyskaryosis (BMD) will be followed by cytology, and will be referred for colposcopy if the smear remains abnormal.[3, 6, 8] Women with moderate and severe abnormalities (>BMD) are referred for colposcopy.[3, 6] However, a substantial proportion of women with abnormal cytology will regress or do not harbour clinically meaningful cervical disease and will therefore be unnecessarily retested or referred.

Infection with a high-risk type of human papillomavirus (hrHPV) is the causative agent in cervical cancer.[9-10] Molecular testing for hrHPV has a higher sensitivity than cytology to detect CIN3+.[4-5, 8, 11-14] In women with abnormal cytology, studies focus on the additional value of hrHPV in triaging women with equivocal or mildly abnormal cytological test results in order to increase efficiency of patient management (i.e. referral for colposcopy) and to identify women with an increased risk for high-grade CIN. Because most of these studies had a restricted follow-up of at maximum six years, little is known on risk profiles with longer periods of follow-up and the effect of hrHPV testing in those situations.[5, 11, 14-25] Only few studies have reported about a follow-up period of over 10 years.[26-28]

In this study we followed a group of women who were diagnosed with an abnormal cytology result of ≥BMD for a maximum of 19 years and evaluated their long-term cumulative risk of developing CIN3+. Also the value of hrHPV testing for risk assessment was established as well as the duration of follow-up needed for women with dyskaryosis.

MATERIALS AND METHODS

Study Population

For this cohort study we followed-up women who had participated in a previous study that studied the association between the presence of hrHPV and the development of high-grade cervical lesions.[16] Detailed methods of recruitment and follow-up until 33 months after intake have been published previously.[16, 29] In short, between June 1990 and December 1992, 353 women were referred to the colposcopic outpatient clinic (VU University medical centre, Amsterdam, The Netherlands) with an abnormal cervical cytology result of mild, moderate or severe dyskaryosis. Until December 1996 each participant had been monitored for cervical disease every 3-4 months by testing for hrHPV, cytology, and colposcopy. Three expert colposcopists assessed serial colpophotographs and gave a consensus impression of the lesion. Only when they suspected a CIN3 lesion covering three or more cervical quadrants, or when a cervical smear result suspect of cervical cancer was found, a biopsy had been taken. At the end of the study in December 1996, all women had a colposcopic examination with mandatory

biopsy (median 36 months, range 1-75). Women identified with high-grade disease (CIN2+) were treated according to Dutch guidelines. In Figure 3.1 follow-up procedures are depicted in a flowchart.

Procedures

Cytology results were originally reported using a classification that predated the currently used classification; therefore all cytological referral slides were retrieved from the archives for blind review by an expert gynaeco-pathologist (FvK). Dotted slides were scored and dichotomized into (≤)BMD, or >BMD. Women of whom no referral slide could be retrieved were excluded from this study.

Between December 1996 and December 2009 all women were monitored by cytological population-based screening once every 5 years. Interim-colposcopies were performed according to national guidelines.[30] To complete the data obtained from routine screening, we invited all women to visit the outpatient clinic (VU University medical centre) for additional cytology and hrHPV testing during 2009 (Figure 3.1). If travel distance was a limitation to participate, women were offered the possibility of performing a hrHPV test at home by self-sampling. These test results are similar to those acquired by a physician.[31-32] Women who had had a hysterectomy were censored at the date of hysterectomy.

Two cervical specimens were obtained from women who visited the outpatient clinic (Cervex-brush, Rovers Medical Devices, Oss, The Netherlands). The first specimen was collected in liquid-based cytology medium (Surepath, Tripath Imaging, Burlington NC, USA), cytologically examined, and classified according to the CISOE-A classification, which is easily translatable into the Bethesda 2001 classification.[7] The second specimen was stored in Universal Collection Medium® (Qiagen Corporation, Gaithersburg, MD, USA) for hrHPV testing. Women who self-sampled returned their cervicovaginal specimen for hrHPV testing by mail. All hrHPV samples were tested with the clinically validated GP5+/6+ PCR with enzyme-immunoassay read-out using a cocktail probe for 14 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), according to established protocols.[33-34] The PCR products of hrHPV-positive women were subsequently genotyped by reverse line blot hybridization. Samples that were negative for any specific probe in this reverse hybridization assay were considered positive for uncharacterized subtypes or variants (HPV X).

A standard colposcopic assessment was performed when a cytological test was abnormal at the threshold of borderline dyskaryosis, or when the hrHPV test was positive (Figure 3.1). Biopsies were taken of all suspect lesions. Histological specimens were graded as CIN0 (no dysplasia), CIN1, CIN2, CIN3, adenocarcinoma *in situ* (AIS) or invasive cancer[35] and classified according to the highest abnormality found in biopsy or treatment specimen. Women who developed CIN2+ were treated according to present guidelines but were censored at time of treatment.

In December 2009 the hospital database and the Netherlands nationwide network and registry of histopathology and cytopathology (PALGA; Bunnik, The Netherlands) were reviewed for all women, irrespective of attendance, to ascertain details of any additional relevant events and procedures. Ethical approval was obtained from the Ethics Board of the VU University medical centre. All women who attended the outpatient clinic or participated by self-sampling

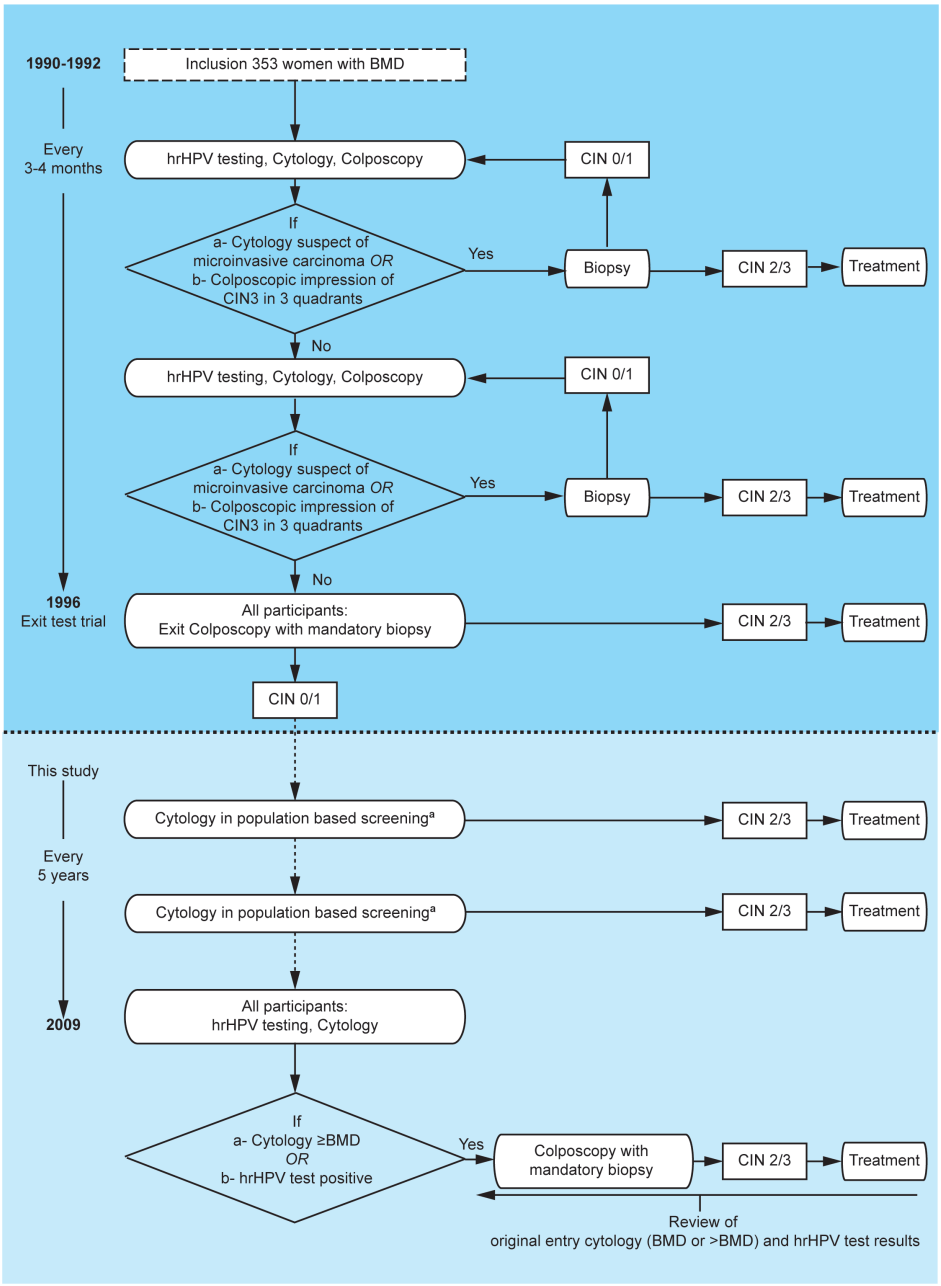


Figure 3.1 Flowchart of follow-up procedures.
BMD, borderline or mild dyskaryosis; hrHPV, high-risk type of the human papillomavirus; CIN, Cervical Intraepithelial Neoplasia.
[a] referral for colposcopy when once a cytology result of >BMD, or twice a result of BMD is detected.

provided additional signed informed consent. The study is registered in the Dutch trial register (NTR1470).

Statistical analysis

In order to report long-term CIN risks in women with dyskaryosis, this study was designed as a follow-up of an observational cohort.[16]

As the original study was designed such that no interference with natural history would occur; a biopsy had only been taken when a colposcopic impression of CIN3 covering three or more cervical quadrants was present, or when a cytology result was suspect of cancer. As a consequence, the exact time at which CIN3+ lesions had developed was difficult to assess. We have calculated the 5-year, 10-year and overall risks until detection of CIN3+ using different approaches. In the first approach we equalled the event time to the time of the first abnormal cytological result of moderate dyskaryosis or worse. In the second approach the event time was equalled to the time of histological diagnosis. As the difference between the risks of these approaches were minimal (data not shown), we applied the second approach in further analyses. In women without an event, data were right-censored at the date of the last registered test.

The primary endpoint was the cumulative risk of CIN3+. We repeated the calculations with CIN2+ as secondary endpoint because treatment of CIN2 is common practice in most western countries. Both CIN3+ and CIN2+ included cases of AIS, adenocarcinoma (AC) and squamous cell carcinoma (SCC).

The cumulative CIN3+ risk was estimated by Kaplan-Meier analysis for the total group as well as for subgroups of different cytological and hrHPV test results at time of referral. In addition we repeated the calculations after dichotomising in younger (<30 years) and older (≥30 years) women. Differences in cumulative risk curves between subgroups were assessed by log-rank tests.

For women who did not develop high-grade CIN within 6 months after inclusion, we reset the time at 6 months to 0 to estimate the value of retesting with cytology, hrHPV or both after 6 months and the risk of persistent hrHPV infection (log-rank tests). For women who had not developed high-grade CIN at 5 years after inclusion, time was reset from 5 years to 0 to estimate the CIN3+-risk from 5 years onwards.

By Cox regression we calculated CIN hazard ratios and 95% confidence intervals (CIs) to compare different test result combinations. Overall cumulative risks were calculated for different hrHPV genotypes to determine whether genotyping has additional value in the follow-up of women with abnormal cytology, focusing on HPV16. All calculations were performed using SPSS (Version 17.0, SPSS Inc., Chicago Illinois, USA). All tests were two-sided and the level of significance was set at 0.05.

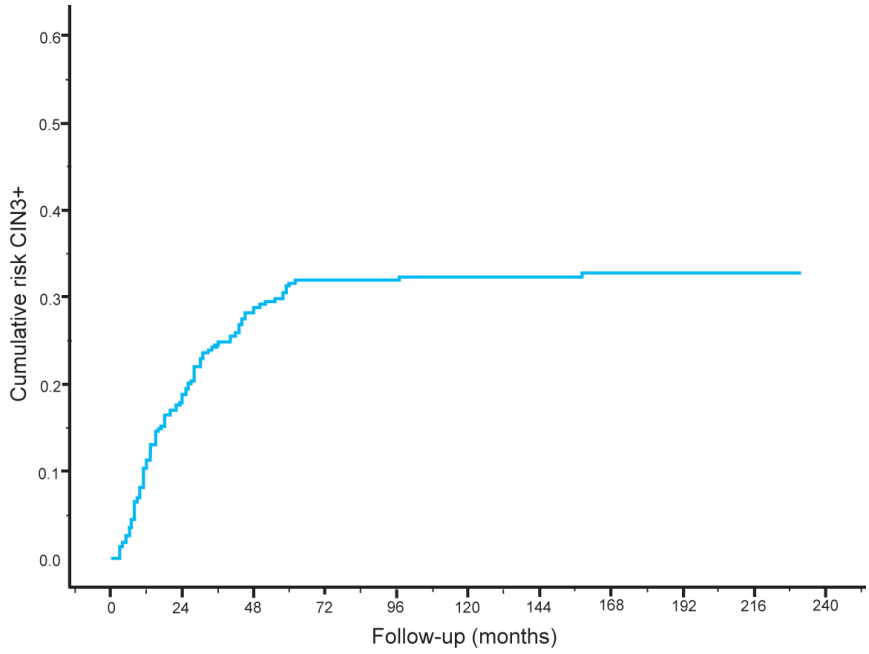
RESULTS

Of the original 353 women, 11 (3.1%) were excluded as no referral slide could be retrieved for review. For the remaining 342 women (median age 31 years, range 17-54) maximum follow-up depended on accrual date and ranged from 17.0 to 19.5 years. The total number of women years in our study was 3152. Overall censoring percentages were 13.2% (45/342) at 5 years,

21.6% (74/342) at 10 years, and 36.5% (125/342) at 15 years after detection of an abnormal cytological test result. During follow-up four women died of unrelated disease, six moved abroad, and 23 had a hysterectomy. None of the women had received prophylactic hrHPV vaccination.

During follow-up 105 (30.7%) CIN3+ cases were identified. Three were invasive cancers, of which two were SCC and one AC; two were AIS and 100 CIN3. CIN2 was diagnosed in another 36 women. The cumulative risk curve of developing CIN3+ after an abnormal cytological test result in our cohort is shown in Figure 3.2. The 5-year CIN3+ risk was 31.1% (95%CI 26.1-36.6) and the risk in the next 5 years was 1.6% (0.5-4.9). Of all CIN3+, 96.2% (101/105) were detected within 5 years of follow-up.

Table 3.1 shows the 5- and 10-year risks of developing CIN3+ in 227 hrHPV-positive women (66.4%) and 115 hrHPV-negative women (33.6%). Only three (2.9%) of 105 CIN3+ lesions were found in women who were hrHPV negative at baseline. These were all CIN3 lesions. HrHPV-negative women had a 5-year CIN3+ risk of 1.9% (95%CI 0.5-7.0) and a risk of 1.1% (0.2-6.4) in the next 5 years. These risks were 45.1% (95%CI 38.4-52.0) and 2.1% (0.5-7.8), respectively, in hrHPV-positive women. Of the hrHPV-positive women 84.6% (192/227) were infected with a single hrHPV type, 31 (13.7%) had a double infection and four (1.8%) were infected with three or



Number at risk	342	265	216	183	174	164	154	129	103	33
Number of CIN3+	60	32	11	0	1	0	1	0	0	0

Figure 3.2 Cumulative risk curve of CIN3+ in 342 women with abnormal cytology (mild to severe dyskaryosis) at baseline.
CIN3+, Cervical Intraepithelial Neoplasia grade 3 or cancer.

Table 3.1 Value of hrHPV testing during follow-up of women with abnormal baseline cytology; 5-year and 10-year risks.

		CIN3+				CIN2+			
		5-year		10-year		5-year		10-year	
Baseline	At risk	Risk (%)	95%CI	Risk (%)	95%CI	Risk (%)	95%CI	Risk (%)	95%CI
All women	342	31.1	26.1-36.6	32.2	26.9-38.0	38.7	33.6-44.1	41.6	36.2-47.2
HPV negative	115	1.9	0.5-7.0	3.0	0.9-9.2	11.8	7.0-19.2	14.7	9.0-23.1
HPV positive	227	45.1	38.4-52.0	47.0	39.9-54.2	52.3	45.7-58.8	55.2	48.4-61.8
HPV16	105	56.5	46.5-66.0	56.5	46.1-66.3	60.0	50.2-69.1	61.1	51.0-70.3
nonHPV16	122	36.5	27.9-46.1	38.7	29.5-48.8	45.7	37.0-54.7	50.2	41.0-59.3
nonHPV16, 18, 31, 33, 45	61	24.4	14.7-37.7	26.6	16.1-40.6	36.4	25.3-49.2	41.8	29.9-54.7
Age < 30 years HPV positive	104	40.4	30.7-51.0	41.8	31.6-52.8	49.5	39.8-59.3	54.0	43.9-63.8
HPV16	49	61.5	46.8-74.4	61.5	46.2-74.8	65.8	51.4-77.8	58.1	43.5-71.4
nonHPV16	55	19.9	10.6-34.3	22.8	12.3-38.3	35.2	23.6-48.9	41.5	28.8-55.4
Age ≥ 30 years HPV positive	123	51.5	42.5-60.4	51.5	42.1-60.8	55.5	46.6-64.1	56.4	47.2-65.2
HPV16	56	52.1	38.9-65.1	52.1	38.3-65.5	55.2	42.0-67.7	55.2	41.5-68.1
nonHPV16	67	51.1	39.1-63.0	51.1	38.5-63.6	55.7	43.8-67.0	57.3	44.9-68.8
Clearance <6-months [a]	50	2.2	0.4-12.2	2.2	0.3-13.1	14.6	7.2-27.3	14.6	7.0-28.0
6-month persistence [a]	166	56.0	45.0-63.7	57.5	49.2-65.4	61.0	53.2-68.2	64.4	56.5-71.6
Persistence HPV16	77	67.2	55.8-76.9	67.2	55.4-77.2	68.4	57.1-77.9	69.9	58.4-79.4
Persistence nonHPV16	89	45.8	35.1-56.9	48.9	37.6-60.4	54.7	44.1-64.9	59.8	48.9-69.8

CIN3+, Cervical Intraepithelial Neoplasia grade 3 and cancer; CIN2+, Cervical Intraepithelial Neoplasia grade 2, 3 and cancer; 95%CI, 95% confidence interval; HPV, human papillomavirus; 6-month persistence, at baseline and at 6 months at least one detected hrHPV type is similar.
[a] All hrHPV-negative women at baseline and all women with a follow-up of less than 6 months were excluded. Time to event is set equal to histological diagnosis of CIN3+ or CIN2+ lesion.

more hrHPV types. All women who developed AIS or invasive cancer had only one hrHPV type: both SCC and one AIS contained HPV16, the AC harboured HPV18, and one AIS was positive for HPV45. In 93.3% (98/105) of CIN3+ cases, the same hrHPV type was present both in the lesion and at baseline, including both AIS, the AC and one SCC. Of one SCC no hrHPV typing information was available.

The most prevalent type was HPV16 (105/227, 46.3%), followed by HPV31 (29/227, 12.8%), HPV18 (22/227, 9.7%) and HPV33 (18/227, 7.9%). The CIN3+ risk of women infected with HPV16 was higher than that of women infected with other hrHPV types (Wald-statistic 6.85, p=0.009). The 5-year risk in HPV16-positive women was 56.5% (95%CI 46.5-66.0) and this was 36.5% (27.9-46.1) in nonHPV16-positive women. The risks in the subsequent 5 years were 0.01% (95%CI 0.0-10.7) and 3.4% (0.8-12.2), respectively. After stratification in two age categories, we found that in younger women (<30 years), HPV16-positive women had a significantly higher CIN3+ risk than nonHPV16-positive women (Wald statistic 13.01, p=0.003, Table 3.1). Their 5-year CIN3+ risks were 61.5% (95%CI 46.8-74.4) and 19.9% (10.6-34.3), respectively. In older women (≥30 years)

we found no difference in CIN3+ risk between HPV16-positive women and nonHPV16-positive women (Wald statistic 0.08, p=0.78). Their respective 5-year risks were 52.1% (95%CI 38.9-65.1) and 51.1% (39.1-63.0).

In women infected with hrHPV types other than HPV16, 18, 31, 33 and/or 45 the CIN3+ risk remained 24.4% (95%CI 14.7-37.7) in the first 5 years and was 2.9% (0.5-15.1) in the following 5 years. These risks were similar for both the age categories.

Women with transient hrHPV infections had a lower CIN3+ risk than women who had a persistent 6-month hrHPV infection (Wald-statistic 17.3; p=0.0003). The 5-year CIN3+ risk was 2.2% (95%CI 0.4-12.2) in women who cleared their infection and 56.0% (48.0-63.7) in women with a persistent infection. Within the persistent group, the risk of developing CIN3+ was higher in women positive for HPV16 (67.2%, 95%CI 55.8-76.9) than in women in whom other hrHPV types persisted (45.8%, 35.1-56.9, Wald-statistic 4.73; p=0.03).

Women were divided into two groups according to referral cytology; 210 (61.4%) women had a smear of BMD and 132 (38.6%) a smear of >BMD. In both these groups the median of cytological screens between 1996 and 2009 was 3.0 (range 1-9; p=0.71, Mann-Whitney).

Borderline and mild dyskaryosis (Table 3.2a)

Forty-seven of 210 (22.4%) women with BMD developed CIN3+. Their 5-year CIN3+-risk was 22.5% (95%CI 17.0-29.1) and their risk in the subsequent 5 years was 0.7% (0.1-4.5). Immediate hrHPV testing clearly stratified these women with regard to cumulative risk (Wald-statistic 11.08, p=0.001, Figure 3.3A). A negative hrHPV-test result, present in 84 (40.0%) women, reduced the 5-year CIN3+ risk to 0.01% (95%CI 0.0-5.1), whereas a positive test result increased this risk to 37.5% (29.0-46.9). The risks for the subsequent 5 years were 0.01% (95%CI 0.0-5.7) and 1.6% (0.2-9.5%), respectively. Women positive for HPV16 had a higher CIN3+ risk than women infected with other hrHPV types (Wald-statistic 5.60; p=0.02). Their 5-year risk was 49.8% (95%CI 36.2-63.4) versus 29.8% (18.4-40.2) in women infected with other hrHPV types. The 5-year risk remained 26.5% (95%CI 14.1-44.3) in women infected with hrHPV types different from HPV16, 18, 31, 33 and 45.

The risk of women who tested hrHPV positive at baseline was further stratified by follow-up testing after 6 months with either cytology or hrHPV (Wald-statistic 8.51; p=0.004 and 37.38; p<0.0001, respectively).

After women with a follow-up shorter than six months had been excluded, the CIN3+ risk of women who complied with the present follow-up algorithm of repeat cytology testing after 6 months was calculated. Women with normal cytology after 6 months had a 5-year CIN3+ risk of 4.9% (95%CI 1.6-13.8), whereas women with an abnormal test result had a risk of 30.9% (23.0-40.1). Risks in the next 5 years were 0.01% (95%CI 0.0-7.1) and 1.4% (0.2-8.5).

After stratification for age, results for both age groups were statistically not different, although the risks in the younger age group were slightly lower than in the older age group (data not shown).

Borderline and mild dyskaryosis (Table 3.2b)

Fifty-eight of 132 (43.9%) women with baseline moderate to severe dyskaryosis developed CIN3+ and their risk was 45.1% (95%CI 36.4-54.1) in the first 5 years and 3.5% (0.9-12.2) in the

Table 3.2 Risk (%) of cytology and hrHPV testing at baseline and at 6-month follow-up, stratified according to baseline cytology in BMD and >BMD.

A Women with borderline and mild dyskaryosis (BMD)

			CIN3+				CIN2+			
			5-year		10-year		5-year		10-year	
Baseline	**Follow-up (month 6) [a]**	**At risk**	**Risk (%)**	**95%CI**	**Risk (%)**	**95%CI**	**Risk (%)**	**95%CI**	**Risk (%)**	**95%CI**
All		210	22.5	17.0-29.1	23.1	17.4-30.4	31.0	25.0-37.8	33.0	26.6-40.1
	Cytology negative [b,c]	65	4.9	1.6-13.8	4.9	1.6-14.3	12.5	6.4-22.9	12.5	6.3-23.4
	Cytology positive [b,d]	127	30.9	23.0-40.1	31.8	23.3-41.7	38.0	29.7-47.0	41.5	32.7-50.9
HPV negative		84	0.0	0.0-5.1	0.0	0.0-5.7	9.9	5.1-18.5	11.2	5.8-20.5
	Cytology negative [b]	37	0.0	0.0-10.2	0.0	0.0-10.7	5.5	1.5-18.1	5.5	1.5-18.6
	Cytology positive [b]	40	0.0	0.0-10.7	0.0	0.0-12.5	10.9	4.3-25.1	13.7	5.7-29.5
	HPV negative	70	0.0	0.0-6.1	0.0	0.0-6.6	10.4	5.1-20.1	10.4	5.1-20.5
	HPV positive	9	0.0	0.0-35.4	0.0	0.0-43.4	12.5	2.2-47.1	25.0	6.6-61.1
	Double negative	36	0.0	0.0-10.4	0.0	0.0-11.0	5.6	1.5-18.5	5.6	1.5-19.0
	Cytology and/or HPV positive	48	0.0	0.0-9.2	0.0	0.0-10.4	13.4	6.2-26.4	15.7	7.5-30.1
HPV positive [c,d]		126	37.5	29.0-46.9	38.5	29.5-48.4	44.9	37.1-52.9	47.6	38.5-56.9
	Cytology negative [b]	28	11.6	3.9-29.9	11.6	3.6-31.5	22.0	10.5-40.5	22.0	10.0-41.7
	Cytology positive [c,d]	87	44.9	34.2-56.1	46.3	35.0-57.9	50.3	39.6-60.9	54.2	43.1-64.9
	HPV negative	29	0.0	0.0-13.8	0.0	0.0-15.5	11.0	3.8-27.9	11.0	3.6-29.2
	HPV positive [c,d]	91	46.3	35.8-57.1	47.6	36.5-59.0	52.2	41.8-62.4	55.9	45.0-66.3
	Double negative	15	0.0	0.0-22.8	0.0	0.0-25.9	6.7	1.1-30.9	6.7	1.0-33.4
	Cytology and/or HPV positive [c,d]	105	40.6	31.1-50.9	41.2	31.1-52.0	47.4	37.8-57.1	50.6	40.6-60.6
HPV16		55	49.8	36.2-63.4	49.8	35.6-64.0	55.3	41.8-68.1	57.4	43.4-70.3
nonHPV16		71	29.8	18.4-40.2	29.8	19.3-43.0	38.7	28.1-50.5	40.3	29.0-52.7

B Women with moderate and severe dyskaryosis (>BMD)

			CIN3+				CIN2+			
			5-year		10-year		5-year		10-year	
Baseline	**Follow-up (month 6) [a]**	**At risk**	**Risk (%)**	**95%CI**	**Risk (%)**	**95%CI**	**Risk (%)**	**95%CI**	**Risk (%)**	**95%CI**
All		132	45.1	36.4-54.1	47.0	37.8-56.4	51.2	42.6-59.7	55.3	46.4-63.9
	Cytology negative [b]	21	5.0	0.9-23.6	15.0	5.1-36.7	5.0	0.9-23.6	15.0	5.1-36.7
	Cytology positive [b]	98	53.9	43.5-64.0	53.9	42.9-64.5	60.7	50.7-69.9	64.0	53.7-73.2
HPV negative		31	7.3	2.0-23.6	11.2	3.4-31.0	16.9	7.4-34.2	24.2	11.6-43.6
	Cytology negative [b]	10	0.0	0.0-29.9	11.1	1.8-45.6	0.0	0.0-29.9	11.1	1.8-45.6
	Cytology positive [b]	18	13.5	3.7-39.1	13.5	3.0-44.3	29.4	13.3-53.1	36.5	16.9-61.9
	HPV negative	30	7.4	2.0-23.8	11.3	3.5-31.1	14.1	5.6-31.3	21.6	9.7-41.3
	HPV positive	1	0.0	-	0.0	-	100	20.7-100	100	20.7-100
	Double negative	10	0.0	0.0-29.9	11.1	1.8-45.6	0.0	0.0-29.9	11.1	1.8-45.6
	Cytology and/or HPV positive	21	11.2	3.0-33.7	11.2	2.6-37.5	25.0	11.2-46.9	30.8	14.2-54.5
HPV positive [e]		101	56.6	46.4-66.3	57.8	47.2-67.7	61.4	51.5-70.4	64.6	54.6-73.5
	Cytology negative [b]	11	9.1	1.6-37.8	18.2	5.1-47.7	9.1	1.6-37.8	18.2	5.1-47.7
	Cytology positive [b,e]	82	62.2	50.7-72.4	62.2	50.3-72.8	67.3	56.4-76.6	69.1	58.0-78.4
	HPV negative	14	0.0	0.0-24.3	0.0	0.0-25.9	7.7	1.4-33.3	7.7	1.3-34.6
	HPV positive [e]	81	65.1	53.7-75.0	66.8	55.1-76.7	68.8	57.9-77.9	72.8	62.0-81.5
	Double negative	5	0.0	0.0-43.4	0.0	0.0-43.4	0.0	0.0-43.4	0.0	0.0-43.4
	Cytology and/or HPV positive [e]	91	58.0	47.2-68.1	59.4	48.2-69.7	62.7	52.3-72.1	66.3	55.7-75.4
HPV16		50	63.7	49.6-75.8	63.7	49.1-76.2	65.3	51.3-77.1	65.3	50.8-77.4
nonHPV16		51	48.7	34.5-63.1	51.5	36.7-66.0	57.5	43.6-70.3	63.8	49.8-75.8

CIN3+, Cervical Intraepithelial Neoplasia grade 3 and cancer; CIN2+, Cervical Intraepithelial Neoplasia grade 2, 3 and cancer; 95%CI, 95% confidence interval; HPV, human papillomavirus. Time to event is set equal to histological diagnosis of CIN3+ lesion.
[a] all women with a follow-up of less than 6 months were excluded.
[b] cytology divided into negative (normal) and positive (borderline or mild dyskaryosis and worse).
[c] including one adenocarcinoma in situ (AIS).
[d] including one squamous cell carcinoma (SCC).
[e] including one SCC, one adenocarcinoma, and one AIS.

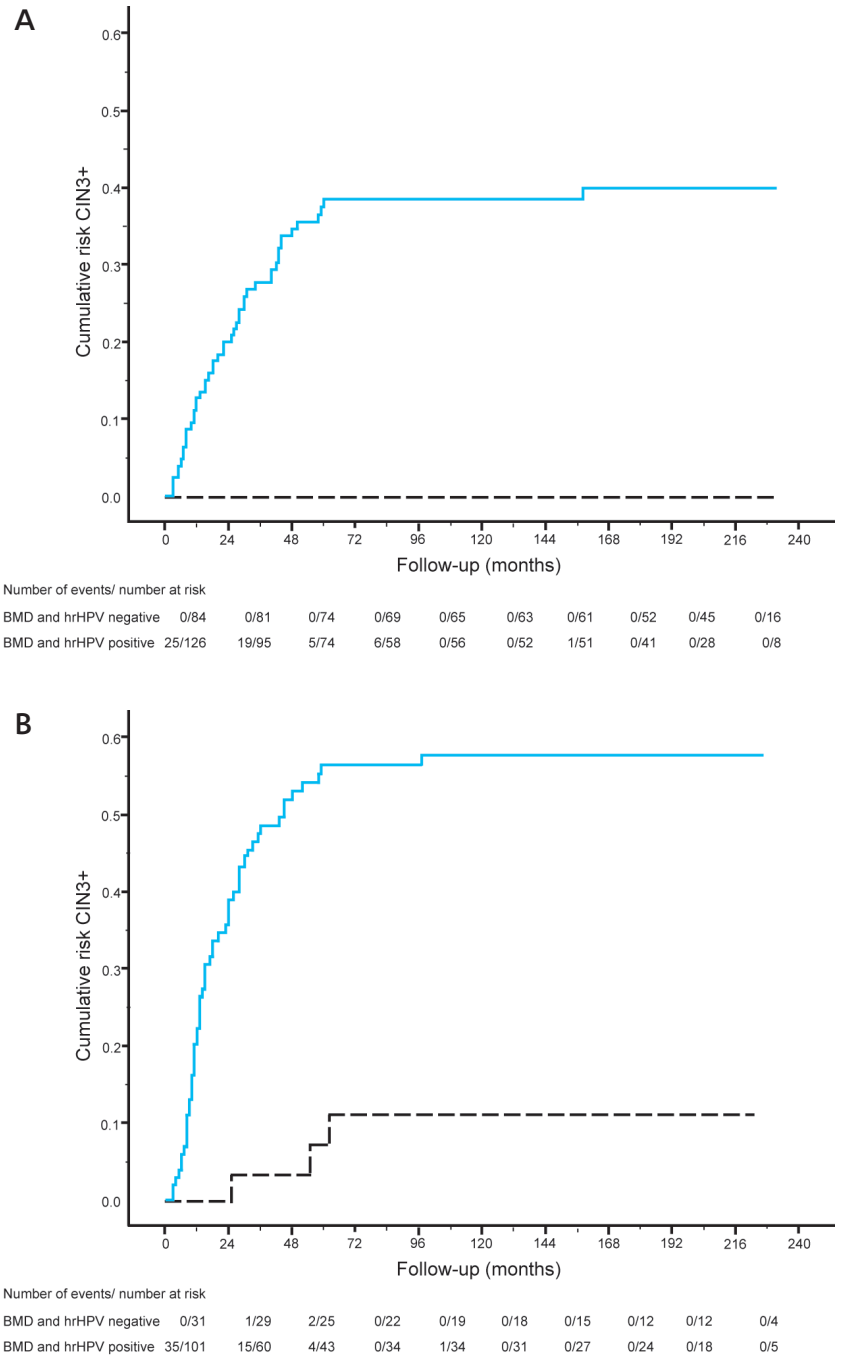


Figure 3.3 Cumulative risk curve of CIN3+ in women with borderline to mild dyskaryosis (BMD, n=210, A) and in women with moderate to severe dyskaryosis (>BMD, n=132; B) at baseline, according to baseline hrHPV status. CIN3+, Cervical Intraepithelial Neoplasia grade 3 or cancer; hrHPV, high-risk type of the human papillomavirus; HPV-positive (continuous) women and HPV-negative (dotted) women.

subsequent 5 years. Also in this group immediate hrHPV testing stratified the CIN3+ risk (Wald-statistic 12.31, p=0.0005, Figure 3.3B). HrHPV-positive women (76.5%) had a 5-year risk of 56.6% (95%CI 46.4-66.3) and this was 2.9% (0.5-15.4) in the subsequent 5 years. The 5-year risk for women positive for HPV16 was similar to the risk for nonHPV16-positive women (Wald-statistic 1.01; p=0.31). Thirty-one (23.5%) women tested hrHPV negative and had a 5-year CIN3+ risk of 7.3% (95%CI 2.0-23.6) and of 4.2% (0.6-23.2) in the subsequent 5 years. Additional testing after 6 months with either cytology, hrHPV, or both did not further stratify the risk (Wald statistic 0.07, p=0.80; 0.02, 0.90 and 0.009, 0.93, respectively). Also after age stratification no groups could be identified with a low enough risk to return to routine screening.

Analyses with CIN2+ as endpoint

Results of analyses with CIN2+ as endpoint were similar to those with CIN3+ as endpoint (Tables 3.1 and 3.2). The 5-year CIN2+ risk for women with abnormal cytology was 38.7% (95%CI 33.6-44.1) and this risk was 4.7% (2.4-9.0) between 5 and 10 years. Of all 141 CIN2+ lesions, 124 (87.9%) were detected in women who were hrHPV positive at baseline. Their 5-year CIN2+ risk was 52.3% (95%CI 45.7-58.8) and 6.1% (2.7-13.1) in the next 5 years. HPV16-positive women had a 5-year CIN2+ risk (60.0%, 95%CI 50.2-69.1) similar to the risk in women infected with other hrHPV types (45.7%, 37.0-54.7, Wald-statistic 3.32; p=0.07). Women who tested hrHPV negative at baseline had a 5-year risk of 11.8% (95%CI 7.0-19.2) and a risk of 3.3% (1.1-9.6) in the subsequent 5 years.

The 5-year CIN2+ risk in women with BMD was 31.0% (95%CI 25.0-37.8), their risk in the next 5 years was 3.0% (1.1-7.8). A negative hrHPV-test result at baseline reduced the 5-year risk to 9.9% (95%CI 5.1-18.5), whereas a positive test increased the risk to 44.9% (37.1-52.9). HPV16-positive women had a significantly higher risk than women infected with other hrHPV types (55.3%, 95%CI 41.8-68.1 *versus* 38.7, 28.1-50.5).

The CIN2+-risk in women with >BMD was 51.2% (95%CI 42.6-59.7) in the first 5 years and 8.4% (3.5-18.8) in the next 5 years. A negative hrHPV test result reduced the 5-year risk to 16.9% (95%CI 7.4-34.2) and a positive hrHPV test result increased this risk to 61.4% (51.5-70.4%). HPV16 had a similar CIN2+ risk as other hrHPV types in women with >BMD (Wald-statistic 0.01; p=0.91).

DISCUSSION

This study describes the long-term cumulative risk of developing CIN3+ after detection of abnormal cytology. For women with an abnormal smear (≥BMD) the 5-year CIN3+-risk was 31.1% and the risk in the next 5 years was 1.6%. We stratified these risks according to referral cytology and found that both women with BMD and women with >BMD referral cytology had an increased risk of developing CIN3+ within the first 5 years after detection. This risk was twice as high in women with >BMD compared with women with BMD (45% versus 22%). In the subsequent 5 years only for women with >BMD an increased risk (3.5%) remained, while for women referred with BMD this risk was with 0.7% similar to that of the general population.[6]

Immediate hrHPV testing stratified the CIN3+ risk of women with an abnormal smear (≥BMD). Almost all CIN3+ lesions (102/105), including all invasive carcinomas, were found in women testing hrHPV positive. Almost half of all hrHPV-positive women were infected with HPV16; these women had a significantly higher CIN3+ risk than women infected with other

hrHPV types.[17, 27-28, 36-37] This risk difference was only found in younger women (<30 years), while in older women (≥30 years) the risks between women positive for HPV16 and women positive for other hrHPV types were similar. This is in line with another study that found that the mean age of women with HPV16-associated cancer was significantly lower than of nonHPV16-associated cancer.[38] All CIN3+ in HPV16-positive women were identified within 5 years after detection of abnormal cytology, while lesions associated with other hrHPV types were also found in the 5 years hereafter. This suggests that HPV16 has its main oncogenic effect within a shorter timeframe and at a younger age than other hrHPV types.[38-39]

The CIN3+-risk was lower in women who cleared the virus than in women with persistent hrHPV infections, with the highest risks for women with a persistent HPV16 infection.[17, 37, 40-41]

Women with BMD

In correspondence with another Dutch study[36], almost 25% of women with BMD developed CIN3+. The majority was diagnosed within the first 5 years, while in the subsequent 5-year period their CIN3+ risk was similar to the risk of the general population.[2, 6] This implicates that women with BMD who did not develop CIN3+ within 5 years may return to routine screening. Other studies also found a negligible increase in high-grade CIN cases between 5 and 10 year after diagnosis of BMD cytology.[26-28]

Meta-analyses concerning women with BMD have found that immediate hrHPV testing better identifies women at risk of developing CIN3+ than repeat cytology after 6 months.[4, 14] Our study confirms these findings. Women with baseline BMD and normal cytology after 6 months (31%) had a 5-year risk of 5%, while this risk was less than 0.1% in women with BMD and a negative hrHPV test at baseline (40%). Therefore we support the referral of hrHPV-negative women with BMD to routine screening.[2, 15, 18, 21, 24] In both these groups the 5-year CIN2+ risk remained approximately 10%. After revision, all these lesions remained CIN2 and we believe most of them would regress over time. However, additional testing with either cytology or hrHPV after 6 months may be considered to minimize the risk of CIN2.

Women with BMD who tested hrHPV positive at baseline had a 5-year CIN3+ risk of almost 40% and are in need of additional testing and/or colposcopy.[2, 5-6, 15] Although hrHPV genotyping did identify HPV16-positive women to have the highest risk, the risk of women positive for other hrHPV types remained so high (28%) that colposcopic referral was required, leaving hrHPV genotyping without additional value.

Another strategy to identify women at risk for CIN3+ is hrHPV testing after 6 months, allowing viral clearance.[22, 42] In our study, almost half (99/210) of the women with BMD tested hrHPV negative after 6 months. As none of them developed CIN3+, this confirms the usefulness of this alternative strategy. However, also with this strategy the 5-year CIN2+ risk in hrHPV-negative women remains 10%.

Women with >BMD

As almost half of the women with >BMD cytology developed a CIN3+ lesion, we support referring all women with >BMD to colposcopy.[2, 6] Although immediate hrHPV testing did stratify the risk of developing CIN3+, no group was identified with a risk low enough to refrain from colposcopy. Therefore we do not advocate hrHPV testing in this group.

Study limitations

Our study has several limitations. First, the initial study was designed such that no interference with natural history would occur and therefore had a "wait and see" period to allow the development of real precursor lesions (CIN3), instead of transient lesions (CIN2). When CIN2+ lesions were detected women were treated, which is in contrast to another observational study.[43] The waiting period is also an explanation for the later diagnosis of CIN3+ lesions in our study than found in a joint European cohort study in which the majority of disease was diagnosed within 12 months.[44]

Second, our study comprises a relatively small cohort of 342 women. Although the censoring percentage at 10 years was only 20%, just one event was diagnosed after 10 years of follow-up. Therefore, we describe the risks up to 10 years and presented 95%CI to assess all risks as precisely as possible, providing a general impression on the long-term CIN-risk of women with an abnormal smear (≥BMD). Our results corroborate and extend the data of other (long-term) cohorts.[19-21, 23, 26-27, 41, 44] As most CIN3+ were detected within 5 years of referral [26], the presented overall 5- and 10-year CIN3+-risks are with 31.1% and 32.2% nearly similar. These risks were higher than reported previously by Dillner (6-year risk 19%) and Sherman (10-year risk 10.2%; 95%CI 7.6-12.9).[26, 44] Possible explanations include differences in the study population, that is the relatively large proportion (39%) of women with >BMD, and the strict endpoint of biopsy taking in the initial study. Other studies often acted on less severe suspicions, thereby increasing the detected number of lower grade CIN lesions. [12, 26-27] A number of CIN2 lesions that would have been detected in countries with less conservative referral thresholds such as the United States and the United Kingdom, will in The Netherlands have developed into CIN3+ lesions before detection. This explains the relatively higher number of CIN3+ lesions and the relatively lower number of CIN2 lesions.

Finally, the median age was relatively low. However, conclusions did not differ greatly after recalculating the risks for 196 women aged ≥30 years (data not shown).

Conclusions

In conclusion, our study confirms the increased CIN3+ risk in women with dyskaryosis. Bearing in mind the limitations of our study, we recommend the following:

Women with BMD should receive additional hrHPV testing for risk assessment. HrHPV-negative women may be referred to routine screening as their 5-year CIN3+ risk is negligible. HrHPV-positive women should be referred for additional testing and/or colposcopy. When these women do not develop CIN3+ within 5 years, they also may be referred to population-based screening. Women with >BMD should all be referred for colposcopy and as their CIN3+ risk is elevated for at least 10 years long-term monitoring is required.

Trial register

Dutch trial register, NTR 1470

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RISK ASSESSMENT IN WOMEN TREATED FOR CERVICAL DISEASE

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RISK OF RECURRENT HIGH-GRADE CERVICAL INTRAEPITHELIAL NEOPLASIA AFTER SUCCESSFUL TREATMENT; A LONG-TERM MULTI-COHORT STUDY

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SUMMARY

Introduction

15% of women treated for high-grade Cervical Intraepithelial Neoplasia (CIN2/3) develop residual or recurrent CIN2, CIN3 or cervical cancer (CIN2+), most of which are diagnosed within 2 years of treatment. To gain more insight into the long-term predictive value of different post-treatment strategies, we assessed the long-term cumulative risk of post-treatment CIN2+ and different follow-up algorithms to identify women at risk of residual and recurrent disease.

Methods

Women who were included in three studies in The Netherlands and who were treated for CIN2/3 between July, 1988, and November, 2004, were followed up by cytology and testing for high-risk human papillomavirus (hrHPV) at 6, 12 and 24 months after treatment, and subsequently received cytological screening every 5 years. The primary endpoint was the cumulative risk of post-treatment CIN2+ by December, 2009. We also assessed the cumulative risk of CIN2+ in women with three consecutive negative cytological smears and women with negative co-testing with cytology and hrHPV at month 6 and 24.

Results

435 women were included, 76 (17%) of whom developed post-treatment CIN2+, of which 39 were CIN3+. The 5-year risk of developing post-treatment CIN2+ was 16.5% (95%CI 13.0-20.7) and the 10-year risk was 18.3% (13.8-24.0). The 5-year risk of developing post-treatment CIN3+ was 8.6% (95%CI 6.0-12.1) and the 10-year risk was 9.2% (5.7-14.2%). Women with three consecutive negative cytological smears had a CIN2+ risk of 2.9% (95%CI 1.2-7.1) in the next 5 years and of 5.2% (2.1-12.4) in the next 10 years. The 5-year risk of CIN3+ was 0.7% (95%CI 0.0-3.9) and the 10-year risk was 0.7% (0.0-6.3). Women with negative results for co-testing had a 5-year risk of CIN2+ of 1.0% (95%CI 0.2-4.6) and a 10-year risk of 3.6% (1.1-10.7). The 5-year risk of CIN3+ was 0.0% (95%CI 0.0-3.0) and the 10-year risk was 0.0% (0.0-5.3).

Discussion

The 5-year risk of post-treatment CIN2+ in women with three consecutive negative cytological smears or negative co-testing for cytology and hrHPV at 6 and 24 months was similar to that of women with normal cytology in the population-based screening programme and therefore justifies their return to regular screening.

INTRODUCTION

Women diagnosed with high-grade cervical lesions (Cervical Intraepithelial Neoplasia grade 2 or 3 [CIN2/3]) are treated by ablative surgery or by excision to prevent progression to cervical cancer.[1-2] Despite treatment, approximately 15%[3] (range 5-25%)[3-8] of these women will develop residual or recurrent (post-treatment) high-grade disease. Because of this substantial risk close surveillance of these patients is standard practice. In The Netherlands, national guidelines recommend repeat cytological testing at 6, 12 and 24 months after initial treatment and, if necessary, yearly thereafter until three consecutive smears are read as normal.[1] After three consecutive negative smears women return to population-based cytological screening every 5 years. In other countries, such as the United Kingdom (UK) and the United States of America (USA), treated women are screened yearly until 5 or 10 years after treatment.[9-11]

The presence of high-risk human papillomavirus (hrHPV) is a prerequisite not only for development of primary CIN2/3 or cervical cancer [12], but also for development of post-treatment CIN2/3. Effective ablative treatment not only results from removal of the lesion, but is associated with elimination of the responsible hrHPV infection.[6, 13-14] Post-treatment surveillance that combines testing with cytology and hrHPV (co-testing), has a negative predictive value of over 99% to detect women at risk of developing post-treatment CIN2/3.[3, 7, 15] Strategies that include hrHPV testing are therefore suggested as an alternative to conventional surveillance with cytology only.[3-4, 6, 15-16] In one such proposed strategy, the 12-month visit is omitted in women who test negative for co-testing at 6 months, but co-testing is done again at month 24.[3, 14, 17] A simulation model predicted that different co-testing strategies would not result in an increase in use of colposcopy or an increased proportion of missed CIN2/3.[18]

Since most CIN2/3 is diagnosed within 2 years after treatment, studies on identification of women at risk are predominantly confined to this period.[4, 7, 14, 16-17] However, the risk of developing recurrent CIN grade 3 or cancer (CIN3+) is significantly increased for 10 or even 25 years after treatment.[5, 8, 19] This finding, combined with an absence of international consensus on optimum surveillance strategies after initial treatment [6, 9], suggests that more insight is needed into the long-term predictive value of different post-treatment strategies.

We aimed to assess the long-term rate of recurrence of CIN2+ and CIN3+ and the effectiveness of the present cytological algorithm at detecting post-treatment disease. We also investigated whether alternative post-treatment surveillance strategies, which include co-testing, could reduce the number of screens needed after treatment, without reducing the effectiveness of the strategy at identifying women with a long-term risk of post-treatment disease.

MATERIALS AND METHODS

Study population

In this cohort study we included women who had participated in one of three previous studies [14, 17, 20] that monitored women by hrHPV testing and cytology at 6, 12 and 24 months after treatment for CIN2/3. All women had been treated by large loop excision of the transformation zone (LLETZ) or cold-knife conisation. The studies were done in hospitals in the Randstad

region, The Netherlands. All hrHPV tests had been done by a single laboratory and all cytology had been reviewed in one institution (VU University Medical Center, Amsterdam, The Netherlands). Because of these similarities, data were pooled into a single database for joint statistical analysis. Patients were excluded if data were incomplete or if they had been treated for persistent low-grade disease.

Ethical approval was obtained from the ethics board at all hospitals. All women who attended the outpatient clinic or participated by self-sampling provided additional signed informed consent.

The study is registered in the Dutch trial register, NTR1468.

Procedures

To complete the data obtained from population-based screening, we invited all women to visit the outpatient clinic (VU University Medical Center, Amsterdam, The Netherlands or ErasmusMC University Medical Center, Rotterdam, The Netherlands) for additional cytology and hrHPV testing between January and December, 2009. If travel distance was a limitation, women were offered the possibility of doing a hrHPV test at home by self-sampling. Results from these home-based hrHPV tests are similar to those acquired by a physician.[21-22] Women who had had a hysterectomy were censored at the date of hysterectomy. Data on demographics, medical history and lifestyle were collected. Women were asked about smoking habits, oral contraceptive use, condom use, parity, and number of sexual partners. Standard questionnaires were used.

Two cervical specimens were obtained from women who visited the outpatient clinics (Cervex-brush, Rovers Medical Devices, Oss, The Netherlands). The first specimen was collected in liquid-based cytology medium (in Amsterdam: Surepath, Tripath Imaging, Burlington NC, USA; in Rotterdam: Thinprep, Hologic, Marlborough MA, USA), cytologically examined and classified 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.[23] The second specimen was stored in Universal Collection Medium (Qiagen Corporation, Gaithersburg, MD, USA) for hrHPV testing. Women who did hrHPV self-sampling returned their cervico-vaginal specimen for hrHPV testing by mail. All samples were tested with the clinically validated hrHPV GP5+/6+ primer-mediated PCR with enzyme-immunoassay read-out using a cocktail probe for 14 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), according to established protocols.[24-25] The PCR products of hrHPV-positive women were subsequently genotyped by reverse line blot hybridization. Samples that were negative for any specific probe in this reverse hybridization assay, were deemed positive for uncharacterised subtypes or variants.

We did a standard colposcopic assessment when a cytological test was abnormal at the threshold of atypical squamous cells of undetermined significance (or borderline dyskaryosis), or when a hrHPV test was positive. Biopsies were taken of all suspect lesions. Histological specimens were graded as CIN grade 0 (no dysplasia), 1, 2, 3, or invasive cancer [26] and classified according to the highest histological abnormality found in biopsy or LLETZ. Women, who developed post-treatment disease (CIN2+), were treated according to present guidelines [1] and received further follow-up tests but were censored for this study.

In December 2009, hospital databases and The Netherlands nationwide network and registry of histopathology and cytopathology (PALGA; Bunnik, The Netherlands) were reviewed for all women, irrespective of attendance, to ascertain details of any additional relevant events and procedures.

The primary endpoint was the cumulative risk of post-treatment CIN2+, because treatment of CIN grade 2 is common practice in The Netherlands.[1] We repeated the calculations for CIN3+, because this is a more adequate surrogate for precancer. For the purpose of this study residual and recurrent disease were combined and defined as post-treatment CIN. This study was limited to the follow-up of squamous lesions. Post-treatment CIN2+ and CIN3+ included squamous cell carcinoma. Total time at risk was measured as the period between the date of initial treatment and the last registered testing date or the midpoint between the date of detection of post-treatment CIN and the date of the cytological test result before the colposcopic referral.

For women who had not developed CIN at 24 months after treatment, we also estimated the risk of post-24-month CIN. For these analyses, we reset time at 24 months after treatment to 0 months. We wanted to identify women who could be referred to population-based screening after a close surveillance period of 2 years and women who should remain under more intense surveillance. In particular, we tested the association between the post 24-month CIN risks in those women and the cytological and hrHPV test results obtained in the first 24 months after treatment.

Statistical analysis

Because this study was designed as a follow-up study, the maximum sample size was limited to the number of women who had participated in the initial trials.[14, 17, 20] Data were interval-censored in case of a CIN2+ event and right censored otherwise. Data were interval-censored in case of a CIN3+ event and right censored if lost to follow-up, or if a CIN2 lesion was diagnosed.

The cumulative risk of post-treatment CIN was estimated by maximizing the non-parametric likelihood,[27] which adjusts for interval censoring between the dates of successive visits. If the maximum likelihood estimate of a cumulative risk was not unique, the largest risk estimate was reported. 95% Confidence intervals (95%CIs) were calculated with the score test. Differences in cumulative risk curves between subgroups were assessed by exact log-rank tests for interval-censored data with Sun's scores measured from baseline to the date of detection of CIN2+.[28]

By Cox regression, we calculated CIN hazard ratios (HRs) and 95%CIs to compare different follow-up test results in the first 24 months. Similar analyses were done to compare test results 6 months after treatment. Time to CIN was set as the midpoint of the time between detection of the CIN lesion and the cytological test result before the colposcopic referral. The reported HRs were adjusted for treatment centre, original cohort and year of treatment.

The cumulative CIN risks and exact log-rank tests were done with the interval R package[29] within statistical software program R (version 2.12).[30] Exact permutation p-values were approximated by Monte Carlo simulation. The Cox regression analyses were done with SPSS (version 17.0). For all tests, the level of significance was set at 0.05.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all the data and the corresponding author had final responsibility for the decision to submit for publication.

RESULTS

Of the 445 women from the previous studies, five could not be identified because of incomplete patient data and another five from one study [17] had been treated for persistent low-grade disease and were excluded. The remaining 435 women were monitored by population-based screening once every 5 years, as per standard practice in The Netherlands. Table 4.1 provides details of individual study designs, inclusion and exclusion criteria, and location.

Maximum follow-up depended on accrual date and ranged from 5.0 to 21.5 years. The total number of women years in our study was 3464. Of 435 women included, 74 (17%) were censored at 5 years, 216 (50%) at 10 years and 289 (66%) at 15 years after treatment.

Table 4.1 Characteristics of initial cohorts.

	Nobbenhuis et al.[14]	**Hogewoning et al. [20]**	**Bais et al.[17]**
Number of women	184	78	183
Inclusion criteria	Treatment for CIN2/3	Treatment for CIN2/3	Treatment for CIN2/3
Exclusion criteria	Previous cervical treatment Concomitant cancer Previous exposure to diethylstilbestrol	Previous cervical treatment Regular condom use at baseline	Previous cervical treatment Concomitant or previous cancer Immune comprising conditions
Hospitals	VU University Medical Center	Albert Schweitzer Hospital	VU University Medical Center ErasmusMC University Medical Center Albert Schweitzer Hospital
Years of Inclusion	1990-1996	1995-2002	2002-2004
Cytology[37]	CISOE-A	CISOE-A	CISOE-A
hrHPV-test	GP5+/6+ PCR	GP5+/6+ PCR	GP5+/6+ PCR
Median follow-up (range; months)	24 (3–76)	15 (3–85)	24 (3–24)
Tests done at month	3, 6, 9, 12 and 24	3, 6, 12, 18 and 24	6, 12 and 24
Median age (range; years)	31 (21–70)	34 (22–54)	35 (22-56)
Women with CIN2+ ≤24 months after treatment (%)	27 (15%)	6 (8%)	16 (9%)

CIN, Cervical Intraepithelial Neoplasia; CISOE-A, Composition, Inflammation, Squamous epithelium, Other and endometrium, Endocervical columnar epithelium, and Adequacy of the smear; hrHPV, high-risk type of the human papillomavirus.

Of the 435 women (median age 33, range 21-70 years) four died of unrelated disease during follow-up, five moved abroad, and 27 had a hysterectomy. None of the women received prophylactic HPV vaccination during follow-up. At initial treatment 358 (82%) of 435 women had been treated by an LLETZ procedure, the remaining 77 women (18%) had a cold-knife conisation. 344 (79%) of 435 women were treated for a CIN 3 lesion. Before initial treatment, 399 (93%) of 430 women who had an available hrHPV test tested positive for hrHPV. HPV16 was the most prevalent type (255/399, 64%), followed by HPV31 (48/399, 12%), HPV33 (43/399, 11%) and HPV18 (34/399, 9%). Of the 399 hrHPV-positive women, 338 (85%) were infected with one hrHPV type, 55 (14%) had a double infection and six (2%) had a triple infection.

During follow-up 76 CIN2+ cases were identified: two were invasive cancers and were diagnosed after 14 and 28 months, 37 were CIN3 and 37 CIN2. The median time until detection of CIN2/3 was 15.5 months (range 3-153). 41 of 76 CIN2+ lesions were found within 2 years of treatment. Figure 4.1 shows the interval censored cumulative risks of post-treatment CIN2+ and CIN3+. CIN2+ and CIN3+ were detected not only during the post-treatment surveillance period, but also in the subsequent population-based screening programme.

Of all 76 women who developed post-treatment CIN2+, 72 were hrHPV positive at baseline and all 39 women who developed post-treatment CIN3+ tested positive at baseline. 54 women diagnosed with post-treatment CIN2+, and 33 of the women diagnosed with CIN3+, including the two women who developed invasive carcinoma, tested positive for HPV16, In both patients who developed carcinoma and in 45 of 74 patients with CIN2/3, the hrHPV type in the post-treatment lesion was the same as was present at initial treatment.

Table 4.2 shows the 5-year and 10-year risks of CIN2+ for all confounding risk factors. We noted a difference in the risk of developing post-treatment CIN2+ for original cohort (Sun-score statistic 12.1; p=0.05), treatment centre (Sun-score statistic 15.1; p=0.03) and year of treatment (Wald statistic 6.44; p=0.01). Neither in the pooled nor in the separate cohorts (data not shown) were significant differences found in the risk of developing post-treatment CIN2+ for treatment modality (Sun-score statistic 1.76; p=0.58) or severity of the initial lesion (5.56; p=0.10). Women with negative hrHPV tests had a similar risk of post-treatment CIN2+ as those with a positive test (Sun-score statistic 1.15; p=0.66), but none of the hrHPV-negative women developed a CIN3+ lesion. There was no significant difference in 5-year and 10-year risks of developing CIN2+ between women who tested hrHPV negative and those who tested positive (Table 4.2); however the number of women who tested negative was low.

6 months after treatment, 87 (21%) of 424 available hrHPV tests were positive and 65 (15%) revealed the same hrHPV type as detected at baseline. More women who were positive for HPV16 developed CIN2+ than did those infected with other hrHPV types (40 of 56 women *versus* 13 of 31, p=0.02). The 5-year risk of CIN2+ was 66.2% (95%CI 52.6-77.5) in women positive for HPV16 and 39.7% (23.9-57.9) in those negative for HPV16. However, the time to develop post-treatment CIN2+ was similar between these two groups (18 *versus* 17 months, p=0.94, Mann-Whitney test). The 5-year risk of developing post-treatment CIN2+ was 28.6% (95%CI 10.9-56.6) in women infected with hrHPV types different from HPV16, 18, 31, 33, and 45. 46 of 65 women in whom the same hrHPV type was found at baseline and 6 months after treatment developed CIN2+ compared with seven of 22 of those infected with a new hrHPV type at 6

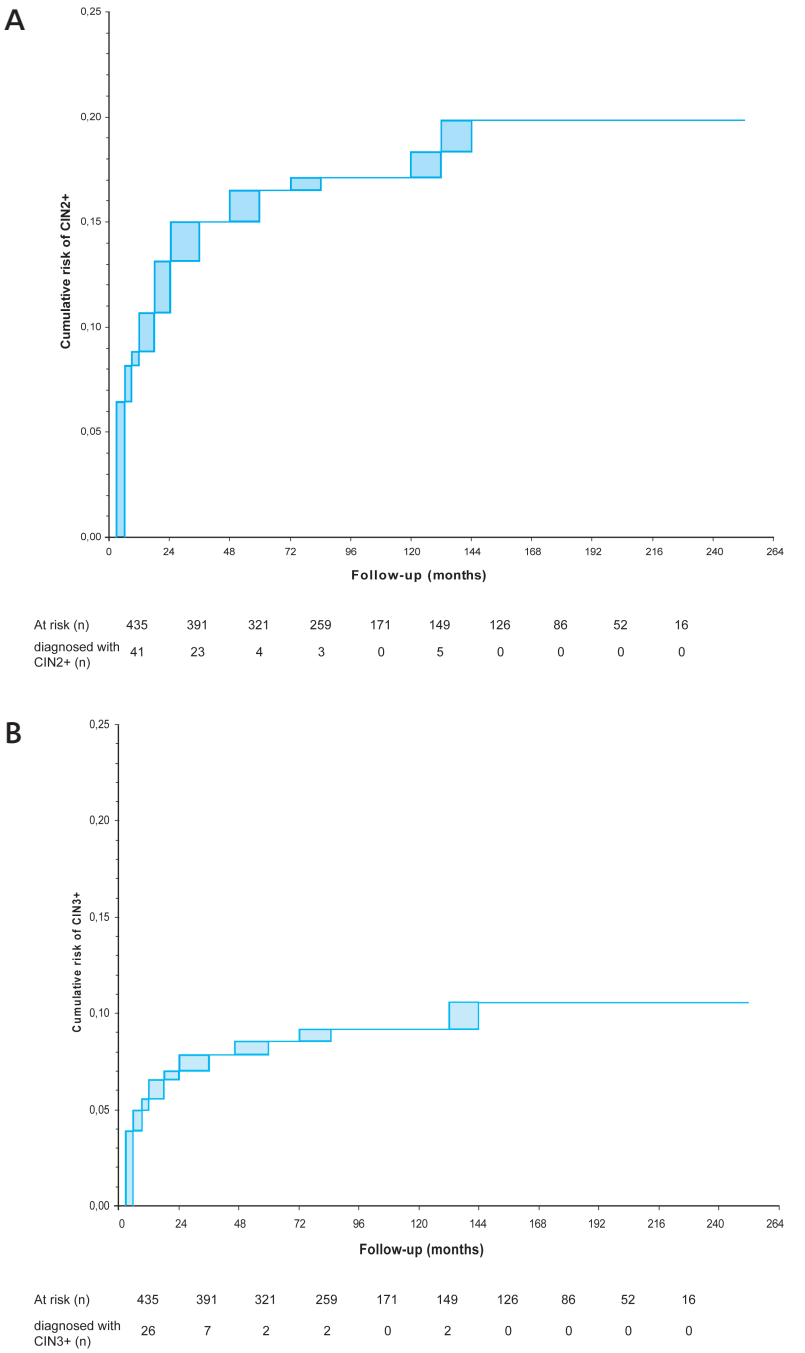


Figure 4.1 Cumulative risk curves of post-treatment CIN2+ and CIN3+.
CIN, cervical intraepithelial neoplasia; Cumulative risk curves of (A) post-treatment CIN2+ and (B) post-treatment CIN3+. Because of interval-censored data the risk curves are not unique. Every cumulative risk curve within the shaded boxes gives the same fit to the data because of interval censoring.

Table 4.2 Risk of developing CIN2+ according to different confounding risk factors.

	5-year risk (95%CI)	10-year risk (95%CI)
Cohort		
Nobbenhuis et al[14]	20.1% (14.8-26.8)	21.9% (16.2-28.9)
Hogewoning et al[20]	12.1% (6.3-22.8)	13.8% (6.2-28.1)
Bais et al[17]	11.4% (6.9-18.2)	29.9% (15.0-50.8)
Treatment centre		
VUmc	18.7% (14.3-24.1)	20.5% (15.2-27.1)
Albert Schweitzer	9.7% (4.9-18.4)	11.5% (4.7-25.3)
ErasmusMC	9.2% (3.8-20.7)	35.1% (13.1-66.0)
Treatment modality		
LLETZ	15.0% (11.4-19.5)	18.1% (13.1-24.5)
Conisation	15.9% (8.8-27.0)	15.9% (7.9-29.4)
Severity of initial lesion		
CIN2	9.3% (4.4-18.5)	11.1% (3.5-29.8)
CIN3	16.7% (12.9-21.4)	19.3% (14.4-25.4)
hrHPV status at initial treatment		
Negative	13.4% (4.6-33.1)	13.4% (1.8-56.8)
Positive	15.5% (12.0-19.8)	18.1% (13.5-23.8)
Smoking at initial treatment		
Never	8.7% (3.7-19.0)	12.6% (4.6-30.3)
Ex-smoker	17.2% (9.8-28.4)	18.9% (9.5-34.2)
1-10 cigarettes per day	9.1% (3.1-23.6)	12.1% (3.3-35.6)
>10 cigarettes perday	27.1% (17.4-39.6)	33.5% (20.0-50.3)
Oral contraceptive use		
Yes	13.0% (6.0-26.0)	13.0% (6.0-26.0)
No	17.3% (12.3-23.8)	22.2% (14.9-31.7)
Condom use		
Yes	16.3% (8.1-30.0)	16.3% (5.1-41.2)
No	16.4% (11.6-22.8)	21.1% (14.2-30.2)
Birth		
None	5.0% (1.7-13.8)	9.0% (2.9-24.7)
≥1	20.8% (15.1-28.0)	24.7% (16.7-34.9)
Sexual partners		
<5	17.5% (10.8-27.1)	18.7% (10.4-31.3)
≥5	15.6% (10.3-22.9)	21.6% (13.1-33.4)

95%CI; 95% confidence interval; LLETZ, large loop excision of the transformation zone; CIN, cervical intraepithelial neoplasia; hrHPV, high-risk type of the human papillomavirus.
Risk was measured by maximum likelihood estimate.

months (p=0.004). The 5-year risk of a 6-month persistent hrHPV infection was 66.2% (95%CI 53.6-76.9) and the risk of a newly detected hrHPV infection at 6 months 27.9% (13.1-49.8).

12 months after treatment 41 (10%) of 400 women tested for hrHPV. 30 women (8%) were also positive after 6 months; all these women were diagnosed with the same hrHPV type as detected at baseline. Nine of the 41 women who were hrHPV positive after 12 months were hrHPV negative after 6 months and in only two of these the same hrHPV type was diagnosed as at baseline. 6-months data were missing for two women. After 24 months 28 of 348 (8%) women were hrHPV positive; 10 (3%) were still infected with their original hrHPV type, eight of whom were positive for HPV16. Of the ten women still infected with their original hrHPV type, one did not develop high-grade post-treatment disease; one was diagnosed with invasive carcinoma, six with CIN3, and two with CIN2. The other 18 women were infected with a new hrHPV type at either 12 (two women) or 24 months (16 women).

In 2009, additional tests were obtained from 215 of 435 (49%) women, of whom 178 (83%) had both cytology and hrHPV test results available. Seven (3%) of 215 women had an abnormal cytology result and 27 (13%) tested positive for hrHPV, of whom 10 had HPV16. 29 (13%) of 215 women had abnormal test results, three of whom had CIN3 and two CIN2.

The 5-year risk of developing post-treatment CIN2+ was 16.5% (95%CI 13.0-20.7) and the 10-year risk was 18.3% (13.8-24.0). The development of CIN2+ could be predicted by 6-month testing with cytology, hrHPV, or both all p<0.0001; Table 4.3). In two studies[14, 20] the first testing point was after 3 months; thus, we also analysed the predictive values of the first test results, acquired after a median of 4 months (range 2-10). These showed similar results to those after 6 months, but with lower HRs (data not shown). The 5-year risk of CIN2+ in women with negative cytology was 5.8% (95%CI 3.6-9.3) and of women with an abnormal cytology result it was 46.2% (36.0-56.7). For women with a negative hrHPV test this risk was 4.4% (95%CI 2.5-7.5) and for those with a positive test 56.9% (46.0-67.2). Women whose co-testing results were negative had a 5-year risk of CIN2+ of 3.0% (95%CI 1.5-6.1) compared with a risk of 41.0% (32.5-50.0) for women who had at least one positive test result.

Women with three consecutive negative smears had a lower risk of developing CIN2+ than women with at least one abnormal cytological test result at 6, 12 or 24 months (p<0.001; Table 4.3; Figure 4.2). The corresponding HR for women with at least one positive test result compared with those with three negative smears was high (Table 4.3). The 5-year risk of developing CIN2+ in women with three negative smears was 2.9% (95%CI 1.2-7.1) and the 10-year risk was 5.2% (2.1-12.4). In women with at least one positive test, corresponding risks were 18.2% (95%CI 11.9-26.8) and 21.9% (14.2-32.1).

Women who tested negative for co-testing at 6 and 24 months after treatment had a lower risk of CIN2+ than women with at least one positive test result (p<0.001; Table 4.3; Figure 4.2). The 5-year risk of developing CIN2+ in women who tested negative for co-testing was 1.0% (95%CI 0.2-4.6) and the 10-year risk was 3.6% (1.1-10.7) In those with at least one positive test, these risks were 17.8% (95%CI 12.0-25.7) and 21.1% (14.0-30.5).

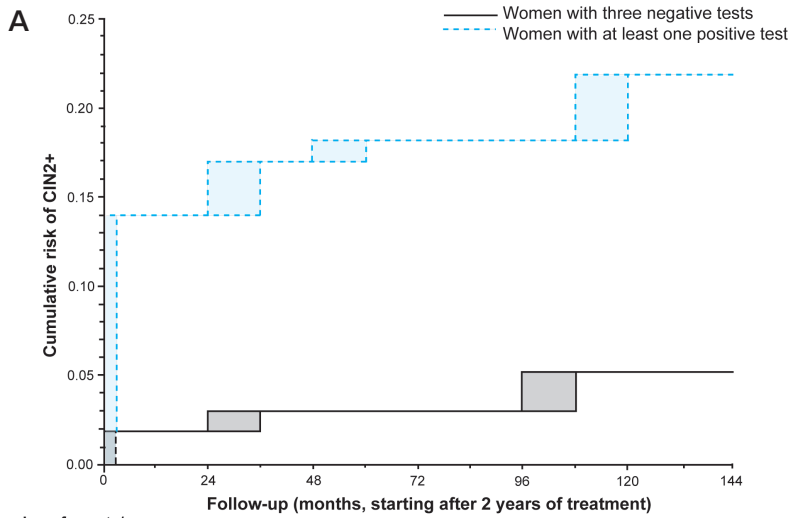
214 (49%) women completed the questionnaires which were analysed to identify additional risk factors for post-treatment CIN2+. The women from whom questionnaires were obtained, had similar properties to the full group in terms of hrHPV-positivity at baseline, proportion with CIN2, and mean age (data not shown); thus we considered this sample to be representative of

Table 4.3 Prediction of recurrent high-grade disease according to different follow-up algorithms.

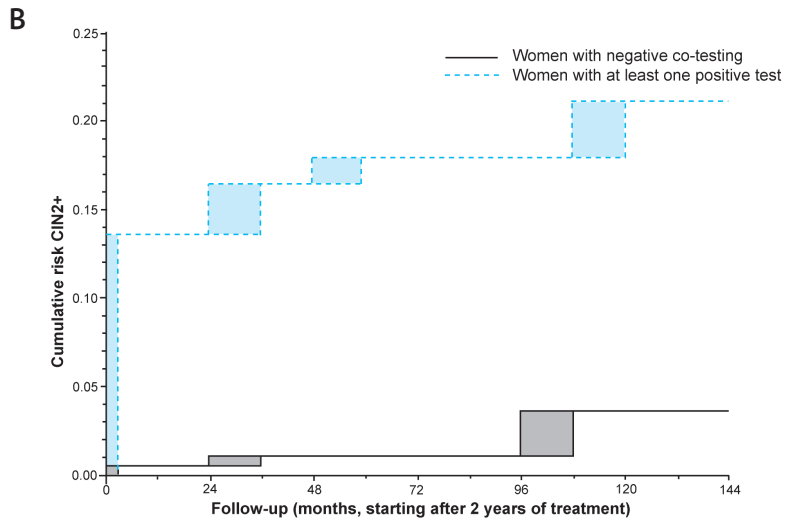
	Number at risk [a]	CIN2+ after treatment		CIN3+ after treatment	
		n (%)	HR (95%CI) [b]	n (%)	HR (95%CI) [b]
Testing at 6 months					
All	435	76 (17.5)		39 (9.0)	
hrHPV negative	337	22 (6.5)		7 (2.1)	
hrHPV positive [c]	87	53 (60.9)	16.84 (10.06 – 28.19)	32 (36.8)	29.30 (12.62 – 68.02)
Normal cytology	326	25 (7.7)		9 (2.8)	
≥ ASC-US [c]	98	49 (50.0)	8.87 (5.36 – 14.68)	30 (30.6)	13.00 (6.00 – 28.19)
Co-testing negative	283	13 (4.6)		4 (1.4)	
hrHPV positive or ≥ ASC-US, or both [c]	135	61 (45.2)	13.70 (7.43 – 25.25)	35 (25.9)	22.50 (7.88 – 64.28)
Testing at both 6 and 24 months					
All	391	35 (9.0)		13 (3.3)	
Cytology triple negative [d]	254	9 (3.5)		1 (0.4)	
At least one result ≥ ASC-US [c]	137	26 (19.0)	6.13 (2.65 – 14.21)	12 (8.8)	26.26 (3.27 – 210.81)
hrHPV negative at 6 months					
Co-testing negative at 24 months	255	7 (2.7)		1 (0.4)	
hrHPV positive or ≥ ASC-US or both at 24 months	136	28 (20.6)	8.05 (3.43 – 18.88)	12 (8.8)	28.51 (3.62 - 222.64)
Co-testing negative at 6 months					
Normal cytology at 24 months	249	8 (3.2)		1 (0.4)	
≥ ASC-US at 24 months	142	27 (19.0)	7.08 (3.03 – 16.51)	12 (8.5)	24.53 (3.14 – 191.54)
hrHPV negative at 24 months	241	6 (2.5)		1 (0.4)	
hrHPV positive at 24 months	150	29 (19.3)	8.51 (3.44 – 21.02)	12 (8.0)	26.02 (3.29 – 205.59)
Co-testing negative at 24 months	221	4 (1.8)		0	
hrHPV positive or ≥ ASC-US or both at 24 months	170	31 (18.2)	10.42 (3.60 – 30.14)	13 (7.6)	

CIN, cervical intraepithelial neoplasia; HR, Hazard Ratio; hrHPV, high-risk type of the human papillomavirus; ASC-US, atypical squamous cells of undetermined significance; co-testing negative, hrHPV testing negative and normal cytology.
[a] data for testing at both 6 and 24 months excludes women who developed post-treatment disease within 24 months or who were follow-up for less than 24 months.
[b] adjusted for treatment centre, original cohort and year of treatment.
[c] including two women with invasive squamous cell carcinoma.
[d] normal cytology results at month 6, 12 and 24 months.

the study population. In this subgroup, we noted that smoking around the treatment date was associated with an additional risk of developing CIN2+ (Sun-score statistic 19.06; p=0.01). In a post-hoc analysis, we found that this risk was only increased in women who smoked more than ten cigarettes per day; their 5-year and 10-year risks of developing CIN2+ were around 30%, whereas the risks for women who had never smoked were much lower (Table 4.2). We did not identify an additional risk of CIN2+ for women who used oral contraceptives (Sun-score statistic



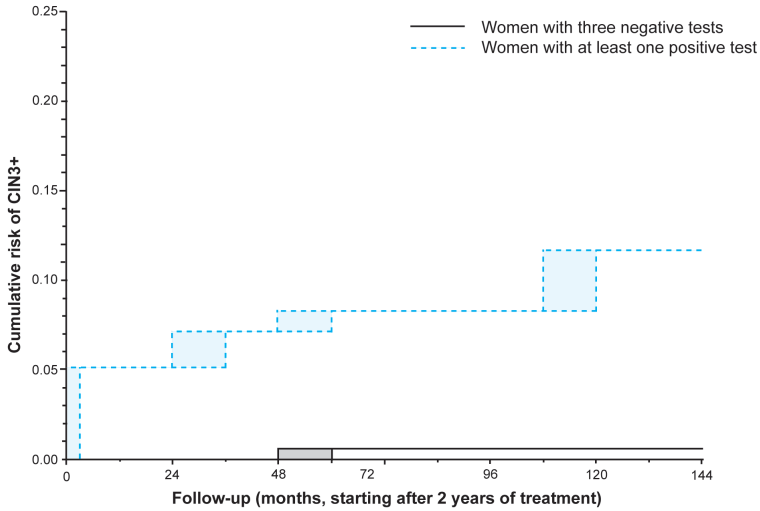
Number of events/ number at risk						
Women with three negative tests	4/254	2/219	1/173	0/103	2/87	0/72
Women with at least one positive test	19/137	2/102	2/86	0/68	3/62	0/54



Number of events/ number at risk						
Women with negative co-testing	1/221	1/189	0/151	0/92	2/78	0/66
Women with at least one positive test	22/170	3/132	3/108	0/79	3/71	0/60

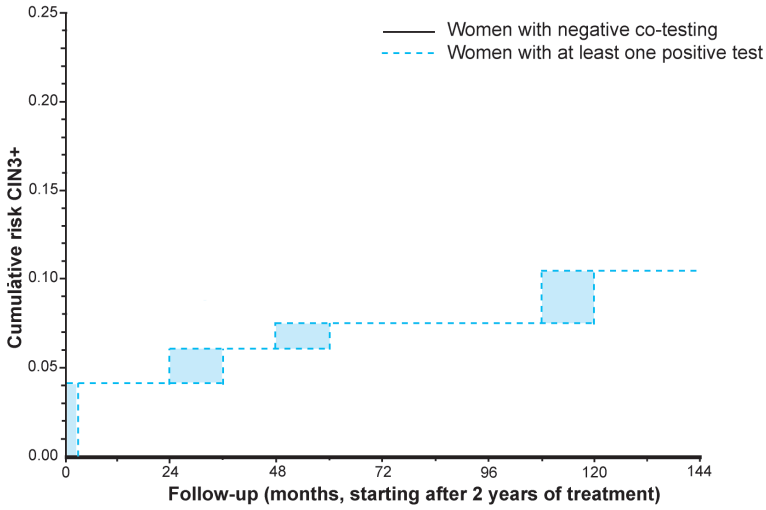
Figure 4.2 Prediction of post-treatment disease according to different algorithms.
CIN, cervical intraepithelial neoplasia; hrHPV, high-risk type of the human papillomavirus.
Post-treatment surveillance by the algorithm of (A) cytological testing at 6, 12 and 24 months for predicting post-treatment CIN2+, (B) co-testing (hrHPV and cytology) at 6 and 24 months for predicting post-treatment CIN2+, (C) cytological testing at 6, 12 and 24 months for predicting post-treatment CIN3+, and (D) co-testing (hrHPV and cytology) at 6 and 24 months for predicting post-treatment CIN3+. Because of interval-censored data the risk curves are not unique. Every cumulative risk curve within the shaded boxes gives the same fit to the data because of interval censoring. For all figures, p<0.0001.

C



Number of events/ number at risk						
Women with three negative tests	0/254	0/219	1/173	0/103	0/87	0/72
Women with at least one positive test	7/137	2/102	1/86	0/68	2/62	0/54

D



Number of events/ number at risk						
Women with negative co-testing	0/221	0/189	0/151	0/92	0/78	0/66
Women with at least one positive test	7/170	2/132	2/108	0/79	2/71	0/60

3.58; p=0.20), did not use condoms (3.58; p=0.38), had given birth (5.15; p=0.06), or had more than five sexual partners (3.19; p=0.57; Table 4.2).

Results of analyses of the risk of post-treatment CIN3+ were similar to those for CIN2+ (Table 4.3; Figure 4.1). The 5-year risk of developing CIN3+ was 8.6% (95%CI 6.0-12.1) and the 10-year risk of developing post-treatment CIN3+ was 9.2% (5.8-14.2).

Women with three consecutive negative smears had a lower risk of developing CIN3+ than women with at least one abnormal cytological test result at 6, 12 or 24 months (p<0.0001; Table 4.3; Figure 4.2). The 5-year risk of CIN3+ in women with three negative smears was 0.7% (95%CI 0.0-3.9) and their 10-year risk was 0.7% (0.0-6.3). The 5-year and 10-year risks for women with at least one positive test were 8.3% (95%CI 4.1-15.9) and 11.6% (5.9-21.6), respectively.

Women who tested negative for co-testing at 6 and 24 months after treatment had a lower risk of developing CIN3+ than women with at least one positive test result (p<0.0001; Table 4.3; Figure 4.2). The 5-year and 10-year CIN3+ risks for women who tested negative for co-testing were 0.0% (95%CI 0.0-3.0) and 0.0% (0.0-5.3), respectively. For those with at least one positive test, corresponding risks were 7.5% (95%CI 3.8-14.3) and 10.5% (5.3-19.6).

Smoking around the treatment date was associated with an increased risk of developing CIN3+ (Sun-score statistic 12.3; p=0.03). The respective 5-year and 10-year risks were 17.6% (95%CI 9.7-29.9) and 21.2% (9.9-39.7) in women who smoked more than ten cigarettes per day compared with 1.7% (0.3-9.8) and 4.1% (0.7-21.7) in women who had never smoked.

DISCUSSION

Women treated for a CIN2+ lesion had a 10-year interval-censored long-term CIN2+ recurrence rate of 18%.The 10-year risk of women with three negative cytological test results was reduced to 5% and the risk for women with negative co-testing results was 4%. We consider women who satisfy these conditions to be successfully treated. The 5-year risks of post-treatment disease in these women are such that they do not need to be followed up more closely than women in population-based screening (every 5 years) and could therefore return to this programme (Panel: *Research in context*).

In our study, over half of the post-treatment CIN2+ lesions were detected within 2 years of follow-up. Hereafter the risk declined, in agreement with Melnikow and colleagues, who found that after 6 years of post-treatment follow-up the risk of CIN is equal to women with negative cytology in population-based screening.[5]

In accordance with Strander and colleagues, our study confirmed that one-time testing with hrHPV is not sufficient to identify women at risk for post-treatment CIN2+.[31] Also, after one-time testing with cytology a 5-year CIN2+ risk of 6% remained.

The present post-treatment surveillance strategy, which consists of three cytological smears, is effective at identifying women at risk of post-treatment disease. Women with three consecutive negative smears had a 3% risk of developing post-treatment CIN2+ in the following 5 years. None of the women developed an invasive carcinoma. According to Dutch guidelines, these women are referred to population-based screening.[1] This is supported by a policy suggested by Castle and colleagues, in which women with a 3-year risk of 2% or less may

PANEL: RESEARCH IN CONTEXT

Systematic review

In 2004, our group published a review and meta-analysis that compared high-risk human papillomavirus (hrHPV) testing with either resection margins or cervical cytology to predict post-treatment disease in women treated for cervical intraepithelial neoplasia (CIN) grade 2 or 3 (CIN2/3).[3] We searched PubMed for articles and reviews on hrHPV testing in the follow-up of women treated for CIN2/3 to gather additional information. Four reviews, published between 2004 and 2009, reported a higher sensitivity and negative predictive value for hrHPV testing than for cytology in prediction of high-grade post-treatment disease and confirmed the additional value of hrHPV testing in the follow-up of these women.[6, 33-35] Most of the studies included in these reviews, as well as the identified published studies, had a maximum follow-up of 5 years. This finding suggested that long-term data on the performance of hrHPV testing in this high-risk population were needed.

Interpretation

Results of previous studies have suggested hrHPV testing is valuable in post-treatment surveillance, because of its high negative predictive value in the first 2 years after treatment. We found that the risk of CIN2+ in women who had three consecutive cytological negative smears or negative co-testing results was similar to the 5-year risk of CIN2+ of women with normal cytology in the general population.[36] Therefore these women can be referred to regular population-based screening. All women with other test result combinations than mentioned above should receive additional testing or colposcopic examination, or both, because they have a substantial risk of developing post-treatment disease within the next 5 years.

For the follow-up of women treated for high-grade disease we advocate the incorporation of hrHPV testing (co-testing) at 6 and 24 months after treatment. If hrHPV testing is unavailable, women should be tested cytologically at 6, 12 and 24 months after treatment.

be followed by regular interval screening [32], and contrasts with the annual cytological follow-up done in other countries.[10-11]

When hrHPV-testing was added to post-treatment surveillance, similar effective recognition of women at risk was obtained by testing at fewer time points. The 5-year risk of 3% after three consecutive negative smears is similar to the risk found in women who have negative co-testing results at 6 months, showing that the high negative predictive value of co-testing is mainly because of a negative hrHPV test. In the algorithm of co-testing at 6 and 24 months only 4 of the 221 women who tested negative for all tests developed post-treatment CIN2+, and only two of these women developed a CIN2+ lesion in the first 5 years after testing negative. This risk is similar to the risk of CIN2+ found in women who tested negative for cytology in the Dutch population-based screening programme.[33]

We confirm that women negative for co-testing at 6 months post-treatment can miss the 12-month testing point and return for co-testing after 24 months [3, 18] because of the high sensitivity of co-testing.[3-4, 16-17] This is not a screening setting, but follow-up of a potentially lethal disease, and thus negative predictive value and sensitivity are valued higher than specificity, and priority is given to co-testing instead of sole hrHPV testing. A benefit of fewer follow-up visits might be that women experience less psychosocial distress.

The population-based screening programme in The Netherlands ends at age 60 years, and thus we would like to add a comment regarding women treated for CIN2/3 after the age of 55 years. For women who have a negative algorithm in the first 2 years after treatment, a check after 5 and 10 years should be done because these women would no longer be invited for tests through the national screening programme. This recommendation is further substantiated by the fact that the risk of post-treatment disease in successfully treated women increases by about 2.5% between 5 and 10 years of follow-up.

Most treated women are free of hrHPV infection within 6 months.[4, 6, 13, 15-16] These women have a lower risk of developing post-treatment CIN2+ than women without clearance.[4, 6-7, 15-16] Women infected with HPV16 have a higher recurrence rate than women infected with other hrHPV types [4], although these lesions did not develop sooner. Despite the higher risk of CIN2+ in HPV16-positive women, women infected with hrHPV types other than 16, 18, 31, 33 and 45 still had a 5-year CIN2+ risk of almost 30% and therefore also need treatment. Thus, we do not advocate inclusion of genotyping in the follow-up.

Study limitations

Our study has several limitations. First, the number of events that were diagnosed after 6 years of follow-up was small. To assess the risks in this study as precisely as possible, we estimated these risks with interval censoring and provided 95%CIs. However, these 95%CIs are wide because of the limited number of women in our study. Second, we might have missed some women with post-treatment CIN2+ because of incompleteness of the registry. The Netherlands nationwide registry covers over 99% of all Dutch laboratories and is judged to be particularly accurate for women attending the outpatient clinic; therefore, we expect such incompleteness to be minimal. Third, we combined three studies, which had partly been undertaken in different hospitals, had slightly different follow-up protocols and had been done over different timeframes. By adjusting our results for treatment centre, cohort and year of treatment, we have corrected for these differences. Finally, our data could have limited applicability to other populations. Because all hospitals in The Netherlands follow the same national guidelines, our conclusions can be extended throughout the country. The main conclusion of referring women with negative post-treatment surveillance algorithms to population-based screening is also applicable to other developed countries that have a population-based screening programme, because in most of these countries screening-intervals are equal to (every 5 years) or shorter than (every 3 years) in The Netherlands.

Ideally, our findings would be confirmed by a randomised controlled trial that would compare our suggested strategies. However, this trial would need to include a large group of treated women who were followed for over 10-15 years. Furthermore, such a study would be difficult to do because of continuous development of new screening methods.

Conclusions

In conclusion, women who have three consecutive negative cytological tests can be referred to population-based screening, because their 5-year risk of developing CIN2+ is less than 3%.[32] By adding hrHPV testing to post-treatment surveillance, testing after 12 months can be omitted in women negative for co-testing at six months. Women negative for co-testing at both 6 and 24 months after treatment had a 5-year risk of developing post-treatment CIN2+ of 1.0% and a negligible risk of developing CIN3+. These risks are similar to the risks of women with normal cytology in population-based screening and therefore these women could be referred to regular screening. However, women who do not have negative screening algorithms post-treatment should receive additional testing or colposcopic examination, or both.

Trial register

Dutch trial register, NTR 1468.

ACKNOWLEDGEMENTS

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4.2

HIGH-RISK HUMAN PAPILLOMAVIRUS TESTING VERSUS CYTOLOGY IN PREDICTING POST-TREATMENT DISEASE IN WOMEN TREATED FOR HIGH-GRADE CERVICAL DISEASE: A SYSTEMATIC REVIEW AND META-ANALYSIS

Gynecologic Oncology, 2012 Jan 18 [Epub ahead of print]

SUMMARY

Introduction

Currently, women treated for high-grade cervical intraepithelial neoplasia (CIN 2/3) are followed-up by cytology to monitor them for residual and recurrent (post-treatment) disease. This systematic review and meta-analysis determine the test performance of testing for high-risk types of the human papillomavirus (hrHPV), cytology and co-testing (combined hrHPV testing and cytology) in predicting high-grade post-treatment disease (CIN2+).

Methods

Studies that compared at least two of three post-treatment surveillance methods, and were published between January 2003 and May 2011, were identified through a bibliographic database search (PubMed, Embase.com and Wiley/Cochrane Library). Identification of relevant studies was conducted independently by two reviewers with a multi-step process. The reference standard used to diagnose post-treatment disease was histologically confirmed CIN2+.

Sensitivity, specificity, diagnostic odds ratios and relative sensitivity and specificity were calculated for each study. Pooled estimates were calculated using a random effects model if heterogeneity among studies was significant, otherwise by using a fixed effects model. Estimates were reported with 95% confidence intervals (95%CIs).

Results

Out of 2410 potentially relevant citations, 8 publications, incorporating 1513 treated women, were included. Pooled sensitivities were 0.79 (95%CI 0.72-0.85) for cytology, 0.92 (0.87-0.96) for hrHPV testing, and 0.95 (0.91-0.98) for co-testing. HrHPV testing was more sensitive than cytology to predict post-treatment CIN2+ (relative sensitivity 1.15; 95%CI 1.06-1.25). Pooled specificities were 0.81 (95%CI 0.74-0.86) for cytology, 0.76 (0.67-0.84) for hrHPV testing and 0.67 (0.60-0.74) for co-testing. HrHPV testing and cytology had a similar specificity (relative specificity 0.95, 95%CI 0.88-1.02).

Discussion

This review indicates that the hrHPV test should be included in post-treatment testing 6 months after treatment, because hrHPV testing has a higher sensitivity than cytology in detecting high-grade post-treatment disease and has a similar specificity.

INTRODUCTION

Women with high-grade cervical precursor lesions (Cervical Intraepithelial Neoplasia grade 2 (CIN2) and grade 3 (CIN3)) are treated by local excision or ablation to prevent progression to cervical cancer.[1-2] Despite treatment, approximately 10.2% (95%CI 6.7-13.8)[3] of these women are diagnosed with residual or recurrent (post-treatment) high-grade disease.[3-9] Because of this substantial risk, many countries use surveillance strategies to identify post-treatment disease. These strategies fluctuate greatly between countries in both content, including follow-up modalities like cervical cytology, testing for high-risk types of the human papillomavirus (hrHPV) and colposcopy, either separately or in combination, and length of post-treatment surveillance.[10-11]

Current Dutch national guidelines recommend cervical cytological testing at 6, 12 and 24 months after treatment, and, if necessary, annually for five years until three consecutive smears are read as normal.[2] After three consecutive negative smears, women return to five-yearly population-based routine screening. In contrast, treated women in the United Kingdom have a cytological examination at six and 12 months after treatment and, irrespective of the results, annual cytology for the subsequent nine years before reconsidering a return to routine screening.[12] In the United States annual cytology is even recommended for at least 20 years.[13] In summary, treated women are followed more closely between two and 20 years before returning to population-based screening.

Besides cytology, several other risk factors, including cone margin status, positive endocervical curettage and age, have been studied to predict recurrent cervical disease. However, these predictors are suboptimal.[11] For excision margins, for instance, previous studies, summarized in a review of Zielinski and colleagues, found that the sensitivity varied between 39 and 100%.[9] Besides, this characteristic has shown to be less sensitive than cytology or hrHPV testing in predicting post-treatment disease.[9, 14]

More and more evidence is gathered concerning the use of hrHPV-testing during the follow-up period, because the presence of hrHPV is not only a prerequisite for the development of primary high-grade CIN [15], but also for the development of post-treatment CIN.[3, 6, 9, 11, 16-20] It is assumed that effective treatment not only removes the pre-malignant lesion, but also eliminates the responsible hrHPV infection.[17, 21] In women who develop post-treatment CIN the hrHPV infection stays present and is therefore associated with disease recurrence.[11, 17, 21] The sensitivity of hrHPV-testing to detect post-treatment CIN outweighs that of cytology (approximately 90% versus 75%) [3, 9, 11, 16, 20], at the cost of a lower [9, 16] or similar [3, 19-20] specificity. Because this is not a screening setting, but post-treatment surveillance of a potentially lethal disease, sensitivity is valued higher than specificity. Strategies which include hrHPV testing are suggested as an alternative for conventional post-treatment surveillance.[4, 9-11, 14, 22] The majority of all treated women clear their hrHPV infection within 6 months [23] and have a significantly lower risk of developing post-treatment CIN3+ than women without hrHPV clearance.[4, 6, 11, 14, 22] In the surveillance of treated women combined testing with cytology and a hrHPV test (co-testing) results in a negative predictive value of over 99% to detect those at risk of developing post-treatment disease.[6, 9, 14]

Previously published systematic reviews have examined the value of hrHPV testing in the context of post-treatment surveillance of CIN. All found a higher sensitivity for sole hrHPV testing or co-testing, compared to sole cytological testing.[3, 9, 11, 16, 19-20] The review most recently published included studies up to 2007.[16]

We conducted this systematic review and meta-analysis to summarize and update current knowledge of the value of cytology, hrHPV testing and co-testing used in post-treatment surveillance. Besides describing the individual studies, we also determined the pooled sensitivity and specificity, diagnostic odds ratio and relative sensitivity and specificity.

MATERIALS AND METHODS

Search strategy

We searched the databases of PubMed, Embase.com, Wiley/Cochrane Library and WHO International Clinical Trials Registry for relevant studies published between January 2003 and April 2011. For this computer-aided search we used the following terms (including synonyms and closely related words) as index terms and free-text words: "vaginal smear" or "human papillomavirus" and "conisation" or "loop excision" or "CIN lesions" and "randomized controlled trials" or "systematic reviews". For the last two concepts we used predefined filters. These searches were not limited by language of publication. The example strategy for PubMed is presented in Supplementary Table 4.1 (S1).

Previous systematic reviews or meta-analyses on the same subject as well as references of retrieved articles were used to search for additional relevant studies that could have been missed by the electronic search. Identification of relevant studies was conducted independently by two reviewers (MK and MU) with a multistep process (Figure 4.3). First, titles of the full list of citations were reviewed, followed by an assessment of abstracts of citations with potentially relevant titles. Disagreements were resolved by consensus. Finally, full-text articles of selected abstracts were considered for introduction in the review. We developed a data extraction sheet based on Cochrane guidelines [24-25] to collect all relevant data from the studies, which was used by two reviewers (MK and MU). Disagreements were resolved after discussion. The following data were extracted: author, year and language of publication, country of study, population characteristics, study design, true positive (TP), false positive (FP), true negative (TN) and false negative (FN) values. The authors of papers which did not state the values to construct a 2x2 table were contacted.

Two independent reviewers (MK and MU) graded the methodological quality of the selected studies with a modified version of the QUality Assessment of Diagnostic Accuracy Studies (QUADAS) tool.[26] This modified version consists of 11 items on methodological characteristics that have the potential to introduce bias and are described in Supplementary Table 4.2 (S2). Items were scored positive (criteria satisfied), negative (criteria not satisfied), or unclear. We kept out two items (index test results blinded and relevant clinical information available) because hrHPV testing is performed by an objective test and is independent of clinical information. Furthermore we added the item "selection bias". Disagreements between the two extracting authors were resolved by consensus. Assessment of quality results was categorized.

Inclusion and exclusion criteria

To be included, both prospective and retrospective studies had to meet several inclusion criteria: (1) women should have been treated for CIN2/3 by either conisation (laser or cold-knife) or LLETZ (Large Loop Excision of the Transformation Zone) procedure; (2) post-treatment surveillance should include at least two out of the following three methods; hrHPV testing, cytology, and/or co-testing (combined testing of cytology and hrHPV) at six months after treatment; (3) the positive endpoint, residual or recurrent high-grade post-treatment disease should be defined as a histological diagnosis of CIN2, CIN 3, adenocarcinoma *in situ* (AIS), adenocarcinoma, or squamous cell carcinoma (CIN2+). A negative endpoint should be defined as either a histological confirmation of no, or low-grade, disease (CIN0/1), or a repetitive negative cytological test result.

Both studies in which hrHPV testing was performed by Hybrid Capture II (HCII), as studies in which this was performed by the polymerase chain reaction (PCR) method were included. Both the sensitivities and negative predictive values of these tests to detect post-treatment CIN3+ seem similar, being 100% and 99% respectively [27] and show a good agreement.[28] In addition, we checked their similarity empirically by performing a bivariate regression analysis with type of hrHPV test as dichotomous covariate.

For a study to be considered for pooling, we required colposcopic evaluation of all positive test results in all women. Positive test results were defined as abnormal cytological test results, characterized as borderline dysplasia or worse, equivalent to atypical cells of unknown significance (ASCUS) or worse, and as hrHPV tests, positive for any hrHPV type.

Studies were excluded from this review, when they concerned the follow-up of women treated for adenocarcinoma *in situ* (AIS) or low-grade cervical disease (CIN1). Other exclusion criteria were studies concerning the follow-up of pregnant women, HIV-infected women, women exposed to diethylstilbestrol (DES) *in utero,* and studies concerning prophylactic HPV vaccination. Also studies with a follow-up of less than 12 months were discarded.

Statistical analysis

Outcome measures were the pooled estimates of sensitivity and specificity, the pooled estimate of the diagnostic odds ratio (DOR), and the pooled estimates of relative sensitivity and relative specificity. The DOR was defined as the odds of a positive test result in subjects with disease divided by the odds of a positive test result in subjects without disease.[29] The relative sensitivity was computed as the ratio of the sensitivity of the hrHPV test tot the cytology test, and the relative specificity was computed analogously.

In order to select the appropriate pooling method, the heterogeneity among the studies of each outcome measure was tested with Cochran's *Q* and quantified by I^2. [30-31] If Cochran's *Q* was significant, the effects were pooled using a random effects model (REM) [32], otherwise a fixed effects model (FEM) was used.

The sensitivity and specificity were pooled after applying the Freeman-Tukey double arcsine transformation [33-34] and presented with a Clopper-Pearson exact 95% confidence interval (95%CI).[35] The 95%CI of the DOR was based on the standard error of the logarithm of the DOR. In all studies, cytology and hrHPV tests had been performed in all women. Therefore the standard error of the relative sensitivity and relative specificity was computed as the standard

error of a relative risk of binary matched-pairs data.[36] A continuity correction of 0.5 was applied if the discordant cell frequencies equalled zero.

The DOR as a measure of the discriminatory power of a test assumes that the thresholds between the different outcome categories remain constant over the included studies.[37] To check this assumption, the Pearson correlation between the logit true positive rate (TPR) and the logit false positive rate (FPR) was calculated for cytology, hrHPV testing and co-testing.[38] As a second check of heterogeneity across studies, Moses' regression model was fitted.[39] Finally, to check whether the type of hrHPV test used, or quality of the study differentially affected the sensitivity or specificity, a bivariate regression analysis of sensitivity and specificity was performed using maximum likelihood estimation with type of hrHPV test or study quality as covariates.[40]

The sensitivity and specificity were pooled using R (version 2.13.0).[41] The DOR was pooled, and the Pearson correlation between the logit TPR and logit FPR and Moses' regression model was fitted using Meta-DiSc (version 1.4).[42] The relative sensitivity and relative specificity were pooled in Review Manager (version 5.1.2)[43], by importing the standard errors from a spreadsheet analysis in Microsoft Excel (2003). The bivariate regression analysis was performed in SAS (version 9.2). An effect with p-value <0.05 was considered statistically significant.

RESULTS

The combined literature search identified 2410 citations. Of these, 2345 were excluded on title and abstracts, leaving 65 citations for full text review. Finally 9 citations met all criteria and were included in this review (Figure 4.3).[4, 14, 28, 44-49] However, one study was later excluded, as the author did not respond to repetitive questioning about study design and results.[28]

The eight studies remaining were heterogeneous in study characteristics, like study design, choice of hrHPV testing methods and the assessment of disease status at entry and end of follow-up (Table 4.4). Concerning design, seven studies were prospective, and one study was a case-control analysis. HrHPV testing was performed using HC2 testing in four studies, and PCR testing in three studies. In one study both techniques were performed. All studies collected samples for cytology and hrHPV-testing six months after treatment. One study had a significant longer follow-up than all other included studies.[46] Therefore, data for this study was recalculated for a follow-up of 2 years.

In all studies combined, 1513 women had been treated for CIN2/3. The number of women per study varied between 63 and 485. The age of the participants ranged between 19 and 83 years. Treatment failure, expressed in terms of residual and recurrent CIN2+, ranged from 4.0% to 11.9%. Recurrence rates of CIN2+, sensitivities as well as DORs of the individual studies and pooled values at six months post treatment are shown in Figure 4.4.

The correlation between the logit TPR and logit FPR was not significant for all diagnostic tests (cytology r=0.024, p=0.96; hrHPV r = 0.49, p = 0.18; and co-testing: r = 0.42, p = 0.26). Moses' linear regression model did not significantly improve the constant model (cytology: p = 0.21, hrHPV: p = 0.80, and co-testing: p = 0.67), giving further support for the assumption of a constant DOR over studies.

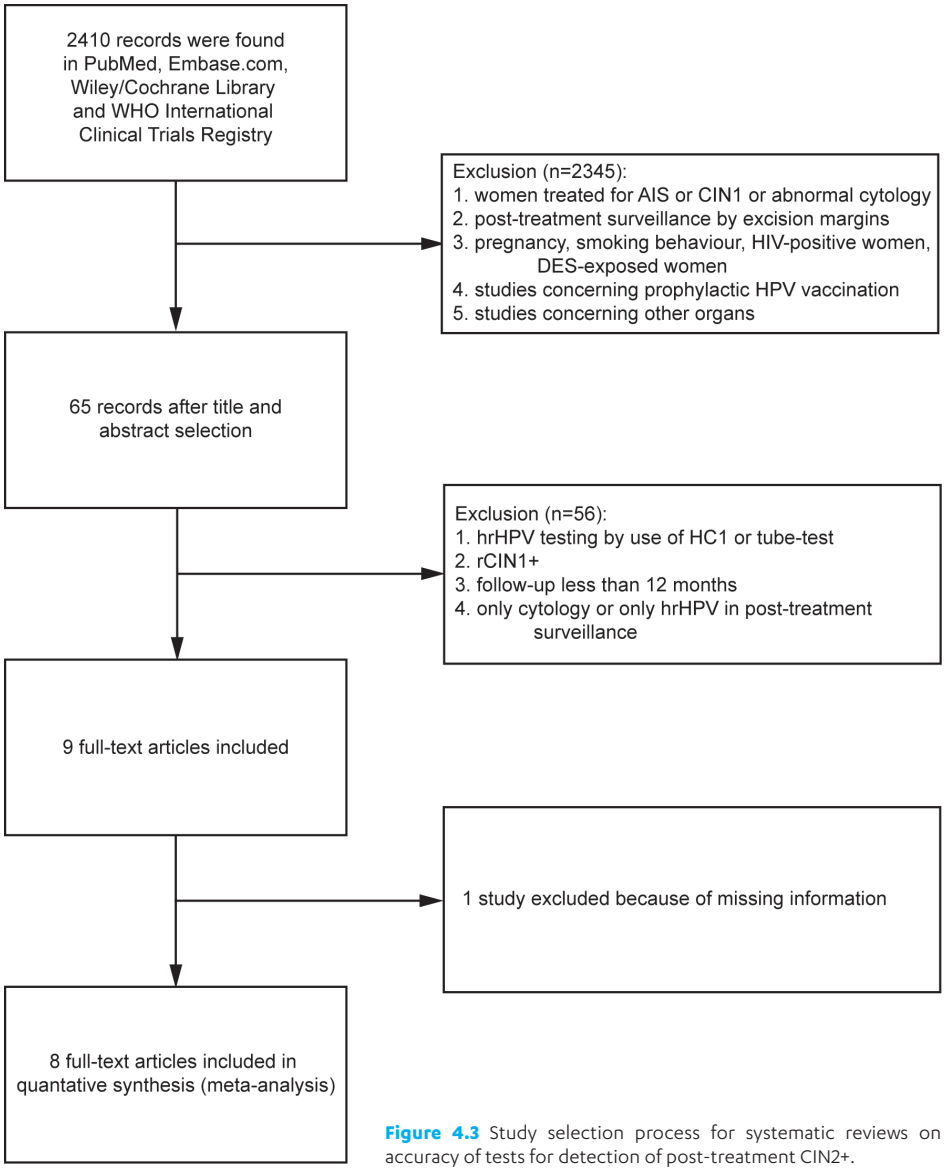

Figure 4.3 Study selection process for systematic reviews on accuracy of tests for detection of post-treatment CIN2+.

Diagnostic accuracy

The sensitivity of cytological testing 6 months after treatment in predicting post treatment CIN lesions varied between the studies between 0.67 and 1.0. For sensitivity, the test for heterogeneity between studies was not significant (Q(7)=5.08, p=0.65; I^2=0.0%). The pooled sensitivity was 0.79 (95%CI 0.72-0.85). The specificity of cytology ranged from 0.64 to 0.91 and heterogeneity between studies was significant (Q(7)=48.76, p<0.0001; I^2=85.6%). The pooled

Table 4.4 Study characteristics of the individual studies that investigated the performance of hrHPV and cytology (6 months after treatment) in predicting residual and recurrent high-grade disease.

Study	Year	Country	Participant final/initial	Recurrence[a] CIN2+ (%)	Follow-up in months (range)	hrHPV test
Cecchini[44]	2004	Italy	84/84	10 (11.9)	23 (11-40)	PCR
Sarian[47]	2004	Brazil	88/107	11 (10.2)	17	HC2
Alonso[14]	2006	Spain	203/224	24 (11.8)	20 (6-66)	HC2
Kreimer[4]	2006	USA	485/610	32 (6.6)	24	HC2 PCR
Verguts[49]	2006	Belgium	72/72	6 (8.0)	24	HC2
Smart[48]	2010	New-Zealand	100/100	4 (4.0)	18	HC2
Heymans[45]	2011	Belgium	63/63	n.a.	>24	PCR
Kocken[46]	2011	Netherlands	435/445	45[b] (10.8)	24[b]	PCR

CIN, cervical intraepithelial neoplasia; hrHPV, high-risk type of the human papillomavirus; LLETZ, large-loop excision of the transformation zone; PCR, polymerase chain reaction; HC2, Hybrid-Capture 2 test; n.a, not applicable

[a] includes all residual and recurrent disease.

[b] for analyses data was limited to 2 years of follow-up.

specificity was 0.81 (95%CI 0.74-0.86). The pooled DOR of cytology was 13.81 (95%CI 9.17-20.80) and there was no evidence for statistically significant heterogeneity (Q(7)=7.16, p=0.41; I^2=2.2%).

In the studies the sensitivity of hrHPV-testing varied between 0.87 and 1.0. For sensitivity, the test for heterogeneity between studies was not significant (Q(7)=6.04, p=0.53; I^2=0.0%). The pooled sensitivity was 0.92 (95%CI 0.87-0.96). The specificity of hrHPV ranged from 0.57 to 0.88 and heterogeneity between studies was significant (Q(7)=91.38, p<0.0001; I^2=92.3%).

Study design
Prospective cohort study including 84 women (mean age 34.3) treated for CIN2/3 by LLETZ between February 1999 and June 2001. Follow-up at six months after treatment included hrHPV-testing, cytology and colposcopy. Method of PCR- testing: type-specific HPVE6/E7 PCR. Method of cytology: not specified
Prospective cohort study including 88 women (mean age 34 years, range 20-60) treated for CIN2/3 by LLETZ between March 2001 and December 2002. Follow-up at six months after treatment for hrHPV-testing, cytology and colposcopy. Biopsies were taken if cytology revealed HSIL, or if a suspect area was present. Method of cytology: glass slide, specimen taken with Ayre spatula and endocervix brush.
Prospective cohort study including 203 women (mean age 38.6 years, range 22-83) treated for CIN2/3 by LLETZ between May 1998 and October 2004. Follow-up at 6 months after treatment included hrHPV-testing, cytology and colposcopy. Biopsies were taken if abnormal cytology (≥ASC-US) or hrHPV–positivity was present. Women with two consecutive negative cytological smears and negative colposcopy were considered negative for recurrence, irrespective of the hrHPV test result. Method of cytology: glass slide, specimen taken with Ayre spatula and cytobrush.
Prospective cohort study including 485 women (median age 24 years, range 21-28) treated for CIN2/3 by LLETZ between January 1997 and December 1998. Follow-up at 6 months after treatment included hrHPV-testing, including genotyping, and cytology. For analyses, data from HC2 testing was used and women with missing hrHPV test results were excluded. Method of PCR- testing: PGMY09/11 PCR. Method of cytology: liquid-based cytology.
Prospective cohort study including 72 women (mean age 40 years, range 22-78) treated for CIN2/3 by LLETZ between February 2000 and February 2003. Follow-up at three to six months after treatment included hrHPV, cytology and colposcopy. Biopsies were taken if any suspected area was present. Method of cytology: liquid-based cytology, taken with Cervex-brush.
Prospective cohort study including 100 women (mean age 32 years, range 19-66), treated for CIN 2/3 by LLETZ or conization between January 2007 and January 2008. Follow-up (mean 9 months; range 3-18) included hrHPV-testing, cytology and colposcopy. Biopsies were taken if any suspect area was seen. One woman with inadequate cytology was excluded from analyses. Method of cytology: liquid-based cytology, taken with Cervex-brush.
Case control study (1:2) including 63 women (median age cases 40.9 years and controls 35.5 years) treated for CIN 2/3 by LLETZ or conization between January 2001 and December 2007. Follow-up at six months after treatment included hrHPV-testing, including genotyping, and cytology. Method of PCR- testing: type-specific HPVE6/E7 PCR. Method of cytology: liquid-based cytology, taken with Cervex-brush.
Prospective cohort study including 435 women (mean age 34.9 years, range 21-70) treated for CIN2/3 by LLETZ or conization between July 1988 and November 2004. Follow-up at six months after treatment included hrHPV-testing, including genotyping, and cytology. Colposcopy was performed if abnormal cytology (≥ASC-US) or hrHPV–positivity was present. For analyses, data was limited to 2 years of follow-up. Method of PCR- testing: GP5+/6+ PCR. Method of cytology: partly glass slides, others not specified.

The pooled specificity was 0.76 (95%CI 0.67-0.84). The pooled DOR of hrHPV testing was 34.68 (95%CI 18.87-63.73) and there was no evidence for statistically significant heterogeneity (Q(7)=3.39, p=0.85; I^2=0.0%).

The sensitivity of co-testing varied between 0.90 and 1.0. For sensitivity, the test for heterogeneity between studies was not significant (Q(7)=5.41, p=0.61; I^2=0.0%). The pooled sensitivity was 0.95 (95%CI 0.91-0.98). The specificity varied between 0.36 and 0.78 and heterogeneity between studies was significant (Q(7)=47.26, p<0.0001; I^2=85.2%). The pooled specificity was 0.67 (95%CI 0.60-0.74). The pooled DOR of co-testing was 35.86 (95%CI 17.59–73.11) and there was no evidence for statistically significant heterogeneity (Q(7)=2.36, p=0.94; I^2=0.0%).

Cytology

Study	Year	TP	FP	FN	TN	Total	Sensitivity	Specificity
Cecchini	2004	8	12	2	62	84	0.80 [0.44 - 0.97]	0.84 [0.73 - 0.91]
Sarian	2004	7	15	0	66	88	1.00 [0.59 - 1.00]	0.81 [0.71 - 0.89]
Alonso	2006	19	24	5	155	203	0.79 [0.58 - 0.93]	0.87 [0.81 - 0.91]
Kreimer	2006	25	140	7	313	485	0.78 [0.60 - 0.91]	0.69 [0.65 - 0.73]
Verguts	2006	4	6	2	60	72	0.67 [0.22 - 0.96]	0.91 [0.81 - 0.97]
Smart	2010	4	15	0	80	99	1.00 [0.40 - 1.00]	0.84 [0.75 - 0.91]
Heymans	2011	16	15	5	27	63	0.76 [0.53 - 0.92]	0.64 [0.48 - 0.78]
Kocken	2011	35	63	10	310	418	0.78 [0.63 - 0.89]	0.83 [0.79 - 0.87]
Summary		**118**	**290**	**31**	**1073**	**1512**	**0.79 [0.72 - 0.85]**	**0.81 [0.74 - 0.86]**

(FEM) pooled sensitivity 0.79 [0.72 - 0.85] Q(7) = 5.08, p=0.65; I² = 0.0%
(REM) pooled specificity 0.81 [0.74 - 0.86] Q(7) = 48.76, p<0.0001; I² = 85.6%
(FEM) pooled DOR 13.81 [9.17 - 20.80] Q(7) = 7.16, p=0.41; I² = 2.2%

hrHPV

Study	Year	TP	FP	FN	TN	Total	Sensitivity	Specificity
Cecchini	2004	9	27	1	47	84	0.90 [0.55 - 1.00]	0.64 [0.52 - 0.74]
Sarian	2004	7	11	0	70	88	1.00 [0.59 - 1.00]	0.86 [0.77 - 0.93]
Alonso	2006	23	43	1	136	203	0.96 [0.79 - 1.00]	0.76 [0.69 - 0.82]
Kreimer	2006	29	164	3	289	485	0.91 [0.75 - 0.98]	0.64 [0.59 - 0.68]
Verguts	2006	6	15	0	51	72	1.00 [0.54 - 1.00]	0.77 [0.65 - 0.87]
Smart	2010	4	11	0	84	99	1.00 [0.40 - 1.00]	0.88 [0.80 - 0.94]
Heymans	2011	21	18	0	24	63	1.00 [0.84 - 1.00]	0.57 [0.41 - 0.72]
Kocken	2011	39	48	6	325	418	0.87 [0.73 - 0.95]	0.87 [0.83 - 0.90]
Summary		**138**	**337**	**11**	**1026**	**1512**	**0.92 [0.87 - 0.96]**	**0.76 [0.67 - 0.84]**

(FEM) pooled sensitivity 0.92 [0.87 - 0.96] Q(7) = 6.04, p=0.53; I² = 0.0%
(REM) pooled specificity 0.76 [0.67 - 0.84] Q(7) = 91.38, p<0.0001; I² = 92.3%
(FEM) pooled DOR 34.68 [18.87 - 63.73] Q(7) = 3.39, p=0.85; I² = 0.0%

Co-testing

Study	Year	TP	FP	FN	TN	Total	Sensitivity	Specificity
Cecchini	2004	9	34	1	40	84	0.90 [0.55 - 1.00]	0.54 [0.42 - 0.66]
Sarian	2004	7	19	0	62	88	1.00 [0.59 - 1.00]	0.77 [0.66 - 0.85]
Alonso	2006	24	51	0	128	203	1.00 [0.86 - 1.00]	0.72 [0.64 - 0.78]
Kreimer	2006	31	168	1	285	485	0.97 [0.84 - 1.00]	0.63 [0.58 - 0.67]
Verguts	2006	6	17	0	49	72	1.00 [0.54 - 1.00]	0.74 [0.62 - 0.84]
Smart	2010	4	21	0	74	99	1.00 [0.40 - 1.00]	0.78 [0.68 - 0.86]
Heymans	2011	21	27	0	15	63	1.00 [0.84 - 1.00]	0.36 [0.22 - 0.52]
Kocken	2011	41	94	4	279	418	0.91 [0.79 - 0.98]	0.75 [0.70 - 0.79]
Summary		**143**	**431**	**6**	**932**	**1512**	**0.95 [0.91 - 0.98]**	**0.67 [0.60 - 0.74]**

(FEM) pooled sensitivity 0.95 [0.91 - 0.98] Q(7) = 5.41, p=0.61; I² = 0.0%
(REM) pooled specificity 0.67 [0.60 - 0.74] Q(7) = 47.26, p<0.0001; I² = 85.2%
(FEM) pooled DOR 35.86 [17.59 - 73.11] Q(7) = 2.36, p=0.94; I² = 0.0%

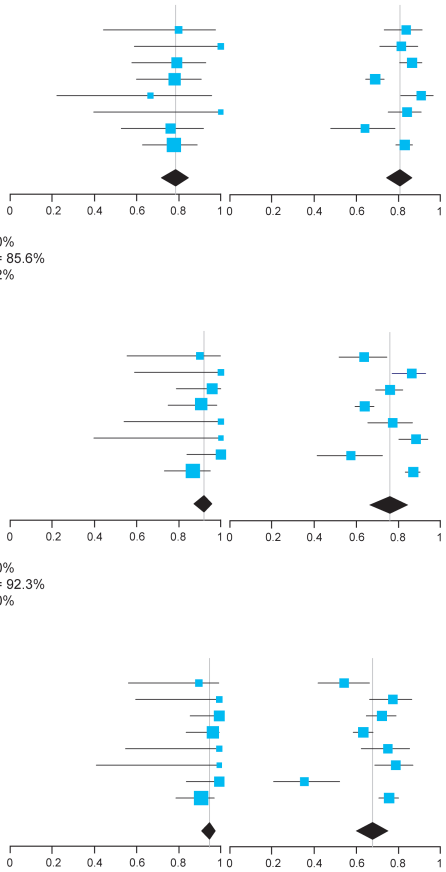

Figure 4.4 Meta-analysis of the sensitivity and specificity, including pooled estimates, of testing 6 months after treatment with cytology, hrHPV or co-testing.
TP, true positives; FP, false positives; FN, false negatives; TN, true negatives; FEM, fixed effects model; REM, random effects model; DOR, diagnostic odds ratio. Forest plots of sensitivity (left) and specificity (right).

A bivariate regression model was fitted to test the influence of the two types of hrHPV tests, HC2 and PCR on sensitivity and specificity. Adding of the hrHPV test type as a covariate explained 11.5% of the between-study variance for cytology, which was not significant (t(7) = 1.25, p = 0.25). Also for hrHPV and co-testing the addition of the hrHPV test type as a covariate explained a not significant part of the between-study variance (hrHPV 7.7%, (t(7) = 0.81, p = 0.45) and co-testing 31.9%, (t(7) = 2.15, p = 0.07)).

Overall, hrHPV-testing after six months predicted post-treatment CIN with significantly higher sensitivity (relative sensitivity 1.15; 95%CI 1.06-1.25 (Z=3.27, p=0.001)) than cytology and a similar specificity (relative specificity 0.95; 0.88-1.02 (Z=1.53, p=0.13)).

Methodological quality of included studies

Table 4.5 summarizes the methodological quality of the 8 included studies. All studies included a representative patient spectrum of women treated for high-grade cervical disease. One case-control study was included and selection bias could not be excluded.[45] We added this criterion and although most studies imply that women were continuously included, this was only explicitly mentioned in one study.[44] The reference standard was adequate in all studies. All studies defined a positive test result of post-treatment disease as a histological finding of CIN2+. A negative test result was verified by colposcopy in five studies. In the three studies remaining a negative test result was verified by consecutive negative cytological smears.[14, 45-46] One of these studies[46] was a multi-cohort study and in two of the three incorporated studies test results were confirmed with a colposcopic examination in all women, and in the third study absence of disease was confirmed by three consecutive negative cytological smears[17]. In every study all patients were assessed within two years. Complete verification with the reference standard was performed in four studies.[4, 14, 47, 49] In three other studies not all patients were examined by colposcopy, but were considered free of disease by cytological examination.[14, 45-46] One study performed a colposcopy in all patients at 6 months after treatment, but did not specify when colposcopies were performed later in follow-up.[48] In none of the studies hrHPV was part of the reference standard. Moreover, in most studies the reference standards were interpreted without knowing the results of the hrHPV-test. Only in two studies women were referred on basis of the hrHPV test result.[14, 46] For the case-control study this item could not be assessed.[45] Of interest might be that only one study explicitly mentions biopsy taking in all

Table 4.5 Summary of methodological quality. Review authors' judgments about each methodological quality item for each included study.

	Kocken 2011 [46]	Heymans 2011 [45]	Smart 2010 [48]	Alonso 2006 [14]	Kreimer 2006 [4]	Verguts 2006 [49]	Sarian 2004 [47]	Cecchini 2004 [44]
Representative spectrum?	+	+	+	+	+	+	+	+
Selection bias	?	-	?	?	?	?	?	+
Acceptable reference standard?	+	+	+	+	+	+	+	+
Appropriate timing of tests and verification of outcome?	+	+	+	+	+	+	+	+
Partial verification avoided?	+	+	+	+	+	+	+	+
Differential verification avoided?	-	-	?	-	+	+	+	+
Incorporation avoided?	+	+	+	+	+	+	+	+
Reference test results blinded?	-	?	+	+	+	+	+	+
Uninterpretable results reported?	+	+	+	+	+	+	+	+
Withdrawals explained?	+	+	+	+	+	+	+	+

Minus sign = negative score, plus sign = positive score, question mark = unclear whether item scores negatively or positively.

women irrespective of any test result[4], in all other studies biopsy taking is dependant of either visual impression[14, 47-49] or abnormal cytological test results.[14, 44, 46-47] If present, uninterpretable results were mentioned in all studies. Although in one study the percentage "missing" was higher in the group without post-treatment disease than in the group diagnosed with post-treatment CIN2+ (13.7% versus 5.9%), this difference was not significant (p=0.295, Fisher's exact).[4] Half of the studies had no withdrawals to explain and the remaining studies dealt with this item appropriately.

The included studies differed in only 3 items of the QUADAS list (Table 4.5); selection bias, differential verification and blinding of reference test results. These three items were each separately added as a dichotomous covariate to the bivariate regression models that were also used to test the effect of the hrHPV test type. A rating of '+' was counted as present, a rating of '?' or '–' as absent. Separate addition of each item as a covariate did not improve the bivariate regression models of cytology, hrHPV-testing or co-testing significantly (data not shown).

DISCUSSION

In this systematic review we described the value of 6-month testing for cytology and/or hrHPV in the surveillance of women treated for CIN2/3 and confirmed the advantage of implementing hrHPV in the follow-up of women treated for high-grade CIN as found in previously conducted meta-analyses.[3, 9, 11, 16, 19-20] HrHPV testing has a significantly higher sensitivity than cytology, indicated by a relative sensitivity of 1.15 (95%CI 1.06-1.25), without decreasing the specificity (relative specificity 0.95, 0.88-1.02).

We measured the DOR to compare the three different tests (hrHPV, cytology and co-testing). The DOR of co-testing testing was higher than the DOR of hrHPV-testing or cytology. This indicates that the overall discriminative power of co-testing is the best. As approximately 10%[3] of women treated for CIN2/3 develop high grade post-treatment disease, it seems logical to choose a test that assures a minimal risk of high-grade disease in this group and to select the test with the highest sensitivity. The pooled sensitivity of co-testing was with 0.95 (95%CI 0.91-0.98) also higher than the sensitivity of sole cytological or hrHPV testing. However, the pooled specificity was only 0.67, resulting in approximately 10% more women referred for repeat testing or colposcopy, or both.

Sources of bias and potential sources of heterogeneity

The purposes of a quality assessment are to identify potential sources of bias and to estimate their impact. The overall methodological quality of the included studies was generally good (Table 4.5), however study characteristics between studies varied. For instance, although the average age in most studies was approximately 35 years, one study included women with a median age of 24 years (range 21-28).[4] As both the prevalence of CIN and of hrHPV varies with age, this factor may influence the test accuracy across the studies. Another difference was that percentages of recurrence were given for the total follow-up period. Most studies measured follow-up until 24 months after treatment and by limiting the follow-up of Kocken *et al*. to two years[46], studies became more homogeneous. Another point to address is that we considered the first hrHPV testing moment to be at 6 months after treatment. Yet, some studies performed

hrHPV tests before [4, 47, 49] or after [14, 48] 6 months. Because hrHPV infections clear over time [17, 21], the risk of developing post-treatment disease will also diminish over time and this could possibly affect the sensitivity of the test.

Other possible reasons for heterogeneity between the studies may be explained by different collection methods of material (e.g. liquid based cytology versus conventional glass slides) and different execution of the analyses (e.g. either single pathologist or review of all cytology and/or histology).

Based on our methodological appraisal the most likely sources of bias are selection bias and differential verification bias. Selection bias arises when women are not included in a consecutive order. Only one of the cohort studies[44] mentions the inclusion of patients to be explicitly consecutive, the other studies only describe to include women within a certain timeframe. However, the populations included in these studies seem to be consecutive and are most likely comparable to one another. But, as for instance, this would have resulted in an exclusion of more difficult cases, it could have resulted in a lower number of false positives and false negatives and hence to increased estimates of sensitivity and specificity.

Differential verification bias arises if two different reference tests are used and the tests have different accuracy. Some of the studies included in our analysis avoided this problem because they performed colposcopic examinations in all women, irrespective of their test results.[4, 44, 47, 49] However, only one study has, besides performing a colposcopy, also taken a biopsy in all subjects to verify the presence, or absence, of cervical disease. Other studies only referred for exit-colposcopy when abnormal test results were found.[14, 46] Some studies did not specify when women were referred for colposcopy.[48] These last studies are therefore prone to (detection) bias. Differential verification bias could have resulted in an overestimation of both sensitivity and specificity. However, women with repetitively negative cytological test results have a low risk of harbouring high-grade disease[46] and therefore we expect this type of bias to be of limited influence.

The included studies remained statistically heterogeneous concerning specificity. Consequently we used a random effect model to calculate the pooled estimates that were heterogeneous. Until better-conducted studies, such as large randomized controlled trials with histological verification of all subjects, independent of hrHPV or cytology results, are available, the pooled estimate provides clinically relevant information.

Study limitations

Our study provides evidence suggesting that hrHPV-testing is more accurate for the diagnosis of post-treatment cervical disease than cytology. Although this is in line with previous reviews, these results are based on a small number of studies.

Also our review is limited to a follow-up of two years as almost all included studies had a follow-up restricted to this period and thereby hampering long-term information of the different test performances. This information is relevant, as the risk of developing recurrent disease is significantly increased for over 10 years after treatment.[5, 7, 18] Only two studies were identified describing hrHPV testing in long-term follow-up.[46, 50] One of these studies excluded residual/recurrent disease developed within two years of treatment and only described the performance of the hrHPV test. Therefore no comparison could be made with the performance

of cytology and this study was excluded for analysis in this review.[50] One study that described the long-term prediction of hrHPV and/or cytology at 6 months after treatment remained.[46] In this study the sensitivities of hrHPV and cytology in detecting post-treatment disease decreased when the total follow-up time increased. For cytology, the sensitivity in the total study with a follow-up up to 21.5 years was 66% (95%CI 55-76) compared to a sensitivity of 78% in the first two years (Figure 4.4). For hrHPV testing, these values were 72% (95%CI 61-81) and 87%, respectively. This could illustrate the acquisition of new hrHPV types or re-infection/ reactivation of the same hrHPV types that will eventually result in high-grade lesions. Another possibility might be the presence of false negative HPV tests, due to integration of the viral DNA targeted in the HPV test in the genome of the host cell.

Another limitation is that only 6-month testing is measured. Several studies indicated that one test moment is insufficient to predict post-treatment disease and that therefore repeat testing should be performed.[46, 50] However, although some authors have described follow-up algorithms for post-treatment surveillance, pooling of these data was not possible.

A final limitation is that although a large number of citations were reviewed, this review might be subject to publication bias. However, the impact that publication bias has on diagnostic accuracy systematic reviews is unknown.[51]

Conclusions

This review clearly indicates that post-treatment testing at 6 months after treatment should include hrHPV testing. HrHPV testing after 6 months has a higher sensitivity than and a similar specificity as cytology. The sensitivity of co-testing is even higher than of the separate individual tests. As women treated for CIN2/3 have a high risk of developing recurrent disease, sensitivity is valued higher than specificity and therefore hrHPV testing (or co-testing) should be incorporated in post-treatment surveillance. As even the sensitivity of co-testing is not sufficiently high to rely on a single test moment, repeat testing is necessary to identify all women at risk for post-treatment disease.[46] Several studies already indicated that women testing negative for co-testing after 6 months could omit the 12-month test moment and return for monitoring at 24 months after treatment. However, more information, especially on long-term recurrence and cost-effectiveness is needed to recommend a definite follow-up algorithm.

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SUPPLEMENTARY DATA

Supplementary Table 4.1 Search strategy using PubMed (April 6th, 2011)

("Vaginal Smears"[Mesh] OR "Papillomaviridae"[Mesh] OR "DNA Methylation"[Mesh] OR "Biopsy"[Mesh:NoExp] OR "Colposcopy"[Mesh] OR methylation*[tiab] OR colposcop*[tiab] OR smear*[tiab] OR papanicolaou[tiab] OR hpv[tiab] OR papillomavir*[tiab] OR biops*[tiab]) AND (conizati*[tiab] OR conisati*[tiab] OR lletz[tiab] OR letz[tiab] OR leep[tiab] OR (loop[tiab] AND (excision*[tiab] OR electroexcision*[tiab])) OR "Conization"[Mesh] OR "Cervical Intraepithelial Neoplasia"[Mesh] OR cin[tiab] OR "cervical intraepithelial"[tiab] OR "Uterine Cervical Dysplasia"[Mesh] OR ((cervical[tiab] OR cervix[tiab]) AND dysplas*[tiab])) AND ((randomized controlled trial[pt] OR controlled clinical trial[pt] OR randomized[tiab] OR placebo[tiab] OR drug therapy[sh] OR randomly[tiab] OR trial[tiab] OR groups[tiab]) OR ((review*[tiab] OR search*[tiab] OR survey*[tiab] OR handsearch*[tiab] OR hand-search*[tiab]) AND (databa*[tiab] OR data-ba*[tiab] OR bibliograph*[tiab] OR electronic*[tiab] OR medline*[tiab] OR pubmed*[tiab] OR embase*[tiab] OR Cochrane[tiab] OR cinahl[tiab] OR psycinfo[tiab] OR psychinfo[tiab] OR cinhal[tiab] OR "web of science"[tiab] OR "web of knowledge"[tiab] OR ebsco[tiab] OR ovid[tiab] OR mrct[tiab] OR metaregist*[tiab] OR meta-regist*[tiab] OR ((predetermined[tiab] OR pre-determined[tiab]) AND criteri*[tiab]) OR apprais*[tiab] OR inclusion criteri*[tiab] OR exclusion criteri*[tiab]) OR (review[pt] AND systemat*[tiab]) OR "systematic review"[tiab] OR "systematic literature"[tiab] OR "integrative review"[tiab] OR "integrative literature"[tiab] OR "evidence-based review"[tiab] OR "evidence-based overview"[tiab] OR "evidence-based literature"[tiab] OR "evidence-based survey"[tiab] OR "literature search"[tiab] OR ((systemat*[ti] OR evidence-based[ti]) AND (review*[ti] OR literature[ti] OR overview[ti] OR survey[ti])) OR "data synthesis"[tiab] OR "evidence synthesis"[tiab] OR "data extraction"[tiab] OR "data source"[tiab] OR "data sources"[tiab] OR "study selection"[tiab] OR "methodological quality"[tiab] OR "methodologic quality"[tiab] OR cochrane database syst rev[ta] OR meta-analy*[tiab] OR metaanaly*[tiab] OR metanaly*[tiab] OR meta-analysis[pt] OR meta-synthesis[tiab] OR metasynthesis[tiab] OR meta-study[tiab] OR metastudy[tiab] OR metaethnograph*[tiab] OR meta-ethnograph*[tiab] OR Technology Assessment, Biomedical[mh] OR hta[tiab] OR health technol assess [ta] OR evid rep technol assess summ[ta] OR health technology assessment[tiab]))

Supplementary Table 4.2 Checklist for the QUality Assessment of Diagnostic Accuracy Studies (QUADAS), modified version.[26]

nr	Item definition	Item question
1	Representative spectrum?	Was the spectrum of included patient's representative of the patients who will receive the test in practice?
2	Selection bias avoided?	Were included women included on a continuous basis?
3	Acceptable reference standard?	Is the reference standard likely to correctly classify the target condition?
4	Acceptable timing of tests and verification of outcome?	Is the time period between reference standard and index test short enough to be reasonably sure that the target condition did not change between the two tests?
5	Partial verification avoided?	Did the whole sample or a random selection of the sample, receive verification using a reference standard of diagnosis?
6	Differential verification avoided?	Did all patients receive the same reference standard regardless or do some the index test result?
7	Incorporation avoided?	Was the reference standard independent of the index test (i.e. the index test did not form part of the reference standard)?
8	Index test results blinded?	Were the index test results interpreted without knowledge of the results of the reference standard?
9	Reference test results blinded?	Were the reference test results interpreted without knowledge of the results of the index test?
10	Clinical data available?	Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?
11	Uninterpretable results reported?	Were uninterpretable or intermediate test results reported?
12	Withdrawals explained?	Were withdrawals from the study explained?

	Assessment
	Yes: patients treated by conisation (either LLETZ or cone) for cervical intraepithelial neoplasia grade 2 or 3 (CIN2/3). Unclear: reporting insufficient to assess this item. No: inclusion of healthy controls.
	Yes: women were explicitly included on a continuous basis. Unclear: continuous selection is not explicitly mentioned. No: women were not continuously included (e.g. case control study).
	Yes: histological confirmation of CIN2+ is considered as presence of disease, absence of disease is either confirmed by colposcopy or by repetitive negative cytology results. Unclear: reporting insufficient to assess this item. No: no histological confirmation of presence of disease or no repetitive negative cytology results.
	'Yes' for all studies because all studies assessed disease status within 2 years of follow-up.
	'Yes' for all studies as all patients performed either a colposcopy in all patients or were considered negative for disease because of ≥2 cytological negative smears.
	Yes: irrespective of the index test result the same reference standard is performed. Unclear: reporting insufficient to assess this item. No: the result of the index test affected the policy of verification (e.g. a positive hrHPV test results in colposcopy) or different reference standards were performed.
	Yes: the hrHPV test is not part of the reference standard. Unclear: reporting insufficient to assess this item. No: the hrHPV test is part of the reference standard.
	Not applicable. The performed hrHPV tests (both HCII and PCR) are objective tests.
	Yes: hrHPV test was performed prior to or simultaneously with reference test of hrHPV test results were blinded. Unclear: reporting insufficient to assess this item. No: hrHPV test was performed and assessed with knowledge of the results of the reference standard.
	Not applicable. The performance of the(automated) hrHPV test is not influenced by the availability of clinical data.
	Yes: the number of patients with indeterminate hrHPV test results has been reported. Unclear: reporting insufficient to assess this item. No: the number of patients with indeterminate hrHPV test results has not been reported.
	Yes: the number of withdrawals has been reported and reasons have been explained or there were no withdrawals. Unclear: reporting insufficient to assess this item. No: the number of withdrawals has not been reported.

5

DISCUSSION

Assessment of women at risk of developing high-grade cervical disease; pre- and post-treatment considerations

The development of cervical cancer is preceded by a long period of well-defined premalignant stages in which these lesions can be diagnosed and treated before they will result in invasive cancer.[1-2] In developed countries this has resulted in nationwide cervical cancer screening programmes which have lead to a substantial reduction of incidence and mortality of cervical cancer.[3-6] Prevention includes a whole spectrum of measurements: screening, triage of equivocal test results, colposcopic examination of abnormal test results, treatment and post-treatment surveillance. Improvements could be made by improving the tests used, by increasing the experience of those performing and interpreting these tests and by increasing the number of women participating in the prevention programme. The health benefit of women could be improved even further when *a priori* thresholds concerning the risk of developing high-grade cervical disease would be adopted for closer surveillance, colposcopy, or treatment.[7] In 2007, Castle and colleagues proposed such an algorithm.[7] In this proposal, a CIN3+ risk of less than 2% in the subsequent 2-3 years justifies referral to the screening programme, a risk between 2 and 10% requires retesting, and a risk of 10% or more referral for colposcopic examination.[7] In the Netherlands the 5-year CIN3+ risk in women with normal cytological test results in the population-based screening programme is 0.7%.[8] This risk is considered acceptable to the Dutch general population, the health authorities, and the professionals.[8]

Hence, in this thesis we use an adapted threshold of a 5-year CIN3+ risk of 0.7% to refer women to routine screening. With the results of the studies described in this thesis, current guidelines on cervical cancer screening and follow-up of women treated for high-grade CIN can be improved. Modifications in the existing guidelines for certain categories of women with abnormal test results or CIN will be discussed in the following sections.

Pre-treatment considerations

Although prevention programmes vary widely by country, all screening programmes should, irrespective of the screening method chosen, suffice to key requirements, being broad coverage of the screening population and adequate follow-up of women with abnormal test results.

In the Netherlands, a cytology-based cervical screening programme is in place in which all women between 30 and 60 years of age are invited every 5 years for cytological examination.[9] The vast majority (96.5%) of these women have normal cytology and a risk of 0.7% of developing CIN3+ in the following 5 years.[8, 10] These women are recalled in the next screening round.[8] In women with abnormal test results a similar risk should be reached before they can be referred back to the population-based screening programme.

Women with abnormal cytology in population-based screening

In the Dutch population-based screening programme approximately 3.5% of the women have an abnormal cytological test result.[8, 11] Most of these abnormalities will not result in cervical carcinoma, however those susceptible to malignant progression should be detected in an early stage. As hrHPV is necessary for the development of cervical cancer, truly progressive lesions may be detected sooner when hrHPV DNA-testing is added to the screening programme.

About 1.0% of screened women have a >BMD test result (equivalent to moderate dyskaryosis or worse, ≥PAP3a2, HSIL, or CISOE-A ≥S5, ≥O4, and/or ≥E6).[8, 11] Currently, these women are referred for a colposcopic examination as their risk of developing high-grade disease within the next 5 years is over 50%.[7-8, 12-13] Additional testing with hrHPV at baseline, or after 6 months, identifies no subgroup that has a low enough risk refraining from colposcopic examination, let alone from referral to population-based screening. Our study (Chapter 3) demonstrated that the CIN3+ risk in the subsequent 5 years was still 5 times higher than the accepted risk of 0.7%. Therefore these women should be monitored more closely, also if their initial colposcopic examination does not reveal high-grade disease.

Conclusions for women having >BMD cytological test results:

All women with >BMD should be referred for colposcopy without additional testing for hrHPV.

The risk of developing high-grade CIN is increased for at least 10 years, and therefore long-term monitoring is required, also in women with negative colposcopy at baseline.

A BMD test result (equivalent to borderline or mild dyskaryosis, PAP2/3a1, ASCUS/LSIL, CISOE-A: S2-4, O3, or E3-5) is found in 2.5% of all screened women.[8] Approximately 10-20% of these women harbour a high-grade lesion that warrants further investigation.[14-15] Therefore Dutch guidelines recommend women with this test result to repeat the smear after 6 months.[16] All women with a ≥BMD repeat smear should be referred for colposcopy, while women with normal cytology will be retested one year later (18 months after the initial BMD smear). The majority of referred women, however, will have insignificant lesions that will regress spontaneously.[14] To reduce the number of referrals and colposcopies, the Netherlands Society of Pathology (NVVP) published updated guidelines in 2006, in which laboratories may choose to include a hrHPV test in the repeat visit after 6 months (see Figure 1.5).[17] Women who test negative for both cytology and hrHPV after 6 months may directly return to population-based screening[12, 18], as negative co-testing has a very high negative predictive value.[14, 19-20] In Chapter 3 we confirm the negligible 10-year CIN3+ risk of this group. Only women with a hrHPV-positive BMD test result and/or cytology of >BMD (irrespective of a hrHPV test result) are referred for colposcopy. All remaining women (having hrHPV positive normal cytology, or hrHPV-negative BMD) are retested after one year.[17] So, by adding hrHPV testing in the follow-up of these women, the number of follow-up visits can be decreased as the risk of women who are one time negative for hrHPV is equal to the risk of women who are two times negative for cytology.

Other studies state that women also could be stratified by baseline hrHPV-testing.[7, 19, 21-22] According to our study this is an appropriate alternative for delayed hrHPV testing. The (long-term) CIN3+ risks after immediate and delayed (after 6 months) hrHPV testing are similar in both groups. The advantage of delayed testing is that approximately 20% of the women will

have cleared their hrHPV infection in these 6 months, which will result in a lower referral rate [18, 23] with similar cost-effectiveness.[24] A disadvantage is that more women will be lost-to-follow-up with delayed testing than with direct testing.

Women testing hrHPV-positive have an increased CIN3+ risk (>30%) and should be monitored more closely with cytology, hrHPV-testing, colposcopy or a combination of these.[7-8] When no CIN3+ lesion has developed within 5 years, their CIN3+ risk in the subsequent 5 years is with 0.7% equal to the risk of women with normal cytology in routine screening and they may therefore return to the screening programme (Chapter 3).[25-26]

Additional hrHPV-testing in the risk assessment of women with a BMD test result is not recommended in women aged 29 and below. In this age group up to 80%[27] of the BMD-women are likely to test positive for hrHPV infections which are mainly transient[28] and will not develop into high-grade disease. Therefore, additional hrHPV-testing is not useful. In contrast, the prevalence of hrHPV is approximately 30% in women with BMD aged 30 years and above.[8] For this group hrHPV-testing can improve the selection of women at risk of developing high-grade CIN lesions for a period of up to 10 years.

Conclusions for women having BMD cytological test results:

The number of visits to the outpatient clinic for women with BMD can be decreased, when hrHPV testing is added to their follow-up schedule.

Women negative for hrHPV (either at baseline or at delayed testing after 6 months) should be referred to the population-based screening programme.

Women positive for hrHPV who do not develop CIN3+ within 5 years may then be referred to the population-based screening programme as their risk in the subsequent 5 years is similar to women with normal cytology in the routine screening programme.

Recent developments

Recently new developments in cervical cancer screening and prevention have been subject of discussion in the Netherlands. Although not the main focus of this thesis, we describe three of these developments, namely prophylactic HPV vaccination, primary screening with hrHPV testing, and self-sampling. As stated previously, the validation and implementation of new techniques should be carefully assessed to the risks accepted on previously determined thresholds.

Prophylactic hrHPV vaccination

The first development is the recent introduction (2009) of a prophylactic hrHPV vaccine for 12-year old girls in the Dutch National Immunisation Programme with a (temporarily) catch-up for 13 to 16-year old girls.[29] Although it will take decades before the full advantages (i.e. reduction of cervical cancer rates) will be apparent[29-30], vaccinated women will be invited to

attend cervical cancer screening in the near future. While cross-protection of these HPV 16/18 L1 vaccines has been described, and even with a theoretical attendance of 100%, these screening programmes will remain in place, because these vaccines do not cover more than 80% of all carcinomas.[30] On the long run screening guidelines might have to be adjusted for vaccinated women. Furthermore, it should also be considered whether vaccinated and non-vaccinated women would need to receive similar, or different, screening programmes. Modelling studies about these issues have been described previously.[31-32] These models demonstrated that by adding vaccination to screening a reduction in cervical cancer mortality between 50 and 81% could be reached without exceeding the cost-effectiveness threshold of 20,000 euro/QALY (quality-adjusted life year).

Population-based hrHPV screening

The second development involves the recent advise of the Health Council of the Netherlands to use hrHPV-testing as the primary test in population-based screening to improve screening efficacy.[11] This will be implemented in the Netherlands in 2013 and comprises primary hrHPV testing in 5 screening rounds (at the ages of 30, 35, 40, 50 and 60 years). Women who test positive for hrHPV at the age of 40, 50, or 60 years, and are negative for cytological triage at baseline and after 6 months, should be screened again 5 years later.

The decision to change from cytological based screening to primary hrHPV screening was based on results of numerous cross-sectional studies and several longitudinal randomised controlled trials. These trials all revealed a higher cross-sectional sensitivity of primary hrHPV-testing than of cytological screening in detecting high-grade cervical lesions (approximately 95% versus 65%).[8, 25, 33-40] Moreover a reduction of 50% of CIN3 and cervical carcinoma was found in the second screening round (3-5 years later) of the women tested with hrHPV if compared to cytology indicating superior protection in the HPV group. Thus a negative hrHPV test provides a greater reassurance against cervical disease than testing negative for cytology.[34, 41] Or, in other words, a negative hrHPV test will result in a lower proportion of cancers occurring in women apparently adequately screened by cytology. Consequently, by using hrHPV-testing the screening interval can be increased, without a rise in high-grade cervical lesions in the meantime, because high-grade disease and cancer are detected sooner.[25, 33, 35-37, 41] In addition, the sensitivity of hrHPV-testing is similar to the sensitivity of combined testing with hrHPV and cytology, which strengthens the decision to use hrHPV as a stand alone screening test.[35, 38]

However, the specificity of hrHPV-testing is approximately 5% lower than the specificity of cytological testing.[33] Because this is a screening setting in which women have a low *a priori* risk of harbouring high-grade disease, the number of false positively tested women should preferably be as low as possible to prevent unnecessary retesting and referral. So in short, screening by hrHPV-testing can detect premalignant lesions that would otherwise grow slowly to the point of detection by less sensitive methods like cytology and colposcopy, but also results in a higher number of abnormal test results. The majority of the approximately 5% of the Dutch women who test positive for hrHPV [8] will have transient infections that would clear without treatment.[36-38] Therefore, some form of triage should be performed in hrHPV-positive women. Rijkaart and colleagues have analysed several triage strategies in these women.[42] The authors have found that baseline triaging with cytology, followed by cytological testing after one year

was an effective option. Women who test negative for cytology at both visits may be referred to routine screening, while those with abnormal test results should be referred for a colposcopic examination.[33, 39, 42] Another effective triage strategy includes cytology with HPV16/18/31/33/35 genotyping, however, a major disadvantage of this strategy was the high overall colposcopy rate. Depending on the screening interval used, different triage options could be preferred. In the Netherlands cytological triage is currently chosen as preferred choice. A modelling study has shown that primary hrHPV-testing with cytological triage can be cost-effective compared to cytology-based screening, provided fewer screening rounds will be present and the price of the hrHPV testing is not exceeding €30,-.[43] In the future more objective markers, such as methylation markers, may be implemented in triaging hrHPV-positive women. Naturally, well-trained colposcopists will remain important for an appropriate risk assessment, also in a hrHPV-based screening programme.

Another benefit of primary hrHPV-testing would be the improved detection of glandular lesions.[44-46] However, although hrHPV-testing seems to be an excellent way to detect both squamous and adenocarcinoma, a special interest should be paid to very rare carcinomas (less than 1% of all cervical carcinomas) which appear to be hrHPV-unrelated, such as minimal deviation carcinoma and cervical clear-cell carcinoma (CCAC).[47] This thesis (Chapter 2) describes the rather weak link between hrHPV and this last type of cancer, and we therefore conclude that hrHPV-testing will be insufficient to detect these tumours. So, when there are indications that a certain group of women has an increased risk of developing these rare hrHPV-unrelated carcinomas, such as women who are prenatally exposed to DES, it is recommendable to offer co-testing, consisting of both hrHPV and cytology to these women. However, since precursor lesions of CCAC are still unknown, the prevention of this cancer subtype will pose a challenge in which the role of cytological screening remains limited to detect the invasive tumour at the lowest possible stage.

Conclusion:

Women with an increased risk of rare and hrHPV-unrelated adenocarcinomas, for instance CCAC in women prenatally exposed to DES, should not only be screened by hrHPV-testing, but should also receive cytological screening.

Self-sampling

In addition to implementing hrHPV-testing as primary test in population-based screening, the Health Council of the Netherlands also recommends to send a device for self-sampling cervico-vaginal material to women, who repetitively not responded to invitations for the screening programme, for hrHPV-testing.[11]

This seems an effective method to increase the coverage of population-based screening, as up to 40% of the non-attendees respond by returning their self-sampler.[48-50] Furthermore, relatively more high-grade cervical lesions are found in this group.[48-50] Testing for hrHPV on

self-collected material has proven to be just as sensitive as hrHPV-testing on physician-collected material.[51-52] A limitation is that the sample is often of insufficient quality to perform reliable cytology on.[53] Therefore women who test hrHPV positive on self-collected vaginal material should have an extra visit to their physician for taking a triage smear. To prevent this extra visit to the physician, alternative and more objective triage markers that are directly applicable on self-collected material, such as DNA methylation markers, are presently evaluated in the triage algorithm of hrHPV positive women detected by self-collected samples.[54]

Post-treatment considerations

Despite all efforts in the screening programme each year approximately 5000 women are treated for high-grade cervical disease by the destruction or excision of the entire transformation zone. These methods are effective in 90% of the cases, which implicates that 10% of treated women will be diagnosed with residual or recurrent (post-treatment) disease.[14] To detect post-treatment disease quickly, treated women are monitored closely so that it is possible to repeat conservative treatment when residual or recurrent disease is detected. Although most post-treatment disease is diagnosed within 2 years of treatment [55-57], the risk of developing post-treatment disease is increased for a much longer period.[58-60] The current surveillance protocol in the Netherlands is cytology-based, and women with three consecutive negative test results (at 6, 12 and 24 months after treatment) return to the population-based screening programme.[61] The 5-year risk of these women to develop CIN3+ is similar to women with normal cytology in the population-based screening programme and is considered acceptable by Dutch health authorities.[8] This is in striking contrast to the annual cytological post-treatment surveillance of these women for at least ten[62] or twenty[63] years in the United Kingdom and United States. This thesis (Chapter 4.1) confirms the effectiveness of the surveillance strategy in the Netherlands, as the 5-year CIN3+ risk of women with three consecutive negative smears was less than 1% and the CIN2+ risk over the same period was less than 3%.

However, a substantial part of treated women do not comply with the complete follow-up schedule of three visits.[64-66] In a Dutch study only half of the women treated for high-grade cervical disease completed the total follow-up programme of three cytological smears in the first two years after treatment.[64] This might be resolved by reducing the number of visits in post-treatment surveillance.

Therefore, the implementation of new techniques, which are at least equal in test characteristics as the current surveillance programme, could be considered. In line with the implementation of hrHPV testing in population-based screening, the use of hrHPV in post-treatment surveillance has been studied extensively.[14, 56, 67-74] Our meta-analysis (Chapter 4.2) confirmed that hrHPV-testing after treatment is more sensitive (relative sensitivity 1.15, 95% CI 1.06-1.25) than cytology with equal specificity (relative specificity 0.95, 0.88-1.02).[14, 68, 70] However, some studies displayed a slightly lower specificity for hrHPV testing, most likely because of different hrHPV prevalences between countries.[69, 71] As treated women have a relatively high *a priori* risk of developing high-grade lesions, sensitivity is in this context considered to be more important than specificity.

As a consequence of its high sensitivity, a single positive hrHPV test assures the early and accurate detection of women with an increased risk for progression to and development of

post-treatment disease. Most women clear their hrHPV infection within 6 months [68, 75] and those testing negative are not at risk of developing post-treatment disease and may therefore return to the population-based screening programme.[68, 76-77] Because sole HPV testing still has a few false negative test results (sensitivity of 95%) and does not detect hrHPV types which are not part of the routinely used test panel, co-testing with both cytology and hrHPV could be performed as alternative in a diagnostic setting.[68, 72, 74] This combination has shown to have the highest sensitivity to detect post-treatment disease and accordingly the highest negative predictive value (Chapter 4).[67-68, 72, 78] Several studies have demonstrated that women who test negative for co-testing 6 months after treatment could omit the 12-month visit during follow-up.[67-68, 72, 74] Our study (Chapter 4.1) confirms the value of hrHPV-testing in post-treatment surveillance; the CIN2+ risk after three consecutive negative cytological tests was with 2.9% similar to the risk after one time co-testing at 6 months after treatment (3.0%), illustrating that the negative predictive value of co-testing is mainly because of a negative hrHPV test.

Although some advocate a single test moment post-treatment[72], we favour a strategy with two recall dates, because a single recall relies too much on the sensitivity of the HPV test. Another reason is that a single recall moment might be too early to detect post-treatment disease developed by re-infection of the cervical epithelium, as women with a history of CIN have higher acquisition rates of HPV.[20]

Coupé and colleagues have performed cost-effectiveness analyses for different follow-up strategies including hrHPV testing. They showed that hrHPV testing at 6 months followed by co-testing at 24 months is more cost-effective than the current surveillance protocol.[79] Co-testing at both 6 and 24 months is slightly more costly than the current algorithm, but detects the most cases of post-treatment disease.[74, 79]

Considering the high negative predictive value of hrHPV-testing in the first two years after treatment and the long-term predictive value described in Chapter 4.1 of this thesis, the current Dutch surveillance guidelines of women treated for high-grade disease (CIN2/3) should be modified.

We advocate monitoring women treated for high-grade premalignant disease by hrHPV-testing and cytology (co-testing) 6 months after treatment. A meta-analysis (Chapter 4.2) showed that 60% of treated women test negative for co-testing and these women can omit the 12-month visit and return for testing at 24 months after initial treatment. However, when one of the tests performed at 6 months is positive, a colposcopy (with biopsy) is indicated. Women who are diagnosed with high-grade disease are treated, while those with no or low-grade disease should be retested by co-testing 12 months after initial treatment. False negative test results are corrected at the 24-month visit. Again, a colposcopy is required in women with an abnormal test result. Women with no or low-grade disease, and those with negative cytology at 12 months are retested 24 months after initial treatment. So, all women are retested by co-testing at 24 months after treatment to avoid missing cervical carcinoma because of detection problems. Only women who test negative for both tests at 6 and 24 months should be referred to population-based screening, as their risk of developing CIN3+ in the next 5 years is negligible (Chapter 4.1). Women with one or both tests positive at 24 months after treatment should be examined with colposcopy and retested. This could be done by co-testing after 30 and 48 months after initial treatment and only women testing negative at 2 consecutive time points are referred to the population-based

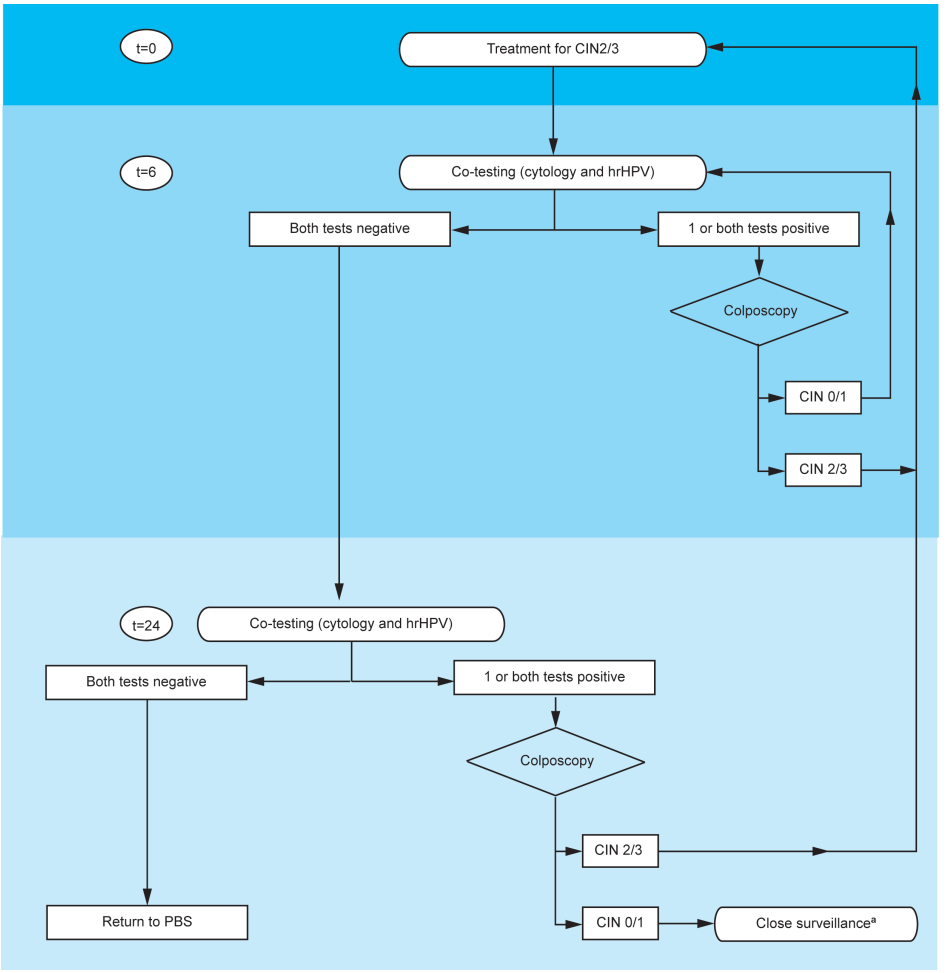

Figure 5.1 Proposed follow-up schedule for women treated for high-grade CIN.
CIN, Cervical Intraepithelial Neoplasia; hrHPV, high-risk type of the human papillomavirus; PBS, population-based screening.
[a] Close surveillance consisting of
a) cytology after 6, 12, and 24 months; referral to population-based screening programme or co-testing once every 5 years when all three tests are negative;
b) co-testing (both cytology and hrHPV) after 6 and 24 months; referral to population-based screening programme or co-testing once every 5 years when both tests are negative at both time points.

screening programme (Figure 1). When hrHPV-testing is not available or possible post-treatment monitoring with cytology at 6, 12 and 24 months is a good alternative, as the 5-year CIN3+ risk in women who test negative at all time points is similar to women with normal cytology in population-based screening (Chapter 4.1). Because population-based screening ends at age 60, women treated after the age of 55, also those with a negative post-treatment algorithm, should receive additional testing after 5 and 10 years, because they will no longer be invited for tests

through the regular programme, while the risk of post-treatment disease in successfully treated women increases by about 2.5% between 5 and 10 years of follow-up.

It is important to keep in mind that current adjustments only apply to women treated for CIN2/3. Until more research has been performed, we support the current guideline to follow women treated for AIS for 5 consecutive years because of the endocervical location and increased risk of multifocality. However, also these women could benefit from including hrHPV-testing in post-treatment surveillance.

It is possible that in the future cytology will be replaced by a more objective and reproducible molecular test. An alternative follow-up algorithm may include HPV16 genotyping, as women infected with this hrHPV type have a higher risk of developing post-treatment disease than women infected with other hrHPV types.[80-81] However, in Chapter 4.1 we demonstrate that the risk for women positive for hrHPV types other than HPV16 remains considerable.[74, 80] Therefore, other options for post-treatment surveillance are investigated. One of these options could be including additional molecular markers (i.e. CADM1 and MAL). Currently a Dutch multicenter prospective trial "Simplified Monitoring of post-treatment CIN2/3 women by molecular testing for hrHPV and methylation markers" (SIMONATH) investigates whether addition of these markers could result in a model in which women negative for a panel of markers at 6 months can be referred to population-based screening directly.

Conclusions for women treated for high-grade cervical disease:

For optimal post-treatment surveillance hrHPV-testing should be included in the follow-up algorithm of treated women.

Women with two negative co-testing results of both hrHPV-testing and cytology at 6 and 24 months after treatment, or three negative cytology results (6, 12 and 24 months) have a 5-year CIN2+ risk that is at least similar to women with normal cytology in the population-based screening programme and should be referred to population-based screening (or should be seen once every 5 years).

Overall, as long as there is no flawless instrument to prevent cervical cancer, the search for the perfect prevention tool will continue. Despite developments as prophylactic vaccination and better screening techniques, cervical cancer will not disappear in the future. Therefore the screening and monitoring of women at risk of developing cervical cancer will remain important. This applies especially for developing countries, which have the highest burden of disease, and where the most important achievement would be that (affordable) prevention will become available for all.

RECOMMENDATIONS

Recommendations for women having > BMD cytological test results:

1. All women with > BMD should be referred for colposcopy.
Additional hrHPV testing will not identify women with a low enough risk (CIN2+ risk of <10%) to refrain from colposcopy. So, additional hrHPV testing has no clinical value.

2. Also for women diagnosed with > BMD and a negative colposcopy result (CIN0/1) close surveillance using co-testing (both cytology and hrHPV) at 6 and 24 months after diagnosis, or cytology at 6, 12, and 24 months after diagnosis is required.
The CIN3+ risk of women having > BMD is increased for at least 10 years, even for women with negative colposcopy at baseline. The CIN3+ risk is 45% in the first 5 years after diagnosis of > BMD and 3.5% in the subsequent 5 years. Therefore, they should receive a surveillance programme similar to other women with an increased risk of developing CIN3. Monitoring could be performed with co-testing (both cytology and hrHPV) after 6 and 24 months, or with cytology after 6, 12 and 24 months. Only women testing negative at all time points should be referred to population-based screening programme. [a]

[a] Referral to the population-based screening programme, or tested with co-testing once every 5 years.

Recommendations for women having BMD cytological test results:[b]

1. Women having BMD and who test positive for hrHPV should be referred for colposcopy.
These women have a risk of over 30% of harbouring a CIN3+ lesion

2. By adding hrHPV testing in the follow-up of women with BMD test results, the number of follow-up visits can be decreased.
As suggested by the Netherlands Society of Pathology[17], the follow-up period of many women having BMD may be decreased by using hrHPV testing. The risk of women who test negative for both cytology and hrHPV after 6 months is with <1% similar to the risk of women who test negative for cytology after 6 and 18 months.

3. Women negative for hrHPV (either at baseline or at delayed testing after 6 months) should be referred to population-based screening programme.[a]
Women with a BMD test result who test negative for hrHPV at baseline have a negligible risk of <1.0% of developing CIN3+ in the next 10 years, and may return to population-based screening programme (like women having BMD at baseline and a negative hrHPV-test result after 6 months). [a]

4. Women positive for hrHPV who do not develop CIN3+ within 5 years may then be referred to population-based screening programme. [a]
Women having BMD, who have not developed CIN3+ within 5 years, have a CIN3+ risk in the subsequent 5 years of 0.7%, similar to the CIN3+ risk of women with normal cytology in the screening programme.

[a] Referral to the population-based screening programme, or tested once with co-testing every 5 years.
[b] Since up to 80% of women below 30 years of age having BMD test results are likely to encounter a (transient) hrHPV infection, the abovementioned recommendations are only valid for women aged 30 years and above.

Recommendations for women treated for high-grade cervical disease:

1. HrHPV testing improves the sensitivity for residual and recurrent (post-treatment) CIN2/3 and should therefore be included in the follow-up algorithm of treated women.
To detect post-treatment disease, hrHPV testing is more sensitive than cytology (relative sensitivity 1.15, 95%CI 1.06 – 1.25) and has equal specificity (relative specificity 0.95, 0.88 -1.02). So, a positive hrHPV test may better identify women with an increased risk for progression to and development of post-treatment disease.

2. Women treated for CIN2/3 who have three consecutive negative cytological test results post-treatment should return to the population-based screening programme. [a]
Treated women with three consecutive cytological negative test results (at 6, 12 and 24 months post-treatment) have a 5-year CIN3+risk of 0.4%, similar to women with normal cytology in the population-based screening programme.

3. Follow-up of women treated for CIN2/3 should be done by co-testing (both cytology and hrHPV).
In the diagnostic setting the risk of missing residual or recurrent (post-treatment) CIN2/3 should be minimised as much as possible. The highest sensitivity (95%, 95%CI 91 – 98) of detecting post-treatment disease is reached by performing co-testing (both cytology and hrHPV). As the a priori risk of these women is relatively high, the most optimal follow-up algorithm is the strategy that has the highest sensitivity.

4. Women treated for CIN2/3 with negative co-testing (both cytology and hrHPV) results after 6 months may omit the 12-month screening visit
Women testing negative for both cytology and hrHPV have a very low risk of developing post-treatment disease (CIN3+ risk of 1.4%). CIN2+ risk after a single negative co-testing result (5-year CIN2+ risk of 3.0%) was similar to the risk after three consecutive negative cytological test results (5-year CIN2+ risk of 2.9%).

5. Women treated for CIN2/3 with two negative co-testing results (both cytology and hrHPV) should return to population-based screening programme.[a]
Women who test negative for co-testing twice (at 6 and 24 months post-treatment) should be referred to population-based screening programme [1], as their risk of developing CIN3+ in the next 5 years is negligible (0.0%, 95%CI 0.0 – 3.0).

[a] Referral to the population-based screening programme, or tested once with co-testing every 5 years.

The recommendations mentioned above concern the current population-based screening programme with cytology as primary screening method.

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6

SUMMARY / SAMENVATTING

SUMMARY

As cervical cancer is an important health problem worldwide with over a half million patients a year and as it is the fourth most common cause of cancer-related death in women, improving the prevention of this disease is a continuing and important process. A major reduction of cancer incidence and mortality has occurred in countries with cervical cancer screening. Because cervical cancer develops through different premalignant stages it can be detected in a premalignant stage, allowing treatment before these stages would be able to develop into cervical cancer. **Chapter 1** gives a general introduction about the cervix, human papillomavirus (HPV), the model(s) of cervical carcinogenesis and different measures that are taken to prevent cervical cancer. These measures include screening, triaging of abnormal test results, colposcopic examination, treatment and post-treatment surveillance.

In the vast majority of cervical cancers a persistent infection with high-risk HPV types (hrHPV) has been proven to be the causative agent in their carcinogenesis. Besides almost all cervical squamous cell carcinomas, approximately 95% of all cervical adenocarcinomas (ACs) are caused by a transforming infection with a hrHPV type. The remaining ACs are rare and sometimes seem hrHPV-unrelated, which could be caused by detection error or because these tumours are indeed caused by another, not HPV-related carcinogenic mechanism. **Chapter 2** describes the attribution of hrHPV in cervical clear-cell adenocarcinoma (CCAC), which are relatively rare tumours (<<1% of all cervical carcinoma). These tumours have a bimodal age distribution with one peak in the early twenties and another after menopause and are characterised by clear cytoplasm and Hobnail cells. In approximately 60% of the cases this tumour has been associated with intrauterine exposure to diethylstilbestrol (DES), a synthetic oestrogen which has been (falsely) used in the past to prevent miscarriages. In this study of 28 women with CCAC, of whom 15 were DES-exposed *in utero*, hrHPV was found in 13 (46.4%) tumours. However, after performing immuno-histochemistry with $p16^{INK4a}$ and p53 to distinguish transient hrHPV infections from transforming, carcinogenic infections, only three carcinomas remained in which a causal relation of hrHPV and CCAC was plausible. This demonstrated a very limited role of hrHPV in the carcinogenesis of CCAC. None of the hrHPV-associated tumours were found in women prenatally exposed to DES. In DES-unrelated tumours only a minority (20-25%) seemed hrHPV mediated.

In the Dutch population-based screening programme approximately 2.5% of screened women have borderline or mild dysplasia (BMD, PAP2/3a1). These women are retested after 6 months with either cytology of a combination of both cytology and HPV (co-testing), and after 18 months with cytology. If the tests remain abnormal, women are referred for colposcopy. However, not all women with BMD comply with this protocol. Many studies have examined the short-term value of hrHPV-testing in predicting the cumulative risk of CIN3+. In **Chapter 3** we have evaluated the long-term cumulative CIN3+ risk in a group of 342 women with an abnormal cytological test result (≥ BMD). These women were followed for a time period of 17 to 19.5 years after detection. Immediate hrHPV-testing clearly stratified the CIN3+ risk; almost all CIN3+ lesions (97.1%) were found in women who tested hrHPV positive. Almost half of all hrHPV-positive women were infected with HPV16; these women had a significantly higher CIN3+ risk than women infected with other hrHPV types. This risk difference between

HPV16-positive women and women positive for other hrHPV types, was only found in younger women (<30 years). In older women (≥30 years) the risks in both age groups were similar. The 5-year CIN3+ risk was lower in women who had cleared the virus within 6 months than in women with persistent hrHPV infections (2.2% versus 56.0%), with the highest risks for women with a persistent HPV16 infection (67%).

We stratified the CIN3+ risks according to referral cytology and found that both women with BMD and women with >BMD referral cytology had an increased risk of developing CIN3+ within the first 5 years after detection. This risk was twice as high in women with >BMD compared to women with BMD (45% versus 22%). In the subsequent 5 years an increased risk (3.5%) remained for women with >BMD, while for women referred with BMD this risk was with 0.7% similar to that of the general population. Immediate (or delayed, i.e. after 6 months) hrHPV testing clearly stratified the risk in women with BMD; the 5-year risk in hrHPV-negative women was 0.01%, and in hrHPV-positive women 37.5%. Therefore we support the strategy to refer hrHPV-negative women with BMD to routine screening and to refer those who are hrHPV positive for additional testing or colposcopy. When these women do not develop CIN3+ within 5 years, they also may be referred to population-based screening.

Additional (baseline) hrHPV-testing in women with >BMD did not result in a group with a risk low enough to refrain from colposcopy, therefore we do not advocate hrHPV testing in this group and advise to refer all these women for colposcopy. As their CIN3+ risk is elevated for at least 10 years, long-term monitoring is required.

Chapter 4 focuses on women treated for high-grade cervical disease (CIN2/3). As over 10% of treated women will develop residual/recurrent (post-treatment) high-grade cervical disease, they are closely monitored by cytological testing after treatment. Most published studies concern the risk-assessment of developing post-treatment disease up to a maximum of two years. Currently, treated women in the Netherlands are referred to population-based screening when they have three consecutive negative cytological test results after treatment. This means that it would take at least another three years before women are invited for population-based screening again. In order to evaluate the safety of the current regimen, long-term follow up data is essential. Also because in several other countries yearly follow-up for up to 10 years after treatment is common. As successful treatment is associated with the elimination of hrHPV, hrHPV testing has been suggested as an improvement in post-treatment surveillance. In **Chapter 4.1** a multi-cohort study is described that includes 435 women followed between 5 and 21.5 years after treatment. Different post-treatment test algorithms were analysed; sole cytological testing, sole hrHPV-testing and combined testing with both cytology and hrHPV (co-testing). The overall 5-year CIN2+-risk in this cohort was 16.5%. However, in women who tested consecutively negative for cytology (at 6,12 and 24 months after treatment) this risk was lowered to 2.9% and even to 1.0% in women who tested negative for co-testing at both 6 and 24 months after treatment. The risk of developing CIN3+ in treated women with three consecutive negative cytological test results is similar to the risk of developing high-grade cervical disease in women who test negative for cytology (PAP1) in population-based screening. However, by adding hrHPV-testing to post-treatment surveillance, a better risk-assessment could be reached with even fewer visits.

In order to judge the results found in this multi-cohort study, studies which compared different surveillance methods (cytology, hrHPV or co-testing), tested six months after treatment, were systematically reviewed in **Chapter 4.2**. After a bibliographic database search, relevant studies published between January 2003 and May 2011 were identified by two reviewers with a multi-step process. Then the selected studies were methodological assessed with a modified version of the QUADAS tool (QUality Assessment of Diagnostic Accuracy Studies). Eventually, only eight out of 2410 identified studies remained, incorporating 1513 treated women. The sensitivity of hrHPV testing to predict post-treatment CIN2+ was significantly higher than of cytology (relative sensitivity 1.15; 95%CI 1.06-1.25), while the specificity of these tests was similar (relative specificity 0.95, 95%CI 0.88-1.02). The sensitivity of co-testing was the highest (95%), however this combined test had the lowest specificity (67%). In summary, this review supports the inclusion of hrHPV testing in post-treatment monitoring protocols.

The general discussion in **Chapter 5** summarises the findings of this thesis and discusses possible future prospects and clinical consequences.

SAMENVATTING

Met meer dan een half miljoen nieuwe gevallen per jaar wereldwijd is baarmoederhalskanker een belangrijk gezondheidsprobleem. Verbeteringen in de preventie van baarmoederhalskanker is dan ook een continu proces. In landen met een (gestructureerd) bevolkingsonderzoek voor het opsporen van baarmoederhalskanker is de incidentie van en sterfte aan deze vorm van kanker aanzienlijk gedaald. Omdat baarmoederhalskanker in een premaligne stadium kan worden opgespoord, kunnen afwijkingen behandeld worden voordat deze invasief worden.

Hoofdstuk 1 bevat een algemene introductie over de baarmoederhals, het humaan papillomavirus (HPV), de ontstaanswijze van baarmoederhalskanker en de verschillende maatregelen die gebruikt kunnen worden om baarmoederhalskanker te voorkomen. Deze maatregelen omvatten screening (door middel van het bevolkingsonderzoek op baarmoederhalskanker), het triëren van abnormale test resultaten, colposcopisch onderzoek, behandeling en follow-up na behandeling.

Bijna alle gevallen van baarmoederhalskanker worden veroorzaakt door een persistente infectie met een hoog-risico HPV type (hrHPV). Zo goed als alle plaveiselcel carcinomen en circa 95% van alle glandulaire (adeno)carcinomen worden veroorzaakt door een transformerende hrHPV infectie. In de resterende 5% van de adenocarcinomen is geen hrHPV aantoonbaar. Dit kan veroorzaakt worden door een detectie fout of omdat deze tumoren inderdaad worden veroorzaakt door een ander, niet HPV gerelateerd carcinogeen mechanisme.

Hoofdstuk 2 beschrijft de bijdrage van hrHPV in heldercellige adenocarcinomen van de cervix (CCACs). CCACs zijn relatief zeldzame tumoren (<<1%) . Deze tumoren hebben een bimodale leeftijddistributie met een piek rond de leeftijd van 20 jaar en een piek rond de menopauze. Zij worden gekenmerkt door helder cytoplasma en zogenaamde Hobnail cellen. In ongeveer 60% van de gevallen zijn deze tumoren geassocieerd met diethylstilbestrol (DES) gebruik van de moeder tijdens de zwangerschap. DES is een synthetisch vervaardigd oestrogeen dat vroeger (onterecht) werd gebruikt om miskramen te voorkomen. In deze studie van 28 vrouwen met CCAC, waarvan er 15 aan DES in de baarmoeder waren blootgesteld, werd in 13 (46.4%) tumoren hrHPV aangetoond. Door het toepassen van een immuno-histochemische kleuring met $p16^{INK4a}$ en p53 kon onderscheid gemaakt worden tussen productieve, tijdelijke hrHPV infecties en transformerende, oncogene infecties. In slechts 3 van de 21 tumoren was hrHPV een aannemelijke oorzaak. Dit toonde de zeer bescheiden rol van hrHPV aan in de carcinogenese van CCAC. Geen van de hrHPV geassocieerde tumoren werd gevonden in vrouwen die intra-uterien waren blootgesteld aan DES, en in de overige vrouwen bleek slechts een minderheid (20-25%) door hrHPV veroorzaakt.

In het Nederlandse bevolkingsonderzoek op baarmoederhalskanker (BVO) heeft circa 2.5% van alle gescreende vrouwen een lichte cytologische afwijking (PAP2/3a1, BMD, KOPAC-B P2-4, A3 en/of C3-5). Deze vrouwen worden na 6 maanden opnieuw getest door middel van een uitstrijkje, of door een combinatie van een uitstrijkje én een hrHPV test (co-test). Na 18 maanden volgt in beide strategieën wederom een uitstrijkje. Vrouwen bij wie de testen afwijkend blijven, worden verwezen voor colposcopisch onderzoek. Echter, niet alle vrouwen volgen deze adviezen op. In de literatuur is de korte termijn voorspellende waarde van de hrHPV test op het ontstaan van een premaligne afwijking voor deze groep vrouwen uitgebreid beschreven.

In **Hoofdstuk 3** hebben wij het cumulatieve lange-termijn risico op het ontwikkelen van CIN3+ beschreven in een groep van 342 vrouwen met een afwijkend uitstrijkje (≥ BMD, ≥ PAP2/3a1, KOPAC-B ≥P2, ≥A3 en/of ≥C3). Deze vrouwen werden gevolgd voor een periode tussen 17 en 19.5 jaar na het detecteren van een afwijkende uitstrijk. Direct testen op hrHPV stratificeerde het CIN3+ risico; bijna alle CIN3+ laesies (97.1%) werden gevonden in vrouwen met een positieve hrHPV test. Bijna de helft van deze vrouwen was geïnfecteerd met HPV16. Zij hadden een significant hoger risico om CIN3+ te ontwikkelen dan vrouwen die geïnfecteerd waren met andere hoog-risico typen. Dit risicoverschil was alleen aanwezig in jonge vrouwen (onder de 30 jaar). In de vrouwen boven de 30 jaar waren de risico's in beide groepen gelijk. Vrouwen wiens immuunsysteem de infectie binnen 6 maanden klaarden, hadden een lager 5-jaars risico om CIN3+ te ontwikkelen dan vrouwen met een persisterende infectie (2.2% versus 56.0%). Vrouwen met een persisterende HPV 16 infectie hadden met 67% het hoogste 5-jaars risico. Naar aanleiding van de ernst van de cytologische afwijking bij verwijzing werden vrouwen verdeeld in twee groepen, BMD en >BMD. Beide groepen vrouwen hadden een verhoogd risico om CIN3+ te ontwikkelen in de eerste 5 jaren na het vinden van een afwijkend uitstrijkje. Dit risico was met 45% twee keer zo hoog in de vrouwen met een >BMD resultaat dan vrouwen met een BMD uitslag (22%). In de hierop volgende 5 jaren behielden vrouwen met een >BMD uitslag een verhoogd risico (3.5%), terwijl dit risico met 0.7% voor vrouwen met een BMD resultaat identiek was aan het 5-jaars risico van vrouwen met een normaal uitstrijkje in het BVO. Het risico op het ontwikkelen van CIN3+ kon voor vrouwen met BMD door middel van het direct (of na 6 maanden) testen op hrHPV gestratificeerd worden; het 5-jaars CIN3+ risico in vrouwen met een negatieve hrHPV test was nihil (0.01%), terwijl het risico voor vrouwen met een positieve test 37.5% bedroeg. Deze bevinding ondersteunt het voorstel om hrHPV-negatieve vrouwen met BMD te verwijzen naar het BVO, terwijl vrouwen met een positieve hrHPV test verder onderzocht moeten worden door middel van een herhaaltest of een colposcopisch onderzoek. Als deze vrouwen in de eerste 5 jaren na detectie geen afwijkingen ontwikkelen, dan kunnen ook zij hierna naar het BVO worden verwezen.

Het toevoegen van een hrHPV test in de groep vrouwen met een >BMD resultaat (KOPAC-B: ≥P5, ≥A4 en/of ≥C6) leidde niet tot het identificeren van een groep vrouwen die kon afzien van aanvullend onderzoek. Derhalve is het niet nuttig om een hrHPV test te verrichten in de follow-up van vrouwen met een >BMD uitslag, maar moeten zij allen verwezen worden voor colposcopisch onderzoek. Aangezien hun CIN3+ risico minimaal 10 jaar verhoogd is, moeten deze vrouwen langdurig vervolgd worden.

Hoofdstuk 4 beschrijft onderzoek dat vrouwen betreft die behandeld zijn voor een hoog-gradige premaligne cervix afwijking (CIN2/3). Aangezien circa 10% van de behandelde vrouwen opnieuw wordt gediagnostiseerd met een hoog-gradige afwijking, worden deze vrouwen door middel van cytologisch onderzoek nauwgezet in de gaten gehouden. De meeste gepubliceerde studies beschrijven het risico dat deze vrouwen hebben om in de eerste twee jaar na behandeling opnieuw een hoog-gradige laesies te ontwikkelen. Indien behandelde vrouwen drie achtereenvolgende normale uitstrijkjes hebben gehad (6, 12 en 24 maanden na behandeling), worden zij in Nederland terugverwezen naar het BVO. Dit houdt in dat het minimaal 3 jaar zal duren voordat deze vrouwen weer worden uitgenodigd voor het BVO.

Daarom is het essentieel om, ter evaluatie van het huidige beleid en met betrekking tot de risico inschatting voor deze vrouwen ook over lange-termijn data te beschikken. In andere landen worden deze vrouwen namelijk soms tot wel 10 jaar na behandeling met jaarlijkse uitstrijkjes gevolgd. Aangezien succesvolle behandeling geassocieerd is met de klaring van hrHPV, is het toevoegen van testen op hrHPV als verbetering in de follow-up van behandelde vrouwen voorgesteld.

In **Hoofdstuk 4.1** wordt een multi-cohort beschreven waarin 435 vrouwen worden gevolgd voor een periode tussen 5 en 21.5 jaar na behandeling. Er werden verschillende follow-up algoritmes geanalyseerd; enkel testen met cytologie, enkel testen met hrHPV, of het gecombineerd testen met hrHPV én cytologie (co-test). Voor het totale cohort vrouwen was het 5-jaars CIN2+ risico 16.5%. Echter, in vrouwen met drie opeenvolgende normale cytologische uitstrijkjes (6, 12 en 24 maanden na behandeling) daalde dit risico tot 2.9%. Voor vrouwen die op 2 momenten (6 en 24 maanden na behandeling) negatief testten voor zowel cytologie als HPV (co-test) daalde dit risico zelfs tot 1.0%. Het 5-jaars CIN3+ risico voor behandelde vrouwen met drie opeenvolgende normale uitstrijken was gelijk aan het risico van vrouwen met een normale cytologische uitstrijk in het BVO. Door het toevoegen van het testen op hrHPV in de follow-up van behandelde vrouwen kan, met minder bezoeken, een nog betere risico-inschatting gemaakt worden.

Om de resultaten van deze multi-cohort studie in perspectief te plaatsen, is een systematische review verricht, waarvan de resultaten vermeld staan in **Hoofdstuk 4.2**. De waarde van verschillende follow-up methoden (cytologie, hrHPV test of co-test) werden op 6 maanden na behandeling met elkaar vergeleken. Alle tussen januari 2003 en mei 2011 gepubliceerde, relevante studies werden door twee onderzoekers onafhankelijk van elkaar beoordeeld op relevantie via een multi-stap model. De geselecteerde studies werden methodologisch beoordeeld aan de hand van de QUADAS criteria (QUality Assessment of Diagnostic Accuracy Studies). Uiteindelijk werden acht van de 2410 oorspronkelijke studies geïncludeerd, waarin de gegevens van 1513 behandelde vrouwen werden beschreven. De sensitiviteit van het testen met hrHPV om de aanwezigheid van hoog-gradige residuen en recidieven (CIN2+) te voorspellen was significant hoger dan de sensitiviteit van cytologie (relatieve sensitiviteit 1.15; 95%CI 1.06-1.25), terwijl de specificiteit van beide testen gelijk was (relatieve specificiteit 0.95, 95%CI 0.88-1.02). De sensitiviteit van co-testen was met 95% het hoogst, echter de specificiteit hiervan was met 67% het laagst. Samenvattend ondersteunt dit review het includeren van het testen op de aanwezigheid van hrHPV in de follow-up van vrouwen behandeld voor een hoog-gradige premaligne cervix afwijking.

De algemene discussie in **Hoofdstuk 5** vat alle bevindingen uit dit proefschrift samen en bespreekt toekomstige veranderingen en de klinische consequenties hiervan. Verder worden in dit hoofdstuk aanbevelingen gedaan om de huidige protocollen om baarmoederhalskanker te voorkomen, te verbeteren.

LIST OF ABBREVIATIONS

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AC	Adenocarcinoma
AGC	Atypical glandular cells
AIS	Adenocarcinoma *in situ*
ASC-H	Atypical squamous cells, cannot exclude HSIL
ASCUS	Atypical squamous cells of undetermined significance
ASIR	Age-standardised incidence rate
ASMR	Age-standardised mortality rate
BMD	Borderline or mild dyskaryosis
CCAC	Clear-cell adenocarcinoma of the cervix
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
CNR	Central Netherlands Registry for clear-cell adenocarcinoma
Co-testing	hrHPV testing and cytology taken at the same time point
DES	Diethylstilbestrol
DBH	Dot-blot-hybridization
DNA	Deoxyribonucleic acid
DOR	Diagnostic odds ratio
EIA	Enzyme immuno assay
FDA	Food and Drug Administration
FEM	Fixed effects model
FIGO	International Federation of Gynaecology and Obstetrics
FPR	False positive rate
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HR	Hazard ratio
hrHPV	High-risk type of the human papillomavirus
HSIL	High-grade squamous intraepithelial lesion
IHC	Immuno-histochemistry
ISH	In situ hybridization
LiPA	Line probe assay
LLETZ	Large loop excision of the transformation zone
lrHPV	Low-risk type of the human papillomavirus
LSIL	Low-grade squamous intraepithelial lesion
mRNA	Messenger ribonucleic acid
NTR	Dutch trial register (Nederlands Trial Register)
PALGA	The Netherlands nationwide network and registry of histopathology and cytopathology

PCR	Polymerase chain reaction
QALY	Quality adjusted life year
Rb	Retinoblastoma
RCT	Randomised controlled trial
REM	Random effects model
RLB	Reverse line blotting
RLU	Relative light unit
RNA	Ribonucleïnezuur
SCC	Squamous cell carcinoma
SCJ	Squamo-columnar junction
TPR	True positive rate
TZ	Transformation zone
UK	United Kingdom
USA	United States of America
WHO	World Health Organisation

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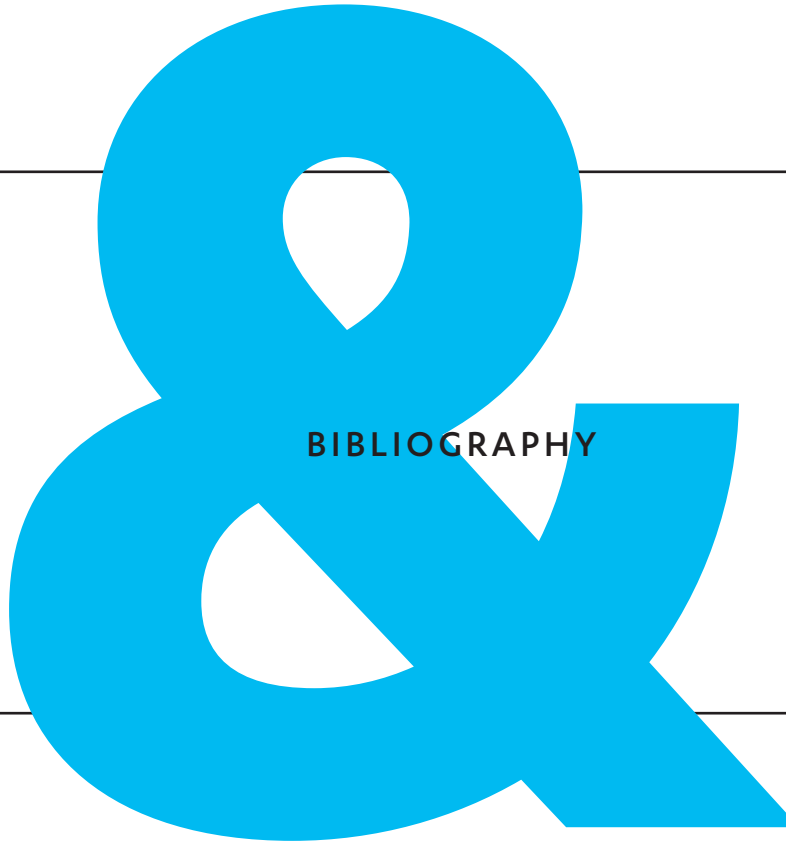

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Courses		
2011	Adobe Photoshop and Illustrator CS5 workshop, *MolMed*	0.3
2010	Short introduction course on Statistics & Survival Analysis for MDs, *MolMed*	0.4
2009	Biomedical English Writing and Communication, *EUR*	4.0
2008	Basic Methods and reasoning in Biostatistics, *Boerhaave Institute, Leiden*	1.2
2008	Introduction to Data-analysis, *NIHES*	0.9
2008	Principles of Research in Medicine and Epidemiology, *NIHES*	0.9
2008	Integrity in Medical Research, *EUR*	2.0
2008	Vulvar pathology, *Stichting OOG*	1.0
2007	Biomedical Research Techniques VII, *MolMed*	1.0
2005	Good Clinical Practice, *Postgrade, Zeist*	1.0
2005	Colposcopy course (basic and advanced), *Stichting OOG*	1.7
National and International presentations		
2010	13th Biennial Meeting of the International Gynaecological Cancer Society, *Prague, Czech Republic* (oral)	2.0
2010	5th European Congress of the European Federation for colposcopy and cervical pathology, *Berlin, Germany* (oral)	2.0
2009	16th International Meeting of the European Society of Gynaecological Oncology, *Belgrade, Serbia* (poster)	1.5
2009	25th International Papillomavirus Conference and Workshops, *Malmö, Sweden* (oral)	2.0
2008	Eurogin 2008, *Nice, France* (oral)	2.0
2008	Patient conference on HPV vaccination, *ErasmusMC* (oral)	0.2
2008	Wetenschapsdag 2008, *Reinier de Graaf Hospital, Delft* (oral)	0.2
2008	Netherlands Society of Medical Microbiology, *Nieuwegein* (oral)	0.2
2007	Comprehensive Cancer Centre Rotterdam, *Zwijndrecht* (oral)	0.2
2007	Workshop at LOVAH-conference, *Utrecht* (oral)	0.5
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2011	Symposium 'Omzien in verwondering; vertrouwen in de toekomst', *ErasmusMC*	0.2

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2006, 2007	Practical course (VO) 'Postmenopausal blood loss', 'Hormonal substitution therapy' and 'Prolepses and urine incontinence', *ErasmusMC*	1.0
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Mariëlle Kocken was born on the 25th of July, 1976 in Rotterdam, the Netherlands. In 1994 she completed her secondary education (athenaeum) at the "Krimpenerwaard College" in Krimpen aan den IJssel. From 1994 to 2001 she studied medicine at the Erasmus University Rotterdam. Her internship at the department of Obstetrics & Gynaecology of the "Lievensberg Hospital" in Bergen op Zoom raised her interest in this specialism. After her registration as Medical Doctor she started working as a resident at the department of Obstetrics & Gynaecology of the "IJsselland Hospital" in Capelle aan den IJssel and later of the Reinier de Graaf Groep in Delft (under supervision of dr. J.C. Kuijpers / dr. W.A. ter Harmsel)

In December 2005 she commenced her PhD project "Risk assessment of cervical disease by hrHPV testing and cytology" in the HumaVac collaboration under supervision of prof. dr. Th.J.M. Helmerhorst and prof. dr. C.J.L.M. Meijer. She presented her work at different international conferences. In 2008 she became a certified diving medical doctor, combining her profession with one of her favourite hobbies

After a sabbatical in which she was traveling the world for four months, she started as a resident at the Pathology department of the VU University Medical Center in Amsterdam (under supervision of prof. dr. P. van der Valk) in November 2011.

Mariëlle lives together with Wilbert van Overloop in Rotterdam.

DANKWOORD

DANKWOORD

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Mariëlle