Clinical value of high-risk HPV detection in the management of cervical intraepithelial neoplasia

Ruud Bekkers

Clinical value of high-risk HPV detection in the management of cervical intraepithelial neoplasia

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CONTENTS

-1-

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

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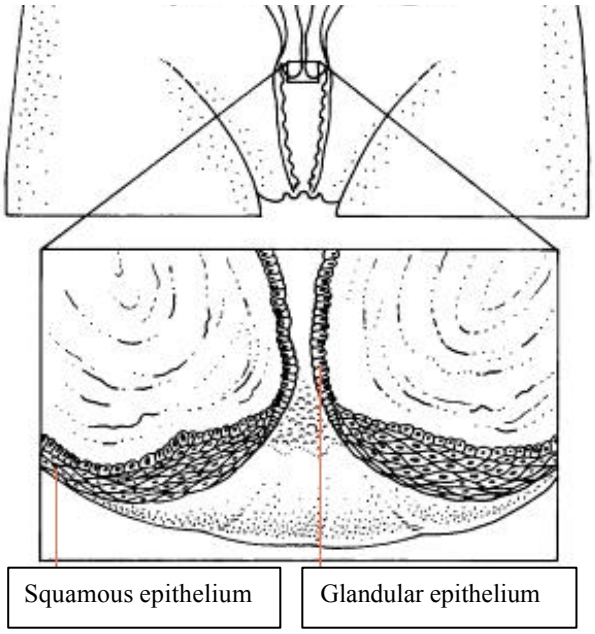
CERVICAL CANCER

The uterine cervix is normally covered with non-keratinizing squamous and glandular columnar epithelium, with the squamous epithelium at the outer surface of the cervix (ecto-cervix), and the glandular epithelium at the inner part of the cervix (endo-cervix) (see Figure 1).

Figure 1. *Epithelial surfaces of the uterine cervix.*



The border between the glandular and squamous epithelium is called the squamo-columnar junction (SCJ). Metaplastic transformation in the SCJ starts from puberty onwards and columnar epithelium is replaced by squamous epithelium, shifting the original SCJ more towards the endo-cervix, forming a new SCJ. The area between the original and new SCJ is called the transformation zone and due to the high cell-turnover, it is assumed to be more susceptible to oncogenic influences [1]. The majority, and maybe even all cervical intraepithelial and invasive lesions develop in this particular zone.

Cancer of the uterine cervix is a major cause of death. It is the second most frequent cancer in women world wide, accounting for 6-10% of all cancer related deaths in women [2]. Each year 470.000 women are diagnosed with invasive cervical cancer world wide, and 230.000 women die of this disease [3]. In the Netherlands, there is an annual incidence of 730 cases, of whom 235 women die each year of invasive cervical cancer. Therefore the mortality rate of cervical cancer is approximately 2.5 per 100.000 women in the Netherlands [4-6].

CIN and exfoliative cytology.

Papanicolaou and Traut were the first to demonstrate that exfoliative cytology could be used to detect invasive and in-situ carcinomas of the uterine cervix [7].

In the late 1960s, Richart was one of the first to assume that cervical cancer develops out of non-invasive pre-malignant stages, which he called cervical intraepithelial neoplasia (CIN) [8]. CIN has been classified as CIN 1, CIN 2, and CIN 3 on the basis of the presence of mitotic activity and nuclear atypia, respectively within the whole thickness of the epithelium [9]. Since that time, different nomenclatures have been used to describe the histological and cytological abnormalities of pre-malignant stages of cervical cancer and these are summarized in Table 1.

Table 1. *Different cytology nomenclature (adapted from DiSaia & Creasman, Clinical Gynecological Oncology 6th ed., Mosby, London, 2002, page 12).*

Dysplasia	CIN	Bethesda	Papanicolaou
Normal	Normal	Within normal limits	Pap 1
Benign atypia	Inflammatory atypia	Benign cellular changes	
Atypical cells	Squamous atypia	ASC-US	Pap 2
Mild Dysplasia	CIN 1	Low-grade SIL	Pap 3A1
Moderate Dysplasia	CIN 2	High-grade SIL	Pap 3A2
Severe Dysplasia	CIN 3		Pap 3B
CIS			Pap 4
AdenoCIS		Glandular abnormality	Pap 4

CIN = Cervical intraepithelial neoplasia, CIS = Carcinoma in situ, Pap = Papanicolaou classification of cervical scrapes, ASC-US = Atypical squamous cells of undetermined significance, SIL = Squamous intraepithelial lesion.

The basis of screening programs for cervical cancer prevention is formed by the assumption that CIN precedes the development of cervical carcinoma via progression through different degrees of CIN, and that cytological abnormalities in cervical scrapes correspond with the different histological degrees of CIN. With cervical scrapes, squamous and glandular cells are ideally scraped from the transformation zone of the uterine cervix.

Detection of abnormal cells in the scrape and subsequent treatment of the underlying pre-malignant CIN prevents progression of lesions towards invasive cervical cancer. The decline in the number of cervical cancer related deaths over the last decades has generally been attributed to the implementation of such screening programs [10-14].

The risk of developing invasive cervical cancer rises with increasing severity of the CIN lesion [15-16]. In review articles, the risk of developing invasive cancer was estimated to be 1% in patients with CIN 1, 5% in patients with CIN 2, and 15% in patients with CIN 3 [15-17]. Most CIN lesions do regress in time and do not need to be treated at all [15-17]. Within 24 months of follow-up, spontaneous regression was observed in 30-62% of the CIN 1 lesions, 17-54% of the CIN 2 lesions, and up to 30% of the CIN 3 lesions. Thus a large part of all women referred and treated for CIN of the uterine cervix, would never have developed cervical cancer [11, 15, 16]. This unnecessary referrals cause unfavorable health effects, as women referred for colposcopy have high anxiety levels [18]. Furthermore, unnecessary treatment reduces the cost effectiveness of cervical cancer screening programs [19]. In order to prevent unnecessary referrals/treatment, new or additional diagnostic methods and/or management strategies are needed, that ideally only identify and treat those patients that have CIN lesions that will progress to invasive cervical cancer.

Cervical screening and treatment of CIN in the Netherlands.

The present cervical cancer-screening program in the Netherlands is carried out by the general practitioners, and consists of 5-yearly conventional cervical scrapes, in women aged 30-60 years, who participate on a voluntary basis. According to the Dutch consensus meeting in 1996 [20], all women with two consecutive atypical squamous cells of undetermined significance (ASC-US) scrapes (with a 6-month interval), or with a single cervical scrape indicating CIN, need to be referred to the gynecologist for colposcopic examination. At colposcopy, biopsies have to be taken from all colposcopically suspect areas of the uterine cervix, and in case of a morphologically confirmed CIN 2 or CIN 3 lesion, treatment should follow in order to prevent the development of cervical cancer [20]. Treatment options for CIN are: Large loop excision of the transformation zone (LLETZ), laser evaporation, cryocoagulation, cone biopsy, or hysterectomy depending on the preference and expertise of the attending gynecologist. In general, LLETZ is preferred because it can be performed as an outpatient procedure, and is followed by a histo-morphological assessment of the tissue removed. In the early 1990s, a see and treat policy has been adopted in many Dutch hospitals, consisting of a colposcopic examination combined with a LLETZ in all

patients with colposcopically suspect lesions [21-23]. Diagnosis and therapy are combined with this policy, and it reduces the number of colposcopic examinations in women, but it has the risk of unnecessary treatment, especially when performed by an inexperienced colposcopist.

With LLETZ a cure rate of > 90 % can been achieved, and close cytological follow-up after 6, 12, and 24 months is advised and necessary to detect any residual or recurrent CIN [20, 21, 23, 24]. However, follow-up cytology has a low specificity in detecting residual or recurrent CIN, and follow-up colposcopy is difficult in a post-treatment cervix [24]. Later it was shown that human papillomavirus (HPV) is cleared from the cervix after treatment of CIN [25-27], and several studies indicated that post-treatment HPV detection may be useful to predict the presence of residual CIN [28-31], but whether HPV detection should be added to follow-up cytology, or may replace follow-up cytology needs further study.

Human papillomavirus.

In the late 1970s, epidemiological studies suggested the role of a sexually transmitted disease in the development of cervical cancer [32-33]. Already in 1973, zur Hausen launched the concept of viral oncogenesis in the development of cervical cancer, and he indicated in 1977 the possible role of HPV infections in the development of squamous cell carcinomas of the uterine cervix [34-35]. After the introduction of modern molecular techniques to detect viral DNA, many studies have indicated a causal relation between HPV infections and cervical cancer [36-43].

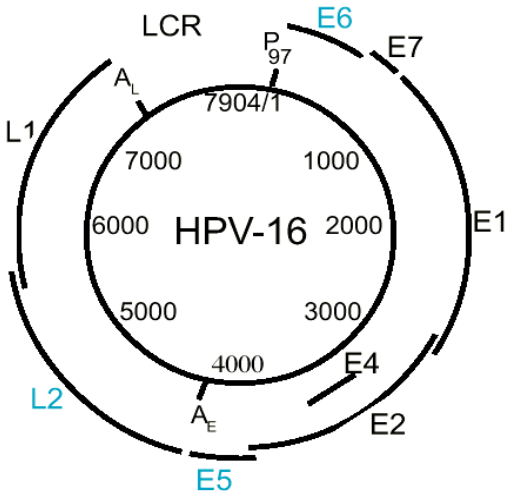
More than 100 different genotypes of HPV have been identified thus far. HPV genotypes can be divided in mucosotropic types, which are mainly found in the mucous epithelium of the oropharynx and anogenital tract, and cutaneous types, which predominantly infect the skin. More than 35 genotypes have been shown to infect mucosal surfaces, and 18 of them have been associated with cervical cancer. Infections with HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, or 82 have been frequently detected in the cervix of patients with cervical cancer, and these genotypes are considered high-risk [40, 44-46]. Infections with HPV 16, 18 and 31 are detected in almost 80% of all squamous cervical carcinomas [40, 44].

Recently it has been estimated that in the developed world about 2.5% of all cancers is associated with HPV, while in the developing world HPV is related to about 7.8% of all cancers. HPV has been detected in almost 100% of cervical cancers, in 80-90% of squamous cell carcinomas of the skin, in 70 % of anal carcinomas, in 50% of basal cell

carcinomas of the skin, in 25% of oropharynx carcinomas, and in 10-35% of oesophagus carcinomas [40, 42, 47-52].

Papillomaviruses belong to the group of Papoviridae, consisting of a double stranded circular DNA genome of approximately 8000 basepairs, enveloped by an icosahedral protein capsule of 45-55 nm in diameter [53] (see Figure 2).
The HPV genome can be divided into an 'early region', encoding proteins that are supposed to be expressed before the onset of viral DNA replication, a 'late region', encoding the viral capsid proteins, and a non-coding long control region [54]. The early and late regions have several open reading frames (ORF) resulting in translation of functional proteins.

Figure 2. *Schematic structure of HPV.*



The early region ORF's (E1, E2, E4-7) are expressed early in the viral life cycle. E1 encodes a protein that maintains the viral genome. E2 is involved in transcriptional regulation and control of the E6 and E7 genes. The E4 gene encodes a protein that finally breaks off the cytoplasmic keratin networks, resulting in koilocytotic cells in the upper layers of the epithelium. The E5 gene encodes a protein that is believed to boost the mitogenic responses of the epithelial host cells to stimulate replication of the virus. E6 and E7 encode multifunctional proteins that control proliferation and transformation. The late region ORF's (L1 and L2) encode the major and minor capsid proteins of the virus, respectively, which form the virus particle. The expression of L1 and L2 is tightly bound with the host cell differentiation into mature keratinocytes in the upper layers of the epithelium [55].

The concept of HPV mediated carcinogenesis.

In vitro studies have shown that infection with HPV is an early event in the multi-step process of cervical carcinogenesis. In benign and low-grade cervical lesions, the HPV genome is maintained in a episomal state, i.e. free in the nucleus. With the progression of CIN, HPV is more often found integrated in the genome of the host cell. The integration process generally disrupts the E2 region and function, which will enhance the expression of E6 and E7, because the E2 suppression is diminished [56].

The E6 and E7 oncoproteins interfere with two pathways of the cell cycle regulation. HPV E6 is able to interact with p53. The function of p53 includes a G1-phase arrest in order to allow DNA repair, and activation of apoptosis to eliminate cells with damaged DNA. Interaction of E6 with p53 leads to p53 dysfunction, thus impairing the ability to block the cell cycle upon DNA synthesis errors [57]. In addition and independent from the p53 effect, E6 also induces telomerase activity preventing telomere shortening of the chromosomes. Thus E6 will keep the telomere length above its critical point and prevents cells in another way from the process of apoptosis [58].

HPV E7 is able to effectively bind and inactivate pRb resulting in the release of the host transcriptional factor E2F from the pRb/E2F-complex. E2F activates many genes that are important for mitosis, will start the cell cycle, and cause hyperproliferation.

The combination of the absence of cell cycle arrest, the absence of apoptosis, and maintenance of telomere length caused by E6, and hyperproliferation caused by E7, is considered a prerequisite for cells to become immortalized and obtain unrestrained growth, which may ultimately result in a malignancy [59-61].

HPV epidemiology.

Parallel with a growing understanding of HPV mediated carcinogenesis in cervical cancer, thousands of studies were providing more insight in the epidemiology of HPV.

Genital HPV infections are sexually transmitted and HPV can normally not be detected in the cervix of virgins [62]. If women are closely followed with HPV detection and cervical scrapes after the onset of sexual intercourse, about 50% will be infected with genital HPV within 2 years of the onset of sexual intercourse [62-64]. It is estimated that the lifetime risk of contracting a genital HPV infection is 80%, leading to genital warts in 5%, abnormal cervical scrapes in 35%, CIN in 25 %, and invasive carcinoma in less than 1% [62, 63, 65]. Most HPV infections and low-grade CIN lesions will disappear within 8 months after the first exposition [62, 66-69].

In women with normal cervical scrapes, the point prevalence of any HPV type is age-dependent, varying from 20% in women aged between 20-25 years, to 6% in women older than 30 years [70]. Studies have shown a second peak in HPV point prevalence in women above the age of 55 [71-74]. Other studies have shown a cumulative prevalence of high-risk HPV (hr-HPV) infections of up to 70% with sequential cervical scrapes, even within one menstrual cycle [69, 75-77].

In CIN lesions both low- and high-risk mucosal HPV genotypes have been detected. Low risk HPV genotypes, like HPV 6, 11, 40, 42, 43, and 44, are predominantly present in condylomata accuminata, ASC-US, and CIN 1. Hr-HPV genotypes are often detected in CIN 2 and 3, and in invasive cancer [40, 43].

The prevalence of hr-HPV genotypes rises with increasing severity of the CIN lesion but depends also on the detection method [78]. HPV tests using polymerase chain reactions (PCR) have proven to be more sensitive in detecting hr-HPV genotypes than in situ hybridization tests, like the Hybrid Capture II®. With sensitive general PCR primers as My 11/09 and GP5+/6+ up to 99% of cervical cancers were hr-HPV positive [42]. To detect the exact HPV genotype, type specific primers are available, but type specific detection requires an elaborate laboratory procedure, especially when multiple genotypes are present. With the introduction of a short fragment polymerase chain reaction in combination with reverse hybridization in a line probe assay (SPF_{10} LiPA PCR), it became possible to detect and subsequently simultaneously type 25 different high- and low-risk HPV genotypes in a two step process [36, 79, 80]. This HPV detection method has been clinically validated and proved to be highly sensitive, specific and reproducible [36, 79-82]. With this assay, hr-HPV was shown to be present in 51% of patients with ASC-US scrapes, 78% of patients with CIN 1, 86% of patients with CIN 2, and 88% of patients with CIN 3 [36].

Detection of hr-HPV genotypes in normal cervical scrapes has been associated with an increased risk of developing CIN [38]. However, especially young women often have transient infections, and more than 80% of all HPV infections will be cleared [66-69, 83]. Several reports have shown that women with hr-HPV positive CIN lesions were more likely to progress than women with hr-HPV negative CIN lesions, and therefore, progression of CIN lesions is assumed to be associated with a persistent hr-HPV infection [84, 85]. Later it was shown that patients with persistent hr-HPV infections have a 100-300 fold increased risk for the development of a CIN 3 lesion [86-90], while clearance of HPV from the uterine cervix was associated with regression of the CIN lesion [91].

Many studies have investigated the addition of HPV detection in cervical scrapes to conventional cytology in cervical cancer screening, in order to improve its sensitivity and specificity in detecting high-grade CIN. A consensus meeting in the United States concluded that only in women with ASC-US scrapes, additional HPV detection increases the sensitivity of the screening [41]. In women with cervical scrapes indicating CIN, the high prevalence of HPV (> 80%) causes a low specificity of HPV detection in the triage of women with abnormal cervical scrapes [41, 81, 92]. Presently, large studies are undertaken to investigate whether the addition of HPV detection to conventional cytology in cervical cancer screening programs may improve the efficacy of screening, and whether screening intervals may become longer with this strategy [93]. As it is still uncertain whether the addition of hr-HPV detection to cervical cancer screening is beneficial, other markers, that specifically detect hyperproliferation, or genetic instability of CIN lesions, must be investigated as well.

Hyperproliferation.

Histomorphological criteria like nuclear atypia, absence of maturation, and frequency and localization of mitosis are used in classifying CIN. In normal cervical epithelium, mitoses are only detected in the basal and parabasal layers of the epithelium, whereas there is an increase in mitotic activity in higher epithelial cell layers in more severe CIN lesions [94]. It is likely that this abnormal mitotic activity is the result of the 'HPV induced' proliferative activity. In the 1990s, several antibodies have been developed that can objectively classify CIN by detecting the proliferative activity. The most promising antibody, MIB1, recognizes an epitope on the Ki-67 antigen. Ki-67 antigen is a non-histone protein that is expressed during the G_1, S, and G_2M phases of the cell cycle, but not in the G_0 phase [95-97]. MIB1 reactivity in histopathological sections of the cervix showed a highly significant relation with the degree of CIN, even when different scoring methods were used [98-100]. It has been suggested in the literature that the MIB1 labeling index (Ki-67 index or percentage of MIB1 positive nuclei) may provide information about what effect HPV is having on particular cells, as it is an excellent measure of a neoplasm's proliferation [101, 102]. MIB1 has been suggested as new marker for the detection of high-grade CIN [103], but its value on cervical scrapes has only been investigated in a few studies [104-106]. Despite the high sensitivity of MIB1 reactivity in cervical scrapes for the detection of high-grade CIN in that study, more studies are needed that can confirm these data.

OUTLINE OF THIS THESIS.

From 1997 to 2001 a prospective non-intervention cohort study was conducted in women referred to the colposcopy clinic of the University Medical Center (UMC) St Radboud Nijmegen, the Netherlands, with an abnormal cervical scrape in the cervical cancer screening program. The study was started as a collaboration of the departments of Obstetrics- Gynecology, Pathology and Medical Microbiology (Virology) of the UMC and financially supported by the Dutch Cancer Society (Koningin Wilhelmina Fonds). The aim of the study was to search for new markers in cervical scrapes that can, either identify all women with high-grade lesions at time of referral, or can predict which CIN lesions will progress towards CIN 3 and/or invasive cervical cancer. Over 800 women were included in the study. They were referred with two consecutive ASC-US scrapes (Pap 2), or a single scrape indicating CIN (Pap $3A_1$ to Pap 4). A liquid based cervical scrape was taken of all women at study entrance, using a Cervex brush® (Rovers Medical Devices b.v., Oss, the Netherlands). These scrapes were subsequently processed into AgarCyto cell blocks, allowing for multiple analysis, as previously described [107]. All women underwent colposcopy within one month of the intake cervical scrape. At colposcopy, all lesions suspect for CIN 3 or a more severe lesion were treated with LLETZ on the basis of a see and treat policy. Women with CIN 1 or 2 lesions or no colposcopic lesions at all were followed under a close surveillance, consisting of follow-up cervical scrapes every 3-6 months, and follow-up colposcopy after 9 and 18 months. Follow-up was ended when a lesion suspect for CIN 3 was detected by follow-up conventional cytology and/or colposcopy, when a lesion persisted for more than 18 months, when women requested to be treated, or requested to exit the follow-up study, or when at least two consecutive normal conventional cervical scrapes in combination with a normal colposcopy were found.

Sections of all Agarcyto scrapes were tested for the presence of high-risk HPV, using the highly sensitive SPF_{10} LiPA PCR assay. Adjacent sections were stained (HE) for conventional assessment, Mib1 for the detection of hyperproliferation, and other markers.

Data of this non-intervention study were used in the chapters 3, 7, and 8 of this thesis.

Objectives of this thesis.

1. To investigate the anxiety level and factors influencing this anxiety in women referred with abnormal cervical scrapes for colposcopy**,** in order to define strategies to reduce this anxiety. **(Chapter 2).**
2. To investigate strategies in the management of patients referred with abnormal cervical scrapes in order to prevent unnecessary referral **(Chapters 3 and 7)** or prevent unnecessary treatment after referral for colposcopy **(Chapter 3).**
3. To investigate the role of hr-HPV detection in the follow-up of patients treated for CIN **(Chapter 8).**
4. To investigate the frequency and distribution of hr-HPV genotypes in patients with CIN, glandular lesions and/or coexisting squamous and glandular lesions in the cervix **(Chapters 4 and 5).**
5. To investigate the changes in hormonal sensitivity in relation to hyperproliferation under the influence of hr-HPV infections **(Chapter 6).**

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VARIABLES INFLUENCING ANXIETY OF PATIENTS WITH ABNORMAL CERVICAL SMEARS REFERRED FOR COLPOSCOPY

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ABSTRACT.

Objectives: The state anxiety scores of women with abnormal cervical smears referred for colposcopy were investigated to identify causes of anxiety, factors influencing anxiety, and emotions involved with anxiety, in order to define strategies to reduce this anxiety.

Methods: Forty-seven women were interviewed and completed a questionnaire and the Spielberger State and Trait Anxiety Inventory (STAI): 22 women prior to their intake consultation at the colposcopy clinic of the University Medical Center St Radboud Nijmegen, and 25 women during their second visit before the actual colposcopy.

Results: The mean State anxiety score was 48.2, without significant differences between the intake and colposcopy consultation. The majority experienced anxiety because of a fear of cancer and/or the colposcopy. The mean State anxiety score was significantly higher in women who considered the level of information provided by the gynecologist/family practitioner insufficient, who experienced a long waiting time, who did not have a partner, and who experienced additional emotions like anger and sadness.

Conclusions: Patients referred for colposcopy after an abnormal cervical smear result, have high levels of anxiety. High levels of anxiety may be reduced by uniform and explicit information about cervical smear results and colposcopy, by reduction of clinic waiting times, by stimulating social support, and by attention to emotions like anger and sadness.

INTRODUCTION.

Screening programs for cervical cancer cause anxiety among the screened population, especially in those 5-10% of patients who have an abnormal cervical smear result [1-5]. Subsequent referral of these patients to colposcopy clinics may further increase anxiety to levels higher than patients undergoing elective surgery or patients undergoing repeat cervical smears [1-6]. Education, provision of information, and a better colposcopy clinic organization have been shown to reduce patient anxiety [5,7,8]. However, more detailed information on factors influencing anxiety may lead to more sophisticated strategies to decrease anxiety levels. Specifically, it remains unclear whether the anxiety of patients referred with abnormal cervical smears is caused by fear of cancer, fear of pain/discomfort of the procedure, fear of losing fertility/sexuality, or a combination of these factors. Furthermore, it is unclear if other emotions, such as sadness and anger, may also play a role. Lastly, more information is needed on variables influencing the level of anxiety.

In the present study, the level of anxiety of patients referred with abnormal cervical smears was established. In order to define at which point in the referral process adjustments are needed, anxiety levels were measured both prior to the first consultation and in anticipation of the colposcopy. In addition, factors that may influence anxiety were explored. To investigate women's subjective emotions during the process, semi-structured interviews were conducted.

METHODS.

In the period May-July 2000, 52 women, referred to the colposcopy clinic of the University Medical Center Nijmegen (UMC) with an abnormal cervical smear result, were asked to participate in the study. Five women refused to participate due to subjectively reported excessive anxiety. Results were available from 47 women. Twenty-five women were investigated prior to the intake consultation at the colposcopy clinic and 22 women prior to the actual colposcopy during their second visit to the clinic. All women with an abnormal cervical smear were first time referrals from the cervical cancer screening program to the colposcopy clinic.

All women were investigated 20-30 minutes prior to the consultation/colposcopy by one of two authors (MvdD and FMK). The investigation consisted of an interview followed by completion of the Dutch version of the State and Trait Anxiety inventory (STAI) and a short questionnaire.

The semi-structured interview consisted of open questions relating to emotions like anxiety, anger, or sadness experienced by the women in the 2-3 weeks prior to the consultation. All interviews were recorded on audiotape and analyzed together with a third author (AvM) regarding these emotions.

Table 1.

Mean State anxiety levels measured with the Spielberger State and Trait anxiety inventory in different populations.

Population	Mean state anxiety (Sd)
Normal Dutch adult women [9]	38.8 (13.2)
Female high school students (England) [5]	39.7 (n.a.)
Before surgery [10]	41.2 (n.a.)
After surgery [10]	31.9 (n.a.)
Pregnant with elevated alpha-FP [5]	47.7 (12.8)
See and treat colposcopy clinic (England) [5]	56.6 (9.9)
Follow-up clinic attendance (England) [5]	50.2 (11.8)
Dutch women undergoing IVF treatment [11]	44.1 (11.7)
First visit colposcopy clinic UMC St Radboud	**50.3 (14.1)**
Second visit before colposcopy UMC St Radboud	**45.9 (17.7)**

n.a. = not available, alpha-FP = alpha-foeto protein, IVF = in vitro fertilization

The STAI was used to establish the level of anxiety [9]. Trait anxiety refers to a general tendency of an individual to be anxious, whereas State anxiety refers to the anxiety level of an individual at a given moment. Both measures include 20 items, the score for each item ranging from 1-4 (total scores range 20-80), higher scores indicating more anxiety.

The short questionnaire consisted of questions regarding their personal situation. The questionnaire recorded age, educational level, cervical smear result, cancer-related family medical history, subjective length of the waiting time, having a partner and children, and the (subjective) level of satisfaction with the information provided. Information was provided at the colposcopy clinic verbally and by leaflets. No data on the method and level of information provision by the primary healthcare workers was available. In order to investigate the present situation the information provision was left unchanged.

Mean State anxiety levels were compared regarding all variables of the questionnaire, as well as the emotions and factors scored in the interview. Independent student's *t*-tests were used to analyze the differences in mean anxiety scores; *p* values of $\leq$ 0.05 were considered significant. The Medical-Ethical Board of the UMC St Radboud Nijmegen approved the study.

RESULTS.

A comparison of mean State anxiety levels measured in the general population and different groups of women undergoing cervical smears has shown that women visiting or being treated at a colposcopy clinic generally experienced higher anxiety levels. The mean State anxiety score of the 47 women was 48.2 (SD 13.4), which is significantly higher than the average of 38.8 (SD 13.2) in normal Dutch adult women ($t(92) = 3.42$, $p<0.001$) (Table 1). The mean Trait anxiety score was 37.4 (SD 8.7), which is comparable with 39.4 (SD 11.2) of normal adult Dutch women ($t(92) = 0.95$, $p > 0.02$) (Table 2) [9]. There were no significant differences between the (State and Trait) anxiety scores of the women prior to the consultation and prior to the colposcopy (see Table 2).

As no significant differences in mean State anxiety scores were observed between these two groups, further analysis includes all 47 women.

Table 2.

Mean State and Trait anxiety scores (SD) of women prior to their intake consultation and prior to the colposcopy.

	All Patients	**Prior to first consultation**	**Prior to Colposcopy**	**Significance t=**
	n = 47	**n = 25**	**n = 22**	
Mean state anxiety	48.2 (13.4)	50.3 (14.1)	45.9 (17.7)	0.93*
Mean trait anxiety	37.4 (8.7)	37.9 (7.7)	37.0 (9.8)	0.35*

** = p > 0.02*

Table 3.

Mean State anxiety scores compared with different factors investigated in the questionnaire (n=47).

Variable	Yes (Sd)	No (Sd)	Significance t
Age < 40 years (n=23)	49.7 (10.2)	46.7 (15.3)	0.87
Education less than college (n=29)	48.8 (11.3)	48.2 (15.7)	0.14
Cx smear mild dyskaryosis or less (n=34)	47.4 (12.9)	48.9 (14.0)	0.15
Cancer among relatives (n=14)	51.4 (12.0)	47.1 (13.5)	1.08
Experienced waiting time as short (n=36)	45.5 (12.9)	57.1 (9.7)	3.23**
Patient has a partner (n=40)	45.3 (11.2)	65.0 (11.0)	4.44***
Patient has children (n=31)	49.9 (14.1)	45.1 (10.5)	1.46
Satisfied with info from GP (n=10 of 25)	42.7 (12.2)	55.2 (12.6)	2.46*
Satisfied with info of gynecologist (n=17 of 22)	38.9 (12.7)	50.3 (7.0)	2.36*
Colposcopy was clearly explained (n=10 of 22)	38.6 (10.0)	53.2 (9.8)	2.43*

*GP = general practitioner * = p < 0.05, ** = p < 0.01, *** = p < 0.001*

The mean State anxiety score was significantly higher for women without a partner, women who subjectively experienced a long waiting time, women who considered the information provided by the family practitioner or gynecologist as insufficient, and women to whom colposcopy was not explained sufficiently (see Table 3). No significant differences in the mean State anxiety score were found regarding age, educational level, cervical smear result, cancer related family history, or women without children (see Table 3).

The semi-structured interviews indicated that 41 women (87%) experienced anxiety. Seven women experienced anxiety at the moment they were notified of the abnormal smear result, two women after being asked to participate in the study, and 32 women already experienced anxiety before the result of the cervical smears was known. 33 women (69%) reported a fear of cancer, 18 women (38%) a fear of the colposcopy, and three women (6%) a fear to lose fertility/sexuality. 13 of these women (28%) reported a fear of both cancer and the colposcopy.

Anger was reported as a strong emotion by 9 women (19%). In most of these women (n=7), their anger was directed at a particular person (general practitioner, assistant).

Sadness was reported as a strong emotion by 13 women (28%). The sadness was reported to be related to the possibility of losing their uterus and feeling incomplete thereafter (n=2), the confrontation with death (n=1), uncertainty about the procedure, which was going to follow (n=1), the loss of sexual libido (n=1), and the postponement of the pregnancy wish (n=1). The remaining seven women could not specifically describe why they were feeling sad.

Table 4.

Emotions in the interview in relation with Mean State Anxiety scores (n=47)

	Present		Not present		Significance
Emotion	**Mean STAI (SD)**	**n=**	**Mean STAI (SD)**	**n=**	**t**
Anxiety	49.5 (13.5)	41	39.5 (9.1)	6	2.34*
Anger	58.8 (13.9)	9	45.8 (12.2)	38	2.58**
Sadness	55.6 (12.4)	13	45.4 (12.9)	34	2.49**
All three emotions	68.5 (11.4)	4	47.0 (11.7)	43	3.58***

*$p < 0.05$, ** $p < 0.02$, *** $p < 0.001$*

Patients reporting subjective feelings of anxiety showed significantly higher State anxiety scores than patients without those feelings. Patients who reported anger or sadness also showed significantly higher State anxiety scores than patients without those feelings. These differences in State anxiety were most pronounced in patients who reported feelings of anxiety, anger, and sadness all together (see Table 4).

DISCUSSION.

This study confirms that women with an abnormal cervical smear referred for colposcopy have high levels of State anxiety. Other patient and population groups, such as patients who had to undergo surgery the next day, patients awaiting *in vitro* fertilization, or healthy volunteers [9,11,12] had significantly lower anxiety levels. In contrast with State anxiety, Trait anxiety scores fell within the range of normal women, indicating that the high levels of anxiety experienced by these women is not due to personality traits, but probably evoked by this specific situation, as was found by others [1, 3, 5, 8]. No significant difference in mean State anxiety was found between women visiting the clinic for the intake consultation and women visiting the clinic for the actual colposcopy. This indicates that the information, presently provided by the gynecologist, is insufficient to reduce State anxiety levels.

Patients with a partner had significantly lower State anxiety scores than patients without a partner, implying that the support of a partner is a protective factor in the emotional reaction to a stressful medical situation like an abnormal cervical smear result or a colposcopy. The role of social support (in general) in relation to anxiety during the period of referral for colposcopy needs further study.

The lack of significant differences in mean State anxiety regarding educational level, the severity of the cervical smear abnormality, or a family history of cancer may confirm women's limited understanding of the relative significance of abnormal cervical smear results. As described by others, they may often incorrectly assume that an abnormal smear result automatically indicates having cancer [13].

Patients who were not satisfied with the information provided by the general practitioner or gynecologist showed significantly higher mean State anxiety scores than satisfied patients. In the literature, conflicting data regarding information provision and anxiety have been reported. In line with our findings, some authors reported that a lack of information was related to higher anxiety scores [2, 5-7, 14, 15]. Other authors, however, did not find a reduction in the level of anxiety after provision of adequate information about abnormal cervical smears and colposcopy [4, 8, 16, 17]. This may be caused by the fact that a woman's high anxiety may obliterate understanding a comprehensive message from the healthcare worker and it may explain why some authors find the timing and method of information provision to be crucial. Neither verbal information nor leaflets reduced anxiety, but information using a videotape or illustrated material did [5, 14]. This study indicates that the information provision in the referral process needs to be standardized. Its method and timing need to be critically evaluated in order to be able to significantly reduce State anxiety

levels of women with abnormal cervical smears. In addition, future research should carefully check how well the provided standardized information is absorbed by the women involved.

Women, who experienced a long delay, before the appointment at the colposcopy clinic could be made, showed significantly higher mean State anxiety scores. The actual delay was not recorded, so the measured higher State anxiety levels may be the result of subjectivity. Women with high anxiety levels may experience the delay as long while women with low anxiety levels may not do so. Whether the actual delay is a cause for more anxiety needs further study.

The majority of women experienced anxiety as a strong emotion. This anxiety was mostly reported to be caused by a fear of cancer and/or a fear of the procedure, which was to follow. The fear of losing fertility or sexuality was present in only a few patients and does not seem to influence anxiety much. A majority of the patients already reported having feelings of anxiety before the cervical smear result was known. Screening for cervical cancer itself probably causes anxiety in these patients, as has been reported before [2,4,13,14]. Other emotions like anger and sadness were present in 19% and 28% respectively of the women. These women had significantly higher State anxiety scores, especially when anxiety, anger and sadness were all present together. Emotions like anger and sadness may submerge due to high levels of anxiety. Anxiety may be a more acceptable emotion for people (including health professionals) surrounding women with an abnormal cervical smear. This facilitates expression of this emotion, especially when a partner is present, and may lead to lower levels of State anxiety. Anger and sadness, on the other hand, may be less acceptable emotions, which are thus neither expressed nor addressed by partners and/or health professionals. These emotions are then not adequately dealt with, leading to higher State anxiety scores. More attention to emotions such as anger and sadness during consultation and in the information provided may be another possibility to reduce state anxiety.

In total, five women refused to participate in this study due to reported high anxiety. This may have caused a bias towards lower anxiety levels in the present study and may explain why, in a previous study, higher State anxiety scores in a colposcopy clinic were found [5]. Two other patients reported that they became anxious after they had been approached to participate in this study. This implicates that studies aimed at anxiety in this setting may in themselves cause anxiety, a factor which future research should take into account.

In conclusion, the present study confirms that patients referred with abnormal cervical smears for colposcopy show relatively high levels of State anxiety, caused by a fear of cancer and/or a fear of the procedure that has to follow. Especially patients without a partner,

patients who were dissatisfied with the provided information and patients who experienced a long waiting time showed elevated anxiety levels, as did patients who reported, in addition to fear, feelings of anger or sadness. In order to reduce anxiety**,** the provision of information by general practitioners and gynecologists needs to be improved, waiting times at the colposcopy clinic need to be kept to a minimum, patients must be advised to bring along a partner or close friend, and emotions such as anger and sadness need to be addressed. Patients without a partner or patients who experience anger and/or sadness need extra attention from the attending health professional regarding their anxiety. Further study is needed to define the ideal method and timing of information provision, to investigate the role of social support in reducing anxiety, and to investigate the role of anger and sadness in response to an abnormal cervical smear result.

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-3-

MANAGEMENT OF PATIENTS WITH TWO CONSECUTIVE ASC-US SMEARS

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ABSTRACT.

Objectives: To determine whether an aggressive or expectative management of patients after two consecutive atypical squamous cells of undetermined significance (ASC-US) smears is preferable. To determine whether triage with high-risk human papillomavirus (hr-HPV) detection will identify all patients with CIN 2 and 3.

Methods: 140 of 282 patients, referred for colposcopy with two consecutive ASC-US smears were only treated when abnormalities suggestive for CIN 3 were present at colposcopy. The other 142 patients underwent excision of all detected colposcopic abnormalities. Both groups were compared regarding the final cytological follow-up, the number of diathermy loop excisions, and the detection of CIN. Retrospectively, the outcome of triage with hr-HPV on the first group was investigated.

Results: There was no significant difference in final cytological follow-up between patients managed by an expectative or by an aggressive management at colposcopy. Significantly less diathermy loop excisions ($p<0.001$) are performed in case of an expectative management. The sensitivity, specificity, negative-, and positive predictive values of triage with hr-HPV detection were comparable with those of colposcopy alone.

Conclusions: Patients referred with two consecutive ASC-US smears may be followed with an expectative management at colposcopy and cytological follow-up. Triage with hr-HPV will reduce the number of referrals and colposcopies, but (cytological) follow-up remains necessary in all hr-HPV negative patients as well.

INTRODUCTION.

The management of patients with cervical smears showing minimally abnormal cells (atypical squamous cells of undetermined significance (ASC-US)) remains a clinical problem [1]. 2-10% of all smears made in the context of screening programs for cervical cancer are graded as minimally abnormal or ASC-US [1,2]. However, more than 70% of these patients do not have (pre)-malignant lesions of the uterine cervix and only a minority (6-12%) will develop cervical intraepithelial neoplasia (CIN) during follow-up [1,3-9]. On the other hand, almost 50% of all patients eventually diagnosed with CIN 2 and 3 initially had an ASC-US smear [10].

In 1992, about 12% of all smears made in the screening program in the Netherlands were graded as ASC-US [11]. After revision of the screening program in 1996, by applying more precise and restrictive diagnostic criteria for ASC-US smears, less than 5% of all cervical smears are graded as ASC-US and a repeat smear after six months is advised [12]. Since 1997, the revised Dutch guidelines advise referral of patients after two consecutive ASC-US smears to the gynecologist for colposcopy. Colposcopy has a high positive predictive value (78%) in detecting CIN lesions, but its specificity is low, causing unnecessary biopsies/excisions [9,10,13,14]. In order to avoid unnecessary excisions, either a more expectative management, or triage prior to referral are considered. Triage with high-risk human papillomavirus (hr-HPV) detection in cervical smears, in which only hr-HPV positive patients are referred for colposcopy, has been recommended by several authors in order to reduce unnecessary treatment of patients with ASC-US smears [5,9,10].

In order to investigate the effects of an aggressive or expectative management on patient outcome, the long-term follow-up of patients referred after two consecutive ASC-US smears treated in two different clinics was compared. Furthermore, the sensitivity, specificity, negative-, and positive predictive values for the detection of patients with CIN 2 or 3 by triage with hr-HPV detection was compared with colposcopy in the group with an expectative management.

MATERIAL AND METHODS.

Patients.

In retrospect, all 295 patients were included who had two consecutive ASC-US smears in the screening program, and who were referred between April 1997 and December 1999 to one of the two hospitals in Nijmegen, the Netherlands. Of the 148 patients referred to the University Medical Center Nijmegen (UMC) and of the 147 patients referred to the Canisius Wilhelmina Hospital Nijmegen (CWZ), respectively eight and five patients were excluded because of cervical polyps. The remaining 140 patients of the UMC and 142 patients of the CWZ were analyzed. Referral of the patients to either the UMC or the CWZ was decided by the referring general practitioner and was not influenced by this study.

Colposcopy.

All 282 patients underwent colposcopy within one month of the intake consultation in either one of the hospitals. If a patient was referred to the UMC only lesions suspected for CIN 3 at colposcopy were treated with diathermy loop excision on the basis of a see and treat policy [15]. If a patient was referred to the CWZ the standard policy, a diathermy loop excision, was performed unless no abnormalities were detected during adequate colposcopy. All patients of both groups had follow-up smears 6 and 12 months after the initial colposcopy, and in case of persistent abnormalities, every 6 or 12 months thereafter. Repeat colposcopy was performed at the UMC when ASC-US smears persisted for more than 12 months, or sooner when follow-up smears indicated CIN. All lesions suspected for CIN were removed with diathermy loop excision during repeat colposcopy at the UMC. Repeat colposcopy in the CWZ was combined with a diathermy loop excision when follow-up smears indicated CIN. The mean duration of long-term cytological follow-up was 41 months (24-54 months) in the UMC and 39 months (24-52 months) in the CWZ.

The management strategies of the two hospitals were compared regarding the number of persistent abnormal cervical smears, the number of diathermy loop excisions, and the occurrence of the different grades of CIN. All patients with two consecutive normal smears, or with one normal follow-up smear and a normal adequate colposcopy, were considered to have regressed to normal and/or to have been treated sufficiently. The diagnosis of CIN was made on histopathological examination of the excised material by an experienced gyneco-pathologist.

Hr-HPV detection.

A liquid based cervical smear was taken from all 140 patients at the UMC during the intake consultation with a cervex brush® (Rovers, Oss, the Netherlands). The cell suspension was processed into an AgarCyto cellblock allowing for multiple analyses as previously described [16]. For HPV detection a highly sensitive short fragment polymerase chain reaction (SPF_{10} PCR) assay was performed on a section of the AgarCyto cellblock. In case of a positive SPF_{10} PCR, reverse hybridization by a line probe assay (LiPA) was performed for simultaneous genotyping of 25 HPV genotypes, including hr-HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. This HPV detection assay was validated before and found to be ultra-sensitive [17-22].

Triage with hr-HPV detection was retrospectively performed on all 140 patients and the sensitivity, specificity, NPV and PPV for the detection of CIN 2 or 3 by respectively, hr-HPV testing and colposcopy was determined. Statistical analysis was performed using Chi-square tests, independent samples (student-t) tests, and calculation of 95% confidence intervals (95% CI). All test results with a probability (p) of < 0.05 were considered significant.

RESULTS.

Colposcopy.

A diathermy loop excision was performed during the initial colposcopy in two patients (1%) at the UMC, and in 119 patients (84%) at the CWZ. Histopathological examination revealed one CIN 3 lesion and one CIN 2 lesion in the UMC patients, and 15 CIN 1 lesions, nine CIN 2 lesions, five CIN 3 lesions, and 90 patients without CIN in the CWZ patients.

Two UMC patients underwent a repeat colposcopy with diathermy loop excision within 12 months of follow-up, because of a follow-up smear and repeat colposcopy indicating CIN. In another nine UMC patients a diathermy loop excision was performed after more than 12 months of follow-up; in seven patients with a smear indicating CIN, and in two patients with persistent ASC-US smears and a lesion suspect for CIN at colposcopy. Histopathological examination of these secondary excisions showed CIN 1 in two patients, CIN 2 in five patients, and no CIN in four patients.

A repeat colposcopy with repeat diathermy loop excision was performed within 12 months of follow-up in five patients of the 119 CWZ patients who had a loop excision before; in four patients with a smear indicating CIN, and in one patient with persistent ASC-US smears at the patients' request. Histopathological examination showed residual CIN 1 in three patients

and no CIN in two patients. The difference in the total number of diathermy loop excisions of respectively 13 in the UMC and 124 in the CWZ was highly significant ($\chi^2 > 100$, $p < 0.001$). CWZ patients had significantly more often ($\chi^2 = 5.80$, $p<0.02$) a normal first follow-up smear than UMC patients (respectively 92 and 70 patients). There were no significant differences in follow-up between UMC and CWZ patients after a mean follow-up duration of respectively 41 and 39 months (see table 1).

Table 1.

Final follow-up after initial and secondary diathermy loop excisions of UMC and CWZ patients (with 95% CI).

Follow-up	**UMC**			**CWZ**		
	n=	**%**	**95% CI**	**n=**	**%**	**95% CI**
No follow-up *	**0**	(0)	0-1%	**2**	(1)	0-6%
Two normal follow-up smears *	**93**	(66)	58-74%	**95**	(68)	60-76%
Norm. colposcopy & 1 norm. FU smear *	**31**	(22)	15-29%	**33**	(23)	16-30%
Persistent ASC-US smears *	**16**	(11)	6-16%	**12**	(8)	4-12%
Total number of patients	140	(100)		142	(100)	

** = no significant difference between UMC and CWZ.*

Hr-HPV triage (UMC).

Hr-HPV genotypes were detected in the smears of 48 of the 140 UMC patients (34%). One CIN 3 lesion and three CIN 2 lesions were detected among the hr-HPV positive patients, and two CIN 1 and three CIN 2 lesions were detected among the hr-HPV negative patients. The sensitivity, specificity, NPV, and PPV for the detection of CIN 2 or 3 by hr-HPV testing and colposcopy are presented in table 2. None of these tests reached 100% sensitivity, and all tests had a NPV above 95%, but the overlapping confidence intervals indicate that the differences in performance are not significant (see table 2).

Table 2.

The sensitivity, specificity, PPV, and NPV for the detection of CIN 2 or 3 in patients of the UMC by hr-HPV detection and colposcopy. (95% CI) (n=140)

	Sensitivity	Specificity	NPV	PPV
Colposcopic lesions suspect for CIN 3 (n=2)	29% (12-46%)	100%	96% (94-98%)	100%
Hr-HPV positive at referral (n=48)	57% (22-92%)	67% (59-75%)	96% (92-100%)	8% (0-16%)
Colposcopic lesions suspect for CIN (n=35)	57% (20-94%)	77% (70-84%)	96% (92-100%)	11% (1-21%)

There were no significant differences in final follow-up between hr-HPV positive and hr-HPV negative patients, while the percentage of patients undergoing a diathermy loop excision (respectively 9% and 10%) was equal in both groups. But also here the 95% CI were large (see table 3).

With the use of hr-HPV detection as triage, 48 patients would have been referred for colposcopy, but two patients with CIN 1 and three patients with CIN 2 would not have been referred.

Table 3.

The relation between hr-HPV triage, the number of loop excisions, and the final cytological follow-up.

	hr-HPV Positive		hr-HPV Negative	
Cytological follow-up	**n= %**	**95% CI**	**n= %**	**95% CI**
Two normal cervical smears *	**31** (65)	52-78%	**62** (67)	57-77%
Normal colposcopy + 1 normal cx smear *	**12** (25)	13-37%	**19** (21)	11-27%
Persistent ASC-US smears *	**5** (10)	2-18%	**11** (12)	5-19%
Total	48 (100)		92 (100)	

** No significant difference between hr-HPV positive and negative patients.*

DISCUSSION.

Colposcopy.

The optimal management of patients with ASC-US smears is still a matter of debate. The presented data confirm that patients with ASC-US smears are at relatively low risk for CIN 2 or 3 lesions [1,5-7,9,23-25]. With an aggressive management (CWZ) CIN was diagnosed at time of referral in 20% of all patients, including 10% with CIN 2 and 3, while with an expectative management (UMC) CIN was diagnosed in only 6% of the patients, including 5% with CIN 2 or 3. This seems to confirm that many lesions, especially low-grade CIN lesions, do regress with an expectative management strategy and that they do not require treatment [3-5,7].

Significantly more patients of the CWZ had a normal first follow-up cervical smear after the initial colposcopy. It seems that a diathermy loop excision in patients with ASC-US smears, independent of the presence or absence of CIN, leads to a faster normalization of cervical smears than without a diathermy loop excision. Eventually, there was no significant difference in long-term cytological follow-up between the hospitals. The large majority of patients referred with two consecutive ASC-US smears had normal follow-up smears after respectively 41 and 39 months. The initial faster normalization of the follow-up smears with an aggressive management did not show any long-term advantage.

Many patients of the CWZ in whom a CIN lesion was suspected at colposcopy did not have CIN on histopathological examination. From the literature it is known that especially CIN 1 and 2 lesions are difficult to identify correctly with colposcopy, while CIN 3 lesions are often correctly identified [14].

The difference in the number of diathermy loop excisions between the hospitals is highly significant. Even if all patients with persistent cervical smear abnormalities would undergo a diathermy loop excision, the number of loop excisions in the CWZ would be almost 5-fold compared with the UMC. In this respect, the expectative management strategy of the UMC seems to be preferable. However, both the patient and the attending gynecologist may prefer a fast normalization of cervical smears and thus follow the aggressive management strategy.

Since this was a retrospective study, we have to be cautious in interpreting the results. A difference in population seems unlikely because all patients are living in the same city, the mean age of both groups is comparable, and all patients were first time referrals from the screening program with two consecutive ASC-US smears. A difference in other factors like sexual behavior, cigarette smoking, social status, and hr-HPV positivity is therefore very unlikely but cannot be ruled out. These factors have all been associated with an increased

risk of developing an abnormal cervical smear/CIN lesion. However, it remains unsure whether these factors still influence the detection of CIN once an abnormal smear has been found. For this reason we consider it less likely that these factors are responsible for the large differences between the groups.

Hr-HPV triage (UMC).

The percentage of hr-HPV positive patients in this study seems lower than in other studies [9,23,25]. However, those studies investigated patients with ASC-US smears as well as patients with smears indicating CIN (with a higher hr-HPV prevalence), or they investigated exclusively patients with a single ASC-US smear. The prevalence of hr-HPV in patients with two consecutive ASC-US smears may be lower than in this last group, because the HPV infection may have been cleared at the time of the second cervical smear, while the abnormality did not (yet) resolve. A lack of sensitivity of the used HPV detection method is unlikely because of the published high sensitivity of the assay [17-22]. In fact, the SPF_{10} PCR assay was shown to be more sensitive in detecting hr-HPV than HPV detection methods using MY09/11 primer sets or the Hybrid Capture II test, especially in cases with multiple hr-HPV infections [20-22].

Hr-HPV detection identified the one patient with CIN 3 and three of the six patients with CIN 2. Several explanations are possible for not detecting hr-HPV in three patients with CIN 2. Firstly it is possible that the CIN 2 lesion developed in the absence of hr-HPV, a possibility that has recently been suggested [26]. Secondly it remains possible that, despite the high sensitivity of the test, the HPV test was false negative, and thirdly it is possible that the hr-HPV infection and CIN lesion developed after the intake cervical smear was taken, but before the loop excision was done. The short time interval (0-18 months) between the intake cervical smear and the final loop excision, however, makes this unlikely.

The sensitivity for detecting CIN 2 or 3 lesions by hr-HPV testing or colposcopy never reached 100%. The performance of hr-HPV testing in this study was less than in other studies, summarized by Wright et al., despite the high sensitivity of the used assay [1]. These other studies included patients with only a single ASC-US smear followed directly by colposcopy with histopathological confirmation of the presence or absence of CIN 2 or 3. These facts may be responsible for the difference in sensitivity between those studies and this study. The low number of patients with CIN 2 or 3 in this study resulting in large 95% CI, may also partly explain that difference.

The performance of hr-HPV testing itself was equal to colposcopy in this study. Several authors have cautioned before for the overestimation of the value of hr-HPV testing [12,27,28]. On the other hand, it is possible that the value of hr-HPV detection is being underestimated in this study. Hr-HPV positive patients, in whom no CIN was detected in this study, may develop CIN lesions at a later stage, while hr-HPV negative patients, in whom CIN was detected, may have clinically irrelevant CIN lesions, because especially hr-HPV negative CIN lesions may regress with a longer follow-up [29].

The high (additional) costs of hr-HPV detection may limit its use in patients with two consecutive ASC-US smears in the Netherlands. Hr-HPV testing in patients with ASC-US smears was shown to be cost-effective in the United States, because of the high costs of colposcopy/histopathology in that country [1,30,31], but this may not be a fact in the Netherlands.

Conclusion.

The present management of patients with two consecutive ASC-US smears in the Netherlands results in a high number of unnecessary referrals and loop excisions. An expectant management strategy that identifies all patients with CIN 3 at colposcopy, but that postpones interventions in the other patients for at least 12 months, leads to significantly less loop excisions and allows for spontaneous regression of most (low-grade) lesions. Triage with hr-HPV detection before referral will reduce the number of referrals/colposcopy with about 60%, but (cytological) follow-up of all hr-HPV negative patients remains necessary to detect all patients with CIN lesions. The cost-effectiveness of hr-HPV triage in patients with two consecutive ASC-US smears needs to be assessed in the Netherlands and should be compared with triage by follow-up conventional/liquid based cytology, and/or with direct referral for colposcopy [1,31].

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LOCALIZED DISTRIBUTION OF HUMAN PAPILLOMAVIRUS GENOTYPES IN THE UTERINE CERVIX

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ABSTRACT.

Objectives: The localization and distribution of single or multiple HPV genotypes in the uterine cervix has not been studied thus far. The present study was undertaken to determine whether single or multiple HPV genotypes, detected in cervical smears, originate from a single (dysplastic) area, or from different areas (dysplastic or normal) of the uterine cervix.

Methods: Of eight patients with moderate or severe dysplasia, 31 colposcopically guided biopsies of different dysplastic lesions of the uterine cervix, as well as of normal epithelium were investigated. A highly sensitive, broad spectrum, short fragment polymerase chain reaction (SPF-10 PCR) HPV detection method in combination with a line probe assay (LiPA) for simultaneous genotyping was used.

Results: In the uterine cervix of four of the eight patients, multiple HPV genotypes were detected. These multiple HPV genotypes were detected in different biopsies as well as within a single biopsy. In three patients, all with carcinoma in situ or microinvasive carcinoma, only a single HPV genotype, HPV 16, was found all over the cervix including in the normal epithelium.

Conclusions: Different HPV genotypes can be detected in different dysplastic lesions as well as within single lesions, especially in patients with severe dysplasia. The severity of the lesion may possibly have a relation with the distribution of the HPV genotypes. The low number of patients and biopsies does not allow definite conclusions. However, the impact of these findings on the outcome of screening and vaccination programs remains to be elucidated.

INTRODUCTION.

Cancer of the uterine cervix ranks number two worldwide in cancers of women, accounting for 6% of all malignancies. Epidemiological and molecular studies over the past two decades have convincingly demonstrated that certain types of human papillomaviruses (HPV) are etiologically related to the development of most cases of cervical cancer. More than 95 genotypes of HPV have been identified so far, of which 35 types were found to infect the genital mucosa [1-3]. Several HPV genotypes, including types 16, 18, 31, 33, and 45 have been implicated in cervical carcinogenesis and are considered high-risk [1-3]. High-risk HPV genotypes are able to disturb cell cycle regulators. Theoretically, these disturbances allow repetitive clonal events to take place which may ultimately, result in cervical carcinoma via the development of pre-malignant stages [4-6].

During the pre-malignant stages, different areas, each with a different degree of dysplasia can be identified in the uterine cervix with colposcopy [7]. Cervical smears, ideally sampled from all these areas, contain in the majority a single genotype of HPV. Recently, more sensitive HPV detection methods have shown to detect multiple HPV genotypes in up to 40% of cervical smears [6, 8-11]. The localization of single or multiple HPV genotypes in the uterine cervix and the distribution of HPV throughout the cervix have not been studied thusfar. Studying the localized distribution of HPV may provide new insights in HPV -infection and -transmission and its complex relation with cervical cancer.

The present study was undertaken to determine whether single or multiple HPV genotypes, detected in cervical smears, originate from a single (dysplastic) area, or from different areas, either dysplastic or normal, of the uterine cervix.

MATERIAL AND METHODS.

Patients referred to the colposcopy clinic of the University Medical Center Nijmegen, with a cervical smear indicating moderate or severe dysplasia, were asked to participate in the study. A total of eight patients were included. One to three weeks prior to colposcopy, a liquid based cervical smear was taken from all patients, using a Cervex brush (Rovers Medical Devices; Oss, The Netherlands). These smears were fixed with Unifix® and processed into AgarCyto cell blocks, allowing for multiple analysis including HPV testing, as described previously [12]. The cervical smears indicated moderate dysplasia in one patient, severe dysplasia in four, and carcinoma in situ in three patients. The mean age of the patients was

37 years (32-46). None of the patients had previously been treated for dysplasia of the uterine cervix.

The cervix was assessed and mapped during colposcopy using acetic acid and lugol. After local anesthesia, three to five biopsies were taken from each patient, using a small electrosurgical loop. All cervical areas with a colposcopically suspected different degree of dysplasia were biopsied, including an area of normal epithelium. Subsequently, the transformation zone was removed by large loop electrosurgical excision in order to complete treatment of all cervical abnormalities. The localization of each biopsy was marked in the patient's record.

All biopsies were embedded in parafin and processed separately to avoid cross-contamination with HPV. Histopathological examination was done on each of the biopsies. Single adjacent sections were tested for the presence of HPV. The results of the HPV test were not known during histopathological examination. For HPV detection a highly sensitive broad-spectrum short polymerase chain reaction fragment (SPF10 HPV-PCR) was used as previously described [8,9]. HPV typing was subsequently performed by reverse hybridization in a line probe assay (LiPA). This LiPA technique identifies 25 different low- and high-risk HPV genotypes simultaneously [8,9].

RESULTS.

HPV was detected in the cervical smears of seven patients. In two of these patients, double infections with respectively HPV genotypes 16/31, and 16/52 were detected. All HPV genotypes detected in the cervical smear of each individual patient were also found in the biopsies of that patient. Additional HPV genotypes were detected in the biopsies of patients 1, 3, 4, and 6. The cervix of patients 1, 3, 5, and 6 contained multiple HPV genotypes. Up to four HPV genotypes could be detected within a single biopsy (patient 3). Table 1 shows the HPV genotypes detected in the smears, the histological grading of each biopsy and the HPV genotypes detected in the different biopsies.

Table 1.

HPV genotypes detected in the cervical smears and in each biopsy, in relation to the histologic grading, shown for each patient.

	HPV in smear	Biopsy	Histologic grading	HPV in biopsy
Pat. 1	X	1	Metaplasia	Neg
		2	Metaplasia	X
		3	Severe dysplasia	58
		4	Moderate dysplasia	33/58
Pat.2	16	1	Carcinoma in situ	16
		2	Moderate dysplasia	16
		3	Normal sq. Epithelium	Neg
		4	Normal endocx. Tissue	16
Pat. 3	33	1	Severe dysplasia	33
		2	Severe dysplasia	31/33/52/66
		3	Inflammation	33/66
		4	Endocx Atypia	66
Pat. 4	Negative	1	Moderate dysplasia	51
		2	Mild dysplasia	Neg
		3	Normal sq. Epithelium	Neg
Pat. 5	16/31	1	Severe dysplasia	16/31
		2	Normal sq. Epithelium	Neg
		3	Metaplasia	16
Pat. 6	16/52	1	Severe dysplasia	16/18
		2	Severe dysplasia and Endocx atypia	16/18/52
		3	Severe dysplasia	16/18
		4	Mild dysplasia	16
Pat. 7	16	1	Carcinoma in situ	16
		2	Mild dysplasia	16
		3	Normal sq. Epithelium	16
		4	Micro invasive carc	16
Pat. 8	16	1	Severe dysplasia	16
		2	Severe dysplasia	16
		3	Normal. sq. Epithelium	16
		4	Micro invasive carc	16
		5	Micro invasive carc	16

Normal sq. epithelium = Normal squamous epithelium, Normal endocx tissue = normal endocervical tissue, X = HPV type not specified.

Figure 1.

Graphical presentation of the localization of the biopsy, the histologic grading, and the HPV genotypes detected within the biopsy.

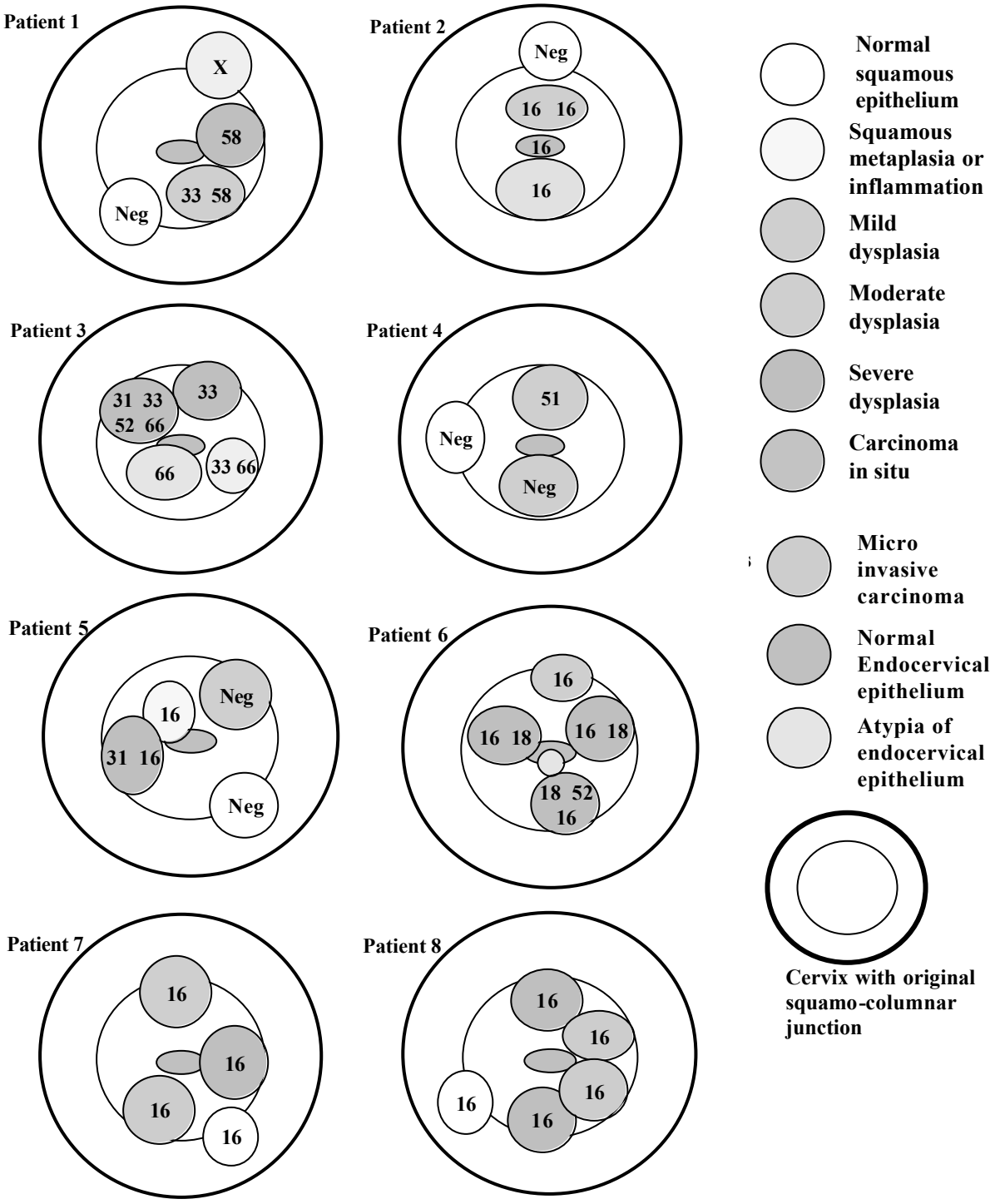

Figure 1 shows a graphic representation of the localization of the biopsies related to the histological grading and the types of HPV detected in the cervix of each patient. HPV was detected in all biopsies showing dysplasia, except for a mild dysplastic lesion in patient 4. HPV was detected in two of the three biopsies with metaplasia, and in two of the five biopsies with normal squamous epithelium. Only in the patients 7 and 8, both with a microinvasive carcinoma, HPV was detected in normal squamous epithelium.

In all patients with severe dysplasia as the most severe lesion (patients 1, 3, 5, and 6), more than one genotype of HPV was detected in the cervix, and multiple HPV genotypes were present within a single biopsy of each of these patients. In the other four patients a single HPV genotype was detected. Two of these patients had invasive carcinoma, and one patient had a carcinoma in situ. In the patients with invasive carcinoma, HPV 16 was detected throughout the cervix, including normal squamous epithelium. Even the severe dysplastic areas in these patients only contained this single genotype of HPV.

DISCUSSION.

This study shows that multiple HPV genotypes can be detected within the uterine cervix in different dysplastic areas as well as within a single dysplastic area. The finding of different dysplastic areas in the uterine cervix, identified by colposcopy, has been described previously, and it was suggested that these areas were not related to each other, nor were part of a developing process of cervical cancer [7]. Our results confirm those of another study, which detected different HPV genotypes in different biopsies of the uterine cervix [13]. However, in contrast with our results, only one biopsy with severe dysplasia in that study contained two HPV genotypes [13]. We detected multiple HPV genotypes in seven of 18 biopsies obtained from patients with severe dysplasia as the highest grade of abnormality, and within a single biopsy up to four different HPV genotypes were detected. It is possible that the detection of different HPV genotypes in different cervical lesions might be attributed to coincident infections causing separate lesions as has been suggested before [13]. In cases with the same HPV genotype detected in different lesions, morphologic progression 'in situ' was more likely [13]. The morphologic progression theory supposes that repetitive clonal events occur early in the development of dysplasia, under the influence of a single HPV genotype [14]. Although it has to be emphasized that the number of patients and biopsies included in our study is very small, the multiple HPV genotypes present in our patients are in contrast with the basics of the morphologic progression theory. On the other hand, it is quite possible that a clonal process attributed to a specific single HPV genotype is in progress, while other HPV genotypes detected are merely superfluous or transient, either newly acquired, or a reactivation of latent HPV infections. The presented data suggest that the detection and distribution of single or multiple HPV genotypes, may possibly have a relation with the stage of the disease. In patients with severe dysplasia as most severe lesion, multiple HPV genotypes were detected within a single biopsy as well as in different biopsies, while in the patients with carcinoma in situ or invasive carcinoma, a single HPV genotype was detected in all biopsies including areas with different degrees of dysplasia and normal epithelium. More studies are required to solve these issues.

We found a lower detection rate of multiple HPV genotypes in cervical smears than in the corresponding biopsies. The assumption that HPV genotypes detected in cervical smears are a true reflection of all types of HPV present in the cervix may therefor not always be true. This observed underestimation in cervical smears may be due to a sampling error, but may also indicate that only the most prominent HPV genotype(s) are detected in a cervical smear. The replication of these genotypes may be more pronounced, or they may be localized in

more superficial layers of the epithelium. This facilitates detection in cervical smears and masks the presence of other, possibly latent genotypes of HPV in the deeper epithelial layers. Indeed, more evidence to support the presence of latent HPV is described in the literature. The prevalence of HPV during pregnancy increases with gestational age to 40% compared with 5-20% prevalence in the non-pregnant population [15-17]. This increase could not be explained by other risk factors [16]. Patients above 55 years and patients with immune suppression also show a higher prevalence of HPV than in a regular population, and there is a strong correlation between the prevalence of HPV and the severity of the immune-suppression [11,18,19].

More sensitive HPV detection methods are able to detect a higher prevalence of genital HPV infection in the population and do more often detect multiple HPV genotypes [6,8,9]. The used highly sensitive SPF-10 LiPA-PCR HPV detection method in this study has been validated before [8]. With this detection method, HPV genotypes were correctly identified in 97.2% of 238 samples, and showed to be more sensitive than other PCR primers (MY 09/11, GP5(+)/6(+)) especially when multiple HPV genotypes were present [8].

As shown in this study, multiple HPV genotypes can be found within a single biopsy of a dysplastic lesion of the uterine cervix, indicating that the relation between HPV and cervical cancer is very complex. The relation between multiple HPV infections and cervical cancer as well as factors influencing latency, recurrence, and clearance of HPV, need further study.

The presence of multiple HPV genotypes may have a direct impact on the outcome of screening and vaccination programs, and a further analysis is required.

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COEXISTING HIGH-GRADE GLANDULAR AND SQUAMOUS CERVICAL LESIONS AND HUMAN PAPILLOMAVIRUS INFECTIONS

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ABSTRACT.

Objectives: The frequency of high-risk human papillomavirus (hr-HPV) genotypes in patients with adenocarcinoma in situ (ACIS) with coexisting cervical intraepithelial neoplasia (CIN), ACIS without coexisting CIN, and high-grade CIN (CIN 2/3) was studied, in order to gain more insight in the relation between hr-HPV infections and the development of coexisting squamous and glandular lesions.

Methods: The SPF_{10} LiPA PCR was used to detect simultaneously 25 different HPV genotypes in biopsies obtained from 90 patients with CIN 2/3, 47 patients with ACIS without coexisting CIN, and 49 patients with ACIS and coexisting CIN.

Results: Hr-HPV was detected in 84 patients (93%) with CIN 2/3, 38 patients (81%) with ACIS without CIN, and in 47 patients (96%) with ACIS and coexisting CIN. 13 different hr-HPV genotypes were detected in patients with CIN 2/3, and only 5 in patients with ACIS with/without coexisting CIN. HPV 31, multiple hr-HPV genotypes, and HPV genotypes other than 16, 18, and 45 were significantly more often detected in patients with CIN 2/3, while HPV 18 was significantly more often detected in patients with ACIS with/without CIN. There were no significant differences in the frequency of specific hr-HPV genotypes between patients with ACIS with-, or without coexisting CIN.

Conclusions: The frequency of specific hr-HPV genotypes is similar for patients with ACIS without CIN and patients with ACIS and coexisting CIN, but is significantly different for patients with CIN 2/3 without ACIS. These findings suggest that squamous lesions, coexisting with high-grade glandular lesions, are etiologically different from squamous lesions without coexisting glandular lesions.

INTRODUCTION.

A causal relation between high-risk human papillomavirus (hr-HPV) infections and cervical cancer has been documented in the literature beyond reasonable doubt [1,2]. Hr-HPV can be detected in almost 100% of squamous- and adenocarcinomas of the uterine cervix [3,4]. Numerous studies have suggested that the development of squamous cervical carcinoma is preceded by cervical intraepithelial neoplasia (CIN) [1,2].

The frequency of hr-HPV genotypes in CIN lesions has been studied previously and its detection rate rises with increasing severity of the CIN lesion [1,2,5]. Only limited studies are available on the frequency of hr-HPV genotypes in pre-malignant glandular lesions and the number of patients in most studies is rather low (see Table 1) [4, 6-14]. Hr-HPV was detected in 66-100% of patients with adenocarcinoma in situ (ACIS) [9,10]. Approximately 48% of all women diagnosed with ACIS have coexisting squamous lesions [4, 6-14], but the frequency of specific hr-HPV genotypes in patients with ACIS has not been studied in relation with the presence or absence of coexisting CIN lesions.

In this study, the frequency of specific hr-HPV genotypes in patients with ACIS and coexisting CIN is compared with its frequency in patients with ACIS without coexisting CIN, and patients with CIN without ACIS, in order to gain more insight in the relation between hr-HPV infections and the development of coexisting squamous and glandular lesions.

MATERIAL AND METHODS.

Glandular lesions.

In the automated databases of the pathology laboratories of the University Medical Center Nijmegen, the Canisius Wilhelmina Hospital Nijmegen, and the Rijnstate Hospital Arnhem, 120 patients diagnosed with ACIS between 1988 and 2000 were identified. No histo-pathological material was left for further study of 9 patients, and 6 patients had a histopathological diagnosis of invasive carcinoma within one month of the diagnosis of ACIS. Of the remaining 105 patients, the histopathological slides of a biopsy-, cone-, or hysterectomy specimen were re-examined independently, by three experienced gyneco-pathologists (JB, AWvT, and MM). Complete agreement on the diagnosis of ACIS was reached in 96 patients (91%), according to the criteria of Brown and Wells [15]. The criteria of Brown and Wells take architectural (glandular irregularity and complexity) and cytological criteria (nuclear enlargement, hyperchromasia, pseudostratification of nuclei, increased or

abnormal mitoses) into account [15]. The three pathologists reached no consensus on the diagnosis of ACIS in nine patients, and these patients were excluded from this study.
Coexisting squamous intraepithelial lesions were detected and confirmed by consensus of the three pathologists (JB, AWvT, MM) in the slides of 49 of the 96 patients (51%); 35 patients (71%) had coexisting CIN 3 (severe dysplasia, or carcinoma in situ), eight patients (16%) had coexisting CIN 2 (moderate dysplasia), and six patients (12%) had coexisting CIN 1 (mild dysplasia). In 41 of these 49 patients (84%), CIN and ACIS were diagnosed in the same slide/section, and hr-HPV detection was done on adjacent sections containing both the squamous- and glandular lesion. In eight patients CIN was diagnosed in a different slide than in which ACIS was diagnosed and on which hr-HPV detection was done. Five of these eight patients had coexisting CIN 1, two had coexisting CIN 2, and one had coexisting CIN 3.
The mean age of the 96 patients was 38.3 years (28-73) at the time of the diagnosis.
A cervical scrape preceding the diagnosis of ACIS less than six months was made in 43 of the 49 patients (88%) with ACIS and coexisting CIN, and in 40 of the 47 patients (85%) with ACIS without coexisting CIN. The cervical scrape indicated a high-grade glandular- (moderate atypia – ACIS), a high-grade squamous lesion (moderate dysplasia – CIS), or both in respectively, 13 patients (30%), 15 patients (35%), and 13 patients (30%) with ACIS and coexisting CIN, and in respectively 24 patients (60%), 4 patients (10%), and 8 patients (20%) with ACIS without coexisting CIN. The remaining six patients had only mild atypia of glandular cells in the cervical scrape.

Squamous lesions.
A control group of 90 patients consisted of patients who consulted the colposcopy clinic at the UMC between 1997 and 1999 and who were diagnosed with high-grade CIN in the biopsy specimen of a large loop excision of the transformation zone. All slides were reviewed by an experienced gyneco-pathologist (JB), and the diagnosis of high-grade CIN was confirmed. Of these 90 patients, 18 patients (20%) were diagnosed with moderate dysplasia (CIN 2), 36 patients (40%) with severe dysplasia (CIN 3), and 36 patients (40%) with carcinoma in situ (CIN 3). The mean age at the time of diagnosis was 37.6 years (28-59).
A cervical scrape preceding the diagnosis of CIN 2/3 less than six months was taken of all 90 patients. The cervical scrape indicated a high-grade squamous lesion in 80 patients (89%), a high-grade glandular and squamous lesion in one patient (1%), and low-grade squamous lesions in 9 patients (10%).

Taking the preceding cervical scrapes of all patients with ACIS and CIN 2/3 together, the cervical scrape indicated a high-grade glandular lesion in 37 patients (21%), a high-grade squamous lesion in 99 patients (57%), both in 22 patients (13%), and only low-grade lesions in 15 patients (9%).

Table 1.

Summary of all studies since 1990 with more than 20 ACIS patients in relation with hr-HPV and/or coexisting CIN.

Author and year	Patients n=	hr-HPV pos	HPV 16	HPV 18	Coexisting CIN
Riethdorf et al. 2002	42	32 (76%)	22 (53%)	10 (24%)	N.D.
Madeleine et al. 2001	82	71 (87%)	32 (39%)	43 (52%)	N.D.
Riethdorf et al. 2000	33	29 (88%)	21 (64%)	8 (24%)	14 (42%)
Pirog et al 2000	23	23 (100%)	10 (43%)	6 (26%)	9 (39%)
Anciaux et al. 1997	21	16 (76%)	6 (29%)	10 (47%)	9 (43%)
Duggan et al. 1994	37	23 (66%)	8 (23%)	15 (43%)	N.D.
Higgins et al. 1992	42	39 (95%)	13 (31%)	28 (67%)	22 (52%)
Leary et al. 1991	30	21 (70%)	10 (33%)	11 (37%)	15 (50%)
Bekkers et al., present study	**96**	**85 (89%)**	**39 (41%)**	**41 (43%)**	**49 (51%)**

N.D. = not documented.

HPV detection.

A 3 µm section of the histopathological specimens, on which ACIS and/or CIN 2/3 was diagnosed, was taken for HPV analysis. In 41 of the 49 patients with ACIS and coexisting CIN, HPV detection was done on a section containing both the glandular and squamous lesion. HPV detection was performed using a broad-spectrum short-fragment polymerase chain reaction (SPF_{10} PCR) as previously described [16-20]. In case of a positive PCR, subsequent genotyping was performed via a reverse hybridization line probe assay (LiPA), allowing for simultaneous typing of 25 HPV genotypes, including hr-HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. This SPF_{10} LiPA PCR HPV detection method is highly sensitive, specific and reproducible and has been clinically validated [4, 16-19]. If more than one hr-HPV genotype was detected with the SPF_{10} LiPA PCR in a single sample of an individual patient, that patient was considered to be infected with multiple hr-HPV genotypes.

Analysis.

The frequency of specific hr-HPV genotypes in patients with ACIS without coexisting CIN, ACIS with coexisting CIN, and CIN 2/3 without ACIS were compared. The frequency of specific hr-HPV genotypes was also investigated in relation with the suspicion of a high-grade glandular and/or high-grade squamous lesion in the preceding cervical scrape. Statistical analysis including Chi-square tests and independent t-tests was performed, considering all values of $p < 0.05$ to be significant.

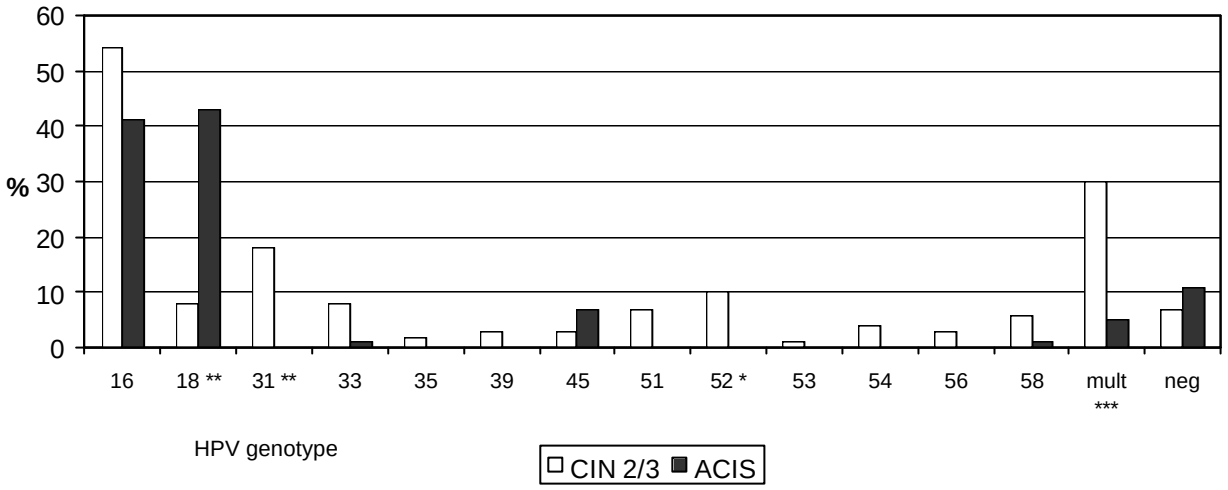
RESULTS.

Hr-HPV genotypes were detected in 93% of the patients with CIN 2/3, 81% of the patients with ACIS without coexisting CIN, and in 96% of the patients with ACIS and coexisting CIN. Figure 1 shows that HPV 31 and multiple hr-HPV genotypes were significantly more often detected in patients with CIN 2/3, while HPV 18 was significantly more often detected in patients with ACIS (with or without coexisting CIN). Among patients with CIN 2/3, 13 different hr-HPV genotypes were detected, compared with only 5 different hr-HPV genotypes in patients with ACIS with or without coexisting CIN.

Figure 1.

The relative frequency of specific hr-HPV genotypes in patients with high-grade CIN (90 patients) and patients with ACIS (96 patients).

** p < 0.05, ** p < 0.01, *** p < 0.001*



HPV 16 was the most frequently detected genotype in patients with CIN 2/3 (54%), while both HPV 16 and 18 showed a high frequency of respectively 41% and 43% in all patients with ACIS (see Figure 1). HPV 16 or 18 were present in 79% of all patients with ACIS (5 patients had a double infection with HPV 16 and 18), and in 61% of the patients with CIN 2/3 (1 patient had a double infection with HPV 16 and 18). There was no significant difference in the frequency of HPV 16, or HPV 45 between patients with CIN 2/3, patients with ACIS and coexisting CIN, and patients with ACIS without CIN (see Table 2 and 3). The frequency of HPV 18 was significantly lower in patients with CIN 2/3 compared with patients with ACIS and coexisting CIN, or ACIS without CIN. This means that the frequency of HPV 18 is significantly higher in CIN lesions coexisting with ACIS than in solitary CIN lesions.

The frequency of HPV 31, and multiple hr-HPV genotypes was significantly higher in patients with CIN 2/3 than in patients with ACIS, either with or without coexisting CIN (see table 2). The frequency of hr-HPV genotypes other than HPV 16, 18, 31, and 45 was 38% and 3% in patients with respectively CIN 2/3 and ACIS (total group), and this difference was highly significant ($?^2 = 11.35$, $p < 0.001$).

Table 2.

The frequency of hr-HPV genotypes in patients with CIN 2/3 compared with patients with ACIS and coexisting CIN.

Hr-HPV genotype	CIN 2/3 n=90	ACIS with CIN n=49	Significance
16	49 (54%)	19 (39%)	N.S.
18	7 (8%)	23 (47%)	$?^2 = 26.49$, $p < 0.001$
31	15 (17%)	0 (0%)	$?^2 = 8.38$, $p < 0.01$
45	3 (3%)	5 (10%)	N.S.
Other	34 (38%)	2 (4%)	$?^2 = 19.73$, $p < 0.001$
Multiple	26 (29%)	2 (4%)	$?^2 = 12.48$, $p < 0.001$
Total hr-HPV positive	84 (93%)	47 (96%)	N.S.
Hr-HPV negative	6 (7%)	2 (4%)	N.S.

N.S. = not significant

There was no difference in the number of hr-HPV negative patients between patients with CIN 2/3 and patients with ACIS.

There were no significant differences in the frequency of HPV 16, 18, 31, 45, multiple hr-HPV, and other hr-HPV genotypes between patients with ACIS without coexisting CIN and patients with ACIS and coexisting CIN. The number of hr-HPV negative patients was significantly higher in patients with ACIS without coexisting CIN (see Table 3). Among the patients with ACIS and coexisting CIN, there were no significant differences in the frequency of specific hr-HPV genotypes regarding the different degrees of coexisting CIN.

The mean age of the patients with ACIS without CIN was 40.1 years, while the mean age of patients with ACIS and coexisting CIN was 36.3 years ($t=2.32$, $p < 0.05$). There was no difference between the mean age of the total group of patients with ACIS and the patients with CIN 2/3 (38.2 and 37.6 years respectively).

Within the group of patients with CIN 2/3, there were no significant differences in the frequency of HPV 16, 18, 31, and 45, multiple hr-HPV, other hr-HPV genotypes, or hr-HPV negative patients between patients with moderate dysplasia, severe dysplasia, or carcinoma in situ. Multiple hr-HPV genotypes were detected in respectively, 22%, 28%, and 33% of these patients.

Table 3.

The frequency of hr-HPV genotypes in patients with ACIS with coexisting CIN compared with patients with ACIS without coexisting CIN.

Hr-HPV genotype	ACIS with CIN n=49	ACIS without CIN n=47	Significance
16	19 (39%)	20 (43%)	N.S.
18	23 (47%)	18 (38%)	N.S.
31	0 (0%)	0 (0%)	N.S.
45	5 (10%)	2 (4%)	N.S.
Other	2 (4%)	1 (2%)	N.S.
Multiple	2 (4%)	3 (6%)	N.S.
Total hr-HPV positive	47 (96%)	38 (81%)	$?^2 = 6.07$, $p < 0.02$
Hr-HPV negative	2 (4%)	9 (19%)	$?^2 = 6.63$, $p = 0.01$

Hr-HPV was detected in 33 of the 37 patients (89%) with a cervical scrape that indicated a high-grade glandular lesion, in 92 of the 99 patients (93%) with a cervical scrape that indicated a high-grade squamous lesion, in all 22 patients (100%) with a cervical scrape that indicated both a high-grade glandular and squamous lesion, and in 13 of the 15 patients (87%) with a cervical scrape that indicated only a low grade lesion. There were no significant differences in the frequency of HPV 16, 18, 31, 45, multiple or other HPV genotypes regarding the presence or absence of a suspicion of a high-grade squamous and/or glandular lesion in the cervical scrapes. This indicates that the detection of a lesion in the cervical scrape is not influenced by the genotype of hr-HPV infecting that lesion.

DISCUSSION.

The number of patients with ACIS and coexisting CIN lesions among all patients with ACIS in this study is similar to the numbers reported in the literature (Table 1). No significant differences in the frequency of different hr-HPV genotypes were observed between patients with ACIS with coexisting CIN, and patients with ACIS without coexisting CIN, as has been reported previously [9,10,21]. However, significant differences in the frequency of HPV 18, HPV 31, multiple hr-HPV genotypes, and hr-HPV genotypes other than HPV 16, 18, 31, and 45 were observed between patients with CIN 2/3 and patients with ACIS, either with or without coexisting CIN. One may hypothesize that coexisting glandular and squamous lesions share a common etiology, different from solitary squamous lesions, under the influence of specific hr-HPV genotypes, as has been previously described for squamous lesions [20,22,23]. Indeed, in 41 of the 49 patients (84%) in the present study the ACIS lesion was adjacent to the coexisting squamous lesion. Furthermore, Colgan et al. found no differences in the linear extend, circumferential extend, multifocality, or the co-presence of invasive adenocarcinoma between patients with ACIS without CIN and ACIS with coexisting CIN lesions [14]. These observations seem to support the hypothesis that ACIS and coexisting CIN lesions share a common etiology.

Only 5 different hr-HPV genotypes (HPV 16, 18, 33, 45, 58) were detected in patients with ACIS (of which HPV 33 and 58 in < 2% of the patients), compared with 13 different hr-HPV genotypes in patients with CIN 2/3. Other authors also found a limited number of hr-HPV genotypes (3-7) among patients with ACIS [4,8]. This may suggest that only certain hr-HPV genotypes are able to infect and/or induce lesions in the glandular mucosa. This fact may be the reason for the significantly lower detection rate of multiple hr-HPV genotypes, and the significantly higher detection rate of HPV 18 in the cervix of patients with ACIS. However, higher rates (13-22%) of multiple hr-HPV infections among patients with ACIS have been described in the literature [4,8]. The lower rate of multiple hr-HPV genotypes in the present study is probably the result of the very restrictive diagnostic criteria, and the consensus agreement by three pathologists, that was used to diagnose ACIS.

The high frequency of a single hr-HPV genotype in patients with ACIS compared with patients with CIN 2/3 may reflect the monoclonal aspect of the ACIS lesion, as is often the case in invasive carcinomas [3]. Patients with (squamous) CIS in this study showed a higher prevalence (33%) of multiple hr-HPV genotypes, as well as a higher number of different hr-HPV genotypes (12), which may be regarded as another indication that ACIS lesions may have a biologic behavior and/or etiology that is different from squamous in situ lesions [24].

The number of hr-HPV-negative patients with ACIS without CIN in this study was significantly higher than in patients with ACIS and coexisting CIN. ACIS may be a precursor of different subtypes of invasive adenocarcinoma (like small cell or endometrioid adenocarcinoma) that have a lower or no prevalence of hr-HPV [4,24,25]. These subtypes are rare, and distinction on a biopsy with only ACIS proved to be difficult [24,25]. It is possible that these hr-HPV-negative subtypes of ACIS are less often associated with coexisting CIN, resulting in a higher rate of hr-HPV-negative patients among patients with ACIS without CIN.

The significant difference in age between patients with ACIS without CIN and patients with ACIS with coexisting CIN, in which the latter are younger, has been described previously [4,14]. Different explanations for this age difference are possible. Firstly, the lesions in patients with ACIS and coexisting CIN may show a faster progression, leading to detection at a younger age. This has been described previously for HPV 18 related lesions [26], but we did not find a difference in HPV 18 prevalence between patients with ACIS alone or ACIS and coexisting CIN. Secondly, the involvement of the squamous epithelium may be the reason for the detection of ACIS with coexisting CIN at a younger age, since ecto-cervical lesions are more easily detected. Thirdly, it may be a coincidental finding especially since we did not find a difference in mean age between patients with CIN 2/3 and the total group of patients with ACIS.

There was no significant difference in the overall sensitivity of the preceding cervical scrape for the detection of either ACIS, or CIN 2/3 in the present study (respectively 93% and 90%). The found sensitivity to detect ACIS was higher than reported in the literature [24,25]. However, these studies used cervical scrapes from a cervical cancer screening program, while in the present study several patients had more than one preceding cervical scrape taken, which may have alerted the pathologist, leading to the higher sensitivity.

We did not find a relation between the presence of certain hr-HPV genotypes in the biopsy, and the sensitivity of the cervical scrape in detecting ACIS and/or CIN 2/3 lesions. This indicates that the detection of a lesion in the cervical scrape is not influenced by the genotype of hr-HPV infecting that lesion.

In conclusion, patients with ACIS have significantly more often HPV 18 infections, while patients with CIN 2/3 have significantly more often infections with HPV 31, HPV genotypes other than 16, 18, 31, and 45, and multiple hr-HPV genotypes. The detection of high-grade glandular- and/or squamous lesion by cervical scrapes is not influenced by the hr-HPV genotype associated with the lesion. Among patients with ACIS, patients with coexisting CIN lesions tend to be younger, but they have a similar frequency of different hr-HPV genotypes

than patients with ACIS without CIN, while the frequency of specific hr-HPV genotypes differs significantly from those of patients with CIN 2/3 without ACIS. These findings suggest that squamous lesions, coexisting with high-grade glandular lesions, are etiologically different from squamous lesions without coexisting glandular lesions. The clinical implication of these findings needs further study.

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DOWN REGULATION OF ESTROGEN RECEPTOR EXPRESSION IN HUMAN PAPILLOMAVIRUS INFECTED CERVICAL DYSPLASIA

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(submitted)

ABSTRACT.

Objectives: To study the alterations in estrogen receptor (ER), and progesteron receptor (PR) expression in relation with proliferative activity (MIB1) during the development of cervical dysplasia in women infected with high-risk human papillomavirus (hr-HPV).

Methods: Three to five biopsies of the cervix of eight patients were taken at colposcopy. Epithelial abnormalities were detected in 21 of the 30 biopsies containing squamous epithelium, and 19 of these 21 biopsies contained hr-HPV. The labelling index (LI) as well as the intensity of staining of the MIB1-, ER-, and PR- expression was assessed in each biopsy, including normal epithelium directly adjacent to the dysplastic lesions.

Results: Statistical analysis showed a significant increase in the MIB1 LI with increasing severity of the dysplasia. The ER LI and ER intensity of staining in dysplastic lesions showed a significant inverse relation with the severity of the dysplasia. The PR LI and intensity of staining did not differ between normal epithelium and dysplasia. The ER-LI/MIB1-LI ratio in dysplastic lesions showed a significant inverse relation with the severity of the dysplasia, while no alterations in this ratio were observed in morphologically normal epithelium adjacent to dysplasia.

Conclusions: The ER but not the PR expression is progressively down regulated during the development of cervical dysplasia in women infected with hr-HPV. This down regulation can be observed in dysplastic lesions but not in normal epithelium adjacent to that dysplasia. The significant decrease in the ER-LI/MIB1-LI ratio in progressively dysplastic lesions may indicate a loss of normal growth control by sex steroid hormones, which is not observed in normal epithelium.

INTRODUCTION.

Cancer of the uterine cervix ranks number two worldwide in cancers of women, accounting for 6% of all malignancies. Epidemiological, clinicopathological, and molecular biological studies over the past two decades have convincingly demonstrated that infections with high-risk human papillomavirus (hr-HPV) are etiologically related to the development of pre-malignant cervical lesions and invasive cervical cancer [1-7]. Hr-HPV infections cause expression of viral oncoproteins, of which especially E6 and E7 disturb cell-cycle regulators by inactivating p53, and pRb, respectively. These disturbances result in hyperproliferation of the epithelium [8,9]. Hyperproliferation shows a strong correlation with the degree of cervical dysplasia and can be visualized with the monoclonal antibody MIB1. MIB1 recognizes the Ki-67 antigen, a nuclear antigen that is expressed in all proliferating cells [9-11].

The long-term use of oral contraceptives, as well as exogenous estrogen suppletion have been identified as independent risk factors for the development of HPV mediated cervical cancer [6,12,13]. A relation between the degree of dysplasia and estrogen receptor (ER), or progesteron receptor (PR) expression has been described previously, but the results of several studies seem to contradict each other [8,14-18].

In the present study, the proliferative activity in relation with ER and PR expression was investigated in normal epithelium and dysplastic lesions within the uterine cervix, in order to study alterations in hormonal sensitivity in relation with proliferative activity during the development of cervical dysplasia in women infected with hr-HPV.

MATERIAL AND METHODS.

Patients.

Eight patients referred to the colposcopy clinic of the University Medical Center Nijmegen with an abnormal cervical scrape were included in this study. Their mean age was 37 years (32-46). None of the patients had previously been treated for dysplasia of the uterine cervix. Colposcopy was performed in the luteal phase of the menstrual cycle in five patients, during the second half of an oral contraceptives cycle (patient 7), and during a daily intake of a progestative (patient 5). In one patient the phase of the menstrual cycle at colposcopy was not registered (patient 8).

The cervix was assessed and mapped at colposcopy using acetic acid. After application of a local anesthetic, three to five biopsies were taken from the uterine cervix of each patient, using a small diathermy loop. All cervical areas with a colposcopically suspected different

degree of dysplasia were biopsied, including an area of colposcopically normal epithelium. Subsequently, the transformation zone was removed by large diathermy loop excision in order to complete treatment of all cervical abnormalities. In total 32 separate biopsies of the eight patients were taken. Squamous epithelium was present in 30 biopsies and these biopsies were further analyzed. The localization of each biopsy was marked in the patient's record.

All biopsies were processed separately and embedded in paraffin to avoid cross-contamination with HPV. Histopathological examination was done on sections of each biopsy. Epithelial abnormalities were detected in 21, mild dysplasia (MiD) in four, moderate dysplasia (MoD) in three, severe dysplasia (SD) in eight, SD with endocervical atypia in one, carcinoma in situ (CIS) in two, and micro-invasive carcinoma (MIC) in three biopsies. In the nine biopsies without dysplasia, three showed squamous metaplasia, and six showed normal squamous epithelium (see Table 1). Subsequent paraffin sections were tested for the presence of hr-HPV, and assessment of MIB1-, ER-, and PR- expression.

High-risk HPV detection.

A 3 µm thick paraffin section of each biopsy was processed for HPV detection with a broad-spectrum short polymerase chain reaction fragment (SPF_{10} HPV-PCR) [19]. In case of a positive result, subsequent HPV genotyping was done in a reverse hybridization line probe assay (LiPA), which identifies 25 different HPV genotypes simultaneously. HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68 were regarded as high-risk, and HPV genotypes 6, 11, 34, 40, 42, 43, 44, 54, 70, and 74 were regarded as low-risk. This detection method was validated before and was found to be highly sensitive, specific, and reproducible, also in cases with multiple HPV infections [19-23].

Immunohistochemistry.

Three µm thick paraffin sections were mounted onto polylysine-coated slides and dried overnight at 37°C. Paraffin sections were dewaxed in xylene and rehydrated in a standard series of graded alcohols. Rehydrated slides were placed in a citrate buffer (10m?, Ph 6.0) and heated in a household microwave oven at 90°C for 20 min. After microwave preprocessing, the sections were allowed to cool down to room temperature. Subsequently, the slides were washed in phosphate-buffered saline (PBS, pH 7.4) [9].

An indirect immunoperoxidase technique was used to visualize the Ki-67 antigen. The sections were incubated with the mouse monoclonal antibody MIB1 (Immunotech S.A.,

France) 1:40 in PBS with 2% normal calf serum overnight at 4°C and subsequently incubated with a rabbit anti-mouse peroxidase (Dako, Denmark) 1:100 in PBS for 60 minutes at room temperature. The peroxidase-labelled complex was developed with diaminobenzidine (DAB; Vector Laboratories) for 4 min at room temperature and intensified with 5% CuSO4 for 5 min at room temperature. All incubation steps were followed by three washes in PBS of 5 min. Subsequently the slides were slightly counterstained with Mayer's haematoxylin, dehydrated in ethanol and xylene, and finally mounted.

In order to detect ER and PR expression, subsequent sections of the biopsy were pretreated as for MIB1, followed by incubation with normal horse serum for 10 min at room temperature. The sections were incubated for 60 min at room temperature with mouse IgG serum (Dako, Denmark) 1:200 in PBS for ER, and 1:900 in PBS for PR, followed by incubation with a biotine-labelled, horse anti-mouse serum (Dako, Denmark) 1:200 for 30 min at room temperature. The peroxidase-labeled complex was developed as for MIB1.

Analysis.

The labelling index (LI) and the intensity of the staining of the MIB1-, ER-, and PR-expression were assessed in each dysplastic squamous lesion, and in normal epithelium. Additionally, in 19 of the 21 sections that contained dysplasia with directly adjacent areas of normal squamous epithelium, the MIB1-, ER-, and PR- expression were also assessed in the normal epithelium. The intensity of the staining was divided in 4 classes (no staining = 0, slight = 0.33, moderate = 0.67, severe = 1) and the fraction of nuclei that were stained (LI) were scored for the full thickness of the epithelium. The relation between the proliferative activity and ER-, and PR- expression was studied by assessing the ratios of the ER-LI/MIB1-LI and PR-LI/MIB1-LI in different degrees of dysplasia as well as in normal epithelium adjacent to dysplasia, as the LI was the most objectively scored parameter.

Statistical analysis consisted of Jonckheere-Terpstra's distribution-free test for ordered alternatives to detect trends of the MIB1-, ER-, PR-expression, and its ratios with the degree of dysplasia. In case of a significant trend with the Jonckheere-Terpstra's test, the Kruskal-Wallis distribution-free test was used to detect differences between the diagnostic subgroups.

RESULTS.

High-risk HPV detection.

Table 1 shows that hr-HPV was detected in all biopsies in which epithelial abnormalities were observed by histopathological examination, except for MiD lesions in patients 4 and 5. Hr-HPV was detected in one of the three biopsies with squamous metaplasia, and in three of the six biopsies with normal squamous epithelium. In all patients with SD as the most severe lesion (patients 1, 3, 5, and 6), more than one genotype of hr-HPV was detected within the cervix, and even within a single biopsy (Table 1). In the other four patients a single hr-HPV genotype was detected. Two of these patients had MIC, and one patient had a CIS. In the two patients with MIC, HPV 16 was detected throughout the cervix, and even the MiD- and SD- lesions in these patients contained only this single genotype of HPV.

MIB1 expression.

The Jonckheere-Terpstra test for ordered alternatives showed a highly significant ($p<0.001$) increase of the mean MIB1 LI, and no relation of the MIB1 intensity of the staining ($p=0.29$) with the severity of the dysplasia in the biopsy (Figure 1a). The MIB1 LI was $\geq$ 33% in all biopsies with SD, CIS, and MIC, and < 33% in all biopsies without dysplasia (Table 1). All areas of normal epithelium adjacent to areas of dysplasia had a MIB1 LI $\leq$ 33%, and in these areas, no significant relation between the severity of the adjacent dysplasia and the MIB1 LI ($p=0.19$), or the MIB1 intensity ($p=0.28$) was found (Figure 2a). There was no significant relation between the MIB1 LI and the presence of more severe dysplastic lesions in the same cervix, the presence of HPV 16, other HPV genotypes, or multiple hr-HPV infections.

Table 1.

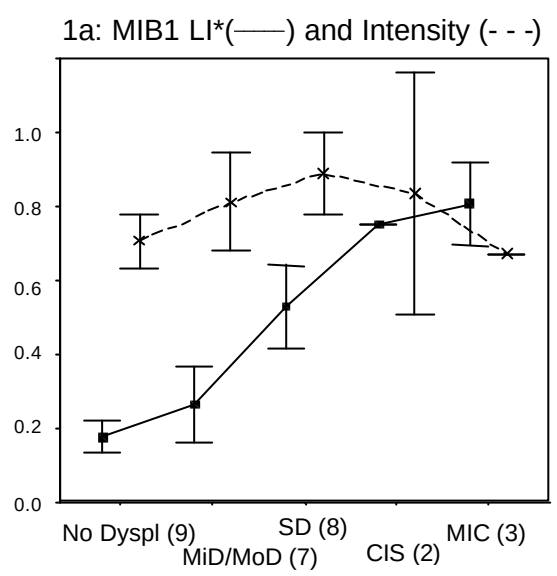
The detected HPV genotypes, histological grading, the LI and intensity of staining for MIB1-, ER-, and PR- expression of each biopsy containing squamous epithelium.

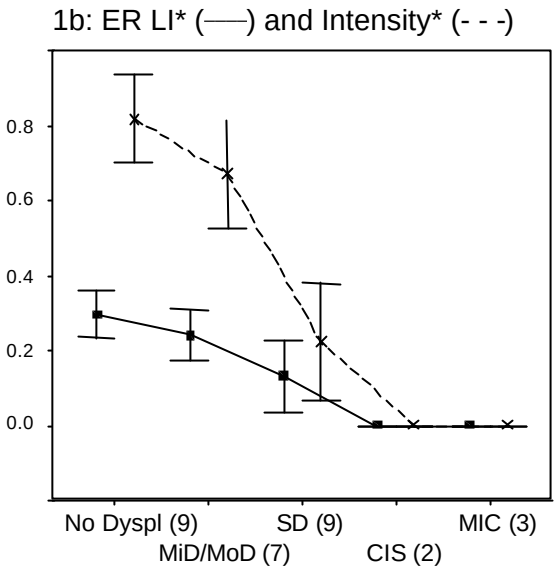
				MIB1		ER		PR	
Pat	**Biopsy**	**HPV**	**Histol. grading**	**LI**	**Int**	**LI**	**Int**	**LI**	**Int**
1	1	Neg	Sq metaplasia	0.25	0.67	0.33	1	0.25	0.67
	2	Neg	Sq metaplasia	0.42	0.67	0.42	1	0.25	0.33
	3	58	SD	0.58	0.67	0.25	0.33	0.17	0.33
	4	33/58	MoD	0.17	0.67	0.33	1	0.08	0.33
2	1	16	CIS	0.75	1	0	0	0	0
	2	16	MoD	0.17	0.67	0.25	0.67	0.17	1
	3	Neg	Nl sq epith	0.08	0.67	0.33	1	0.08	0.33
3	1	33	SD	0.33	1	0.42	0.67	0.17	0.33
	2	31/33/52/66	SD	0.33	1	0.17	0.33	0.17	0.33
	3	33/66	Nl sq epith	0.25	1	0.25	1	0.17	0.33
4	1	51	MoD	0.25	1	0.17	0.33	0	0
	2	Neg	MiD	0.33	1	0.17	0.67	0.25	0.67
	3	Neg	Nl sq epith	0.25	0.67	0.50	1	0.17	0.67
5	1	16/31	SD	0.42	1	0	0	0	0
	2	Neg	MiD	0.08	1	0.25	0.67	0.08	0.33
	3	Neg	Nl sq epith	0.08	0.67	0.17	0.67	0	0
	4	16	Sq metaplasia	0.17	0.67	0.25	0.67	0	0
6	1	16/18	SD	0.75	1	0	0	0	0
	2	16/18/52	SD + endocx atypia	0.50	1	0	0	0	0
	3	16/18	SD	0.42	1	0	0	0	0
	4	16	MiD	0.42	0.33	0.17	0.67	0	0
7	1	16	CIS	0.75	0.67	0	0	0.50	0.33
	2	16	MiD	0.17	0.67	0.25	0.67	0.17	0.33
	3	16	Nl sq epith	0.17	0.67	0.25	0.67	0.17	0.33
	4	16	MIC	0.75	0.67	0	0	0.75	0.33
8	1	16	SD	0.75	0.67	0.17	0.33	0.17	0.33
	2	16	SD	0.67	0.67	0.17	0.33	0.25	0.33
	3	16	Nl sq epith	0.17	0.67	0.25	0.67	0	0
	4	16	MIC	0.92	0.67	0	0	0	0
	5	16	MIC	0.75	0.67	0	0	0	0

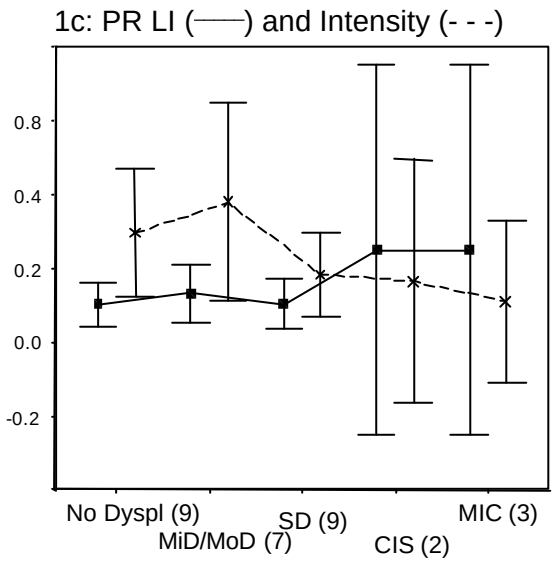
Nl = normal, sq = squamous, epith = epithelium, endocx = endocervical, MiD = mild dysplasia, MoD = moderate dysplasia, SD = severe dysplasia, CIS = carcinoma in situ, MIC = microinvasive carcinoma, ER = estrogen receptor, PR = progesterone receptor, LI = Labelling index, Int = intensity of staining.

Figure 1.

The MIB1-, ER-, and PR- LI (¦) and intensity of staining (x) in relation with the severity of the dysplasia in the biopsy. (Vertical bars represent 2 standard errors of the mean).





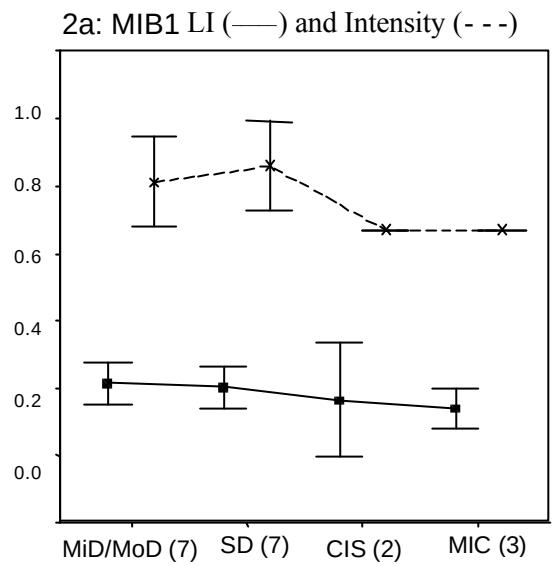


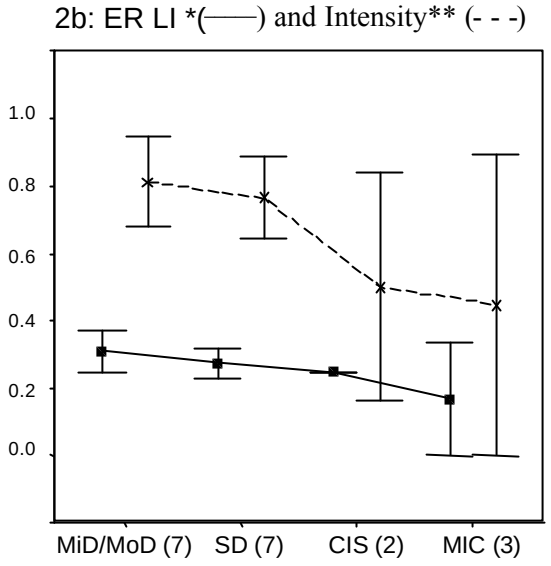
No Dyspl = no dysplasia

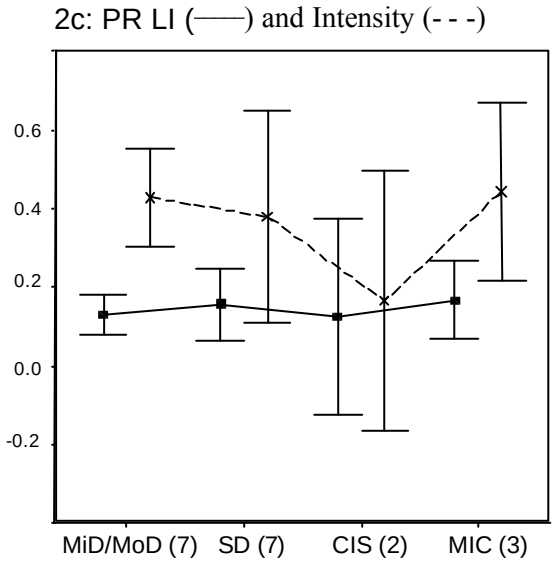
** = Significant relation of the MIB1 LI (p<0.001), and a significant inverse relation of the ER LI (p<0.001 with Jonckheere-Terpstra and p=0.005 with Kruskal-Wallis) and ER intensity of staining (p<0.001), with the severity of the lesion.*

Figure 2.

The MIB1-, ER-, and PR- LI (¦) and intensity of staining (x) in normal epithelium adjacent to dysplasia in relation with the severity of that dysplasia. (Vertical bars represent 2 standard errors of the mean).







** = Inverse relation of the ER LI with the severity of the adjacent dysplasia. (p=0.05 with Jonckheere-Terpstra, p=0.13 with Kruskal-Wallis)*

*** = Inverse relation of the ER Intensity of staining with the severity of the adjacent dysplasia (p=0.03 with Jonckheere-Terpstra, p=0.29 with Kruskal-Wallis).*

ER expression.

The Jonckheere-Terpstra test showed a highly significant decrease of the ER LI ($p<0.001$), and of the ER intensity of staining ($p<0.001$) with increasing severity of the dysplasia in the biopsy (Figure 1b). The ER expression was 0 in all biopsies with MIC or CIS, and in four of the nine biopsies with SD. All biopsies with MoD or less showed ER expression (Table 1). All areas of normal epithelium adjacent to dysplastic lesions, except for one area next to MIC (patient 7), showed a positive ER expression. The Jonckheere-terpstra test showed a weakly significant ($p=0.03$) decrease in the ER intensity of staining in normal epithelium adjacent to dysplasia with increasing severity of that adjacent dysplasia, however the Kruskal-Wallis test disclosed that the differences between the diagnostic subgroups are not significant (Figure 2b). There was no difference in the ER-LI or -intensity of staining regarding the presence of more severe dysplastic lesions in other parts of the cervix, the presence of HPV 16, other HPV genotypes, or multiple hr-HPV infections.

PR expression.

The Jonckheere-Terpstra test for ordered alternatives did not show a significant relation of the PR LI ($p=0.16$) and the PR intensity of staining ($p=0.98$) with the severity of the dysplasia in the biopsy (Figure 1c). No staining for the PR (LI=0) was observed in 3 biopsies with normal epithelium/squamous metaplasia, in 2 biopsies with MiD/MoD, in 4 biopsies with SD, in 1 biopsy with CIS, in 2 biopsies with MIC (Table 1), and 3 areas of normal epithelium adjacent to dysplasia. There was no significant relation of the PR -LI ($p=0.61$) or -intensity of staining ($p=0.60$) in normal epithelium with the severity of the adjacent dysplasia, the presence of HPV 16, other hr-HPV genotypes, or multiple hr-HPV genotypes in the biopsy (Table 1, Figure 2c).

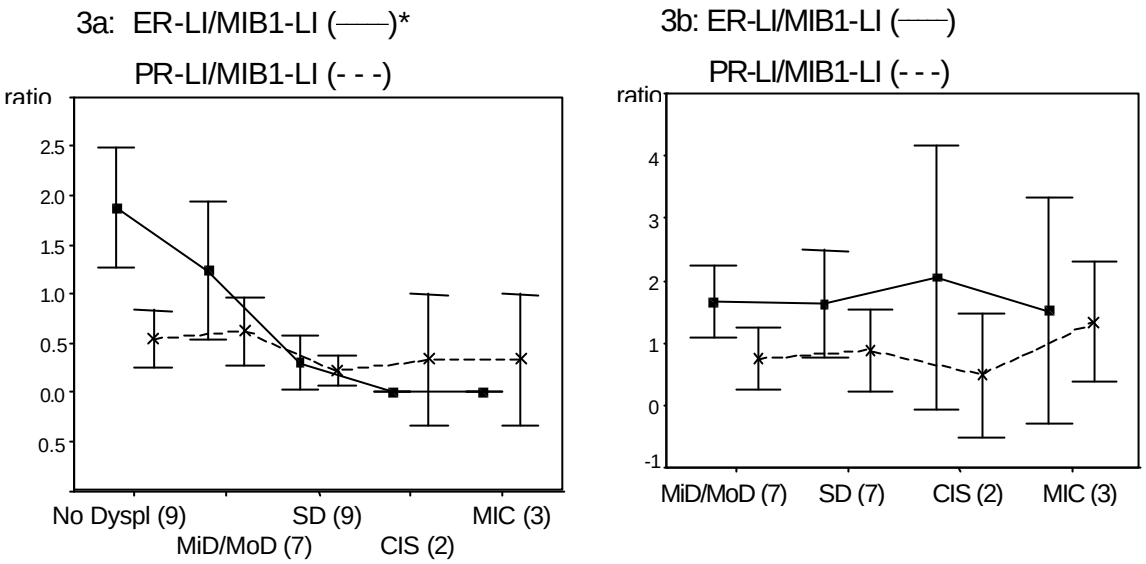
ER-LI/MIB1-LI and PR-LI/MIB1-LI ratios.

Figure 3 shows a highly significant decrease ($p<0.001$) of the ER-LI/MIB1-LI ratio (3a) with increasing severity of the dysplasia. The PR-LI/MIB1-LI ratio did not show any relation with the severity of the dysplasia ($p=0.11$).

In normal epithelium directly adjacent to dysplasia no alterations in the ER-LI/MIB1-LI ($p=0.64$) and PR-LI/MIB1-LI ($p=0.21$) ratios with increasing severity of the adjacent dysplasia were observed (3b).

Figure 3.

The ER-LI/MIB1-LI, and PR-LI/MIB1-LI in relation with the severity of the dysplasia (figure 3a), and in normal epithelium adjacent to dysplasia in relation with the severity of the dysplasia (figure 3b). (Vertical bars represent 2 standard errors of the mean).



** P<0.001 with Jonckheere-Terpstra and with Kruskal-Wallis*

DISCUSSION.

This study confirms the significant increase in MIB1 LI with increasing severity of cervical dysplasia, as described previously [9-11], while no relation between the MIB1 intensity of staining and the severity of the dysplasia was observed.

The ER LI and intensity of staining showed a significant inverse relation with the severity of the dysplasia in this study. In the literature, some studies showed a down regulation of the ER expression in dysplasia in relation with HPV 16 or 18 infections and less with HPV 31, 33, or 35 [8,16,17], while others did not find a relation between hr-HPV infections and ER expression at all [14,18]. Normally, a variation in ER (and PR) expression in relation with hormonal fluctuations can be observed in normal cervical epithelium. ER is expressed in basal cells of the epithelium throughout the menstrual cycle, while the ER expression in parabasal cells is higher in the follicular phase and less in the luteal phase or during pregnancy [8]. Indeed, high levels of progesterone (pregnancy, luteal phase) have been shown to decrease ER expression in endometrium and myometrium, and inhibit the proliferative effect of estrogens on the epithelium [24,25]. Furthermore, estrogens that bind the ER inhibit further expression of that ER, while simultaneously the synthesis of new ER and PR is stimulated [24,25]. The observed association between a higher proliferative activity with a decreased ER expression in cervical epithelium during the luteal phase of the menstrual cycle suggests a regulation of the proliferation of normal cervical epithelium by sex steroid hormones [8, 17]. Down regulation of ER expression in dysplasia, as observed in this study, indicates that malignant transformation of cervical epithelium is associated with loss of normal growth control by sex steroid hormones [8, 17]. Indeed, Bulten et al. found in their study of atypical atrophic post-menopausal cervical scrapes that estrogen therapy did not alter the proliferative activity in patients with high-grade CIN, while there was a significant change in proliferative activity in patients without CIN [27].

The PR LI and intensity of staining did not show any relation with the severity of the dysplasia in this study. One study described a significant relationship between the degree of dysplasia and PR expression especially in HPV 16/18 infected lesions, suggesting that progesterone is a co-factor in HPV mediated cervical neoplasia [15]. Others described a large variation in PR expression in dysplastic lesions as well as in invasive cervical cancer, and did like this study not confirm this relation [8,14,17]. Normally, PR expression is induced by estrogens and decreased by progestatives [24]. The fact that the majority of the patients in this study underwent colposcopy with biopsies during the luteal phase, or under the influence of oral progestatives, may have caused the low expression of the PR, but does in

our opinion not explain the lack of any relation between the PR and dysplasia. Recently, two iso-forms of the PR have been isolated, which are each associated with different proteins that play an important role in the response to sex steroid hormones [24]. Whether these iso-forms are responsible for the large variation in the detection of PR expression in normal and dysplastic cervical lesions needs further study.

The MIB1-, ER-, and PR- expression in a dysplastic lesion did not depend on the presence of other more or less severe dysplastic lesions within the same cervix. This indicates that different dysplastic lesions develop relatively independent from each other under the influence of the same, or different hr-HPV genotypes [23,27,28]. The relation between the detection of single and/or multiple hr-HPV genotypes within a single cervix or within a single lesion of these patients has been described and discussed by us in detail previously [23].

The ER intensity of staining in normal epithelium directly adjacent to dysplasia showed a weakly significant inverse relation with the severity of that dysplasia with the Jonckheere-Terpstra test, but this was not confirmed by the Kruskal-Wallis test. So, no convincing differences were observed for the ER LI and ER intensity of staining as well as for the MIB1 LI, the PR LI, or the PR intensity of staining with the severity of the adjacent dysplasia.

The significant inverse relation of the ER-LI/MIB1-LI ratio with increasing severity of the dysplasia may indicate that there is a progressive loss of control of the proliferative activity by sex steroid hormones. In normal epithelium adjacent to the dysplasia, no such relation was observed, indicating that hormonal sensitivity is still present.

Due to the small number of biopsies and patients in this study, larger studies are needed to confirm these data.

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MIB1 AND HIGH-RISK HUMAN PAPILLOMAVIRUS DETECTION AS MARKERS FOR CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE 3 IN CERVICAL SCRAPES

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(Submitted)

ABSTRACT.

Objectives: To assess whether the MIB1 labeling index (LI) or detection of high-risk human papillomavirus (hr-HPV) in cervical scrapes of patients referred with atypical squamous cells of undetermined significance (ASC-US), or scrapes indicating cervical intraepithelial neoplasia (CIN) grade 1 or 2, can be used to identify patients with CIN 3 among these patients.

Methods: In 108 patients referred with ASC-US, CIN 1, or CIN 2 scrapes a large loop excision of the transformation zone (LLETZ) was performed. The MIB1 LI and detection of hr-HPV were performed on AgarCyto cell blocks of the cervical scrapes of these 108 patients taken during the intake consultation. The results were related to the presence of CIN 3 on histopathology.

Results: Stepwise logistic regression analysis showed a significant relation ($p<0.001$) of the MIB1 LI, but not of hr-HPV detection ($p=0.15$), with the degree of CIN. The mean MIB1 LI of patients with CIN 3 was significantly higher than of patients without CIN 3 ($p<0.001$), independent from the presence of hr-HPV, independent from the fact whether LLETZ was performed initially or during follow-up, and independent from the referral cervical scrape result. The sensitivity of the MIB1 LI and hr-HPV detection for the identification of patients with CIN 3 were respectively 82%, and 80%, and the specificity respectively 62%, and 48%.
In a subgroup of patients with an assessable MIB1 LI, the sensitivity and specificity of the MIB1 LI were 96% and 59% respectively.

Conclusions: A high sensitivity of the MIB1 LI for the identification of patients with CIN 3, among patients referred with ASC-US, CIN 1, or CIN 2 scrapes was found in only a subgroup of patients with an assessable MIB1 LI. However, the large number of patients in whom the MIB1 LI was not assessable in the AgarCyto cell block, make this technique unsuitable for clinical practice.

INTRODUCTION.

The majority of cytological abnormalities found in cervical scrapes made in screening programs for cervical cancer prevention, are classified as atypical squamous cells of undetermined significance (ASC-US), or suspect for CIN 1 or 2 [1]. However, not all of these patients have histopathological abnormalities in their cervical biopsies. Cervical intraepithelial neoplasia (CIN) grade 1-3 can be detected in 30%, 70%, and 90% of patients with respectively ASC-US, CIN1, and CIN 2 scrapes [1,2]. CIN lesions often regress spontaneously and studies have shown that CIN 1, 2, and 3 lesions regress within 24 months without treatment in approximately 68%, 47%, and 35% of the cases, respectively [3,4]. There is general agreement that patients with CIN 3 lesions need to be treated as they have a high risk of progression to invasive cancer [1,4]. CIN 3 lesions are detected in approximately 10%, 25%, and 40% of patients with respectively ASC-US, CIN 1, and CIN 2 cervical scrapes [4]. A marker that can differentiate on cervical scrapes between patients with and without CIN 3 may be able to reduce the number of referrals and unnecessary treatment in patients with ASC-US, CIN 1, and CIN 2 scrapes.

CIN is presumed to be caused among other factors, by human papillomavirus infections and is characterized by hyperproliferation of cervical epithelium [5]. MIB1 is a monoclonal antibody that recognizes and binds to the Ki-67 nuclear antigen, which is expressed in cells in the G1, S and G2/M phases of the cell cycle. MIB1 reactivity indicates proliferative activity in those cells. MIB1 can identify and classify CIN in histological specimens, and can differentiate between high-grade CIN (CIN 2 and 3) and atrophic lesions in cervical scrapes of post-menopausal women [5-10]. Data on the use of MIB1 in abnormal cervical scrapes are limited. One study has reported a high sensitivity (89%) and specificity (65%) in the presence of MIB1 positive abnormal epithelial cells in cervical scrapes and the detection of high-grade CIN in subsequent biopsies, and another study showed a correlation between the MIB1 labeling index (LI) in cervical scrapes and the subsequent biopsies [9,11]. No data to confirm these findings have been published thus far.

High-risk human papillomavirus (hr-HPV) detection has been indicated as another marker to identify patients with high-grade CIN, especially in patients with ASC-US scrapes [1].

The present study was performed to assess whether the MIB1 LI or hr-HPV detection in cervical scrapes of patients referred with two consecutive ASC-US scrapes, or a single scrape suspect for CIN 1 or 2 can be used to identify patients with CIN 3.

MATERIAL AND METHODS.

Patients.

In 108 of in total 338 patients referred to the colposcopy clinic of the University Medical Center Nijmegen between April 1997 and January 2000 with either two consecutive ASC-US scrapes, or a single scrape suspect for CIN 1, or CIN 2, a large loop excision of the transformation zone (LLETZ) was performed. All patients were first time referrals from the screening program on cervical cancer prevention. Of these 108 patients, 13 (12%) were referred with ASC-US, 40 (37%) with CIN 1, and 55 (51%) with CIN 2 scrapes. The mean age of these patients was 40 years (29-61 years).

A liquid based cervical scrape was taken during the intake consultation at the colposcopy clinic. This cervical scrape was made using a Cervex Brush® (Rovers, Oss, the Netherlands), stored in Unifix®, and subsequently processed into an AgarCyto cell block, allowing the analysis of multiple parameters as previously described [12]. All patients underwent colposcopy within one month of the intake consultation. LLETZ on the basis of a see and treat policy was performed at the initial colposcopy in 49 patients, who had lesions suspect for CIN 3. Another 59 patients underwent LLETZ during follow-up, because of persistent or progressive lesions at follow-up colposcopy, and/or cytology. The mean interval between the initial colposcopy and the delayed LLETZ (during follow-up) was 11 months (3-24 months).

Immunohistochemistry.

A 3 µm paraffin section of the AgarCyto cell block was stained with HE for routine examination and an adjacent 3 µm paraffin section was stained with MIB1 by a standardized procedure as described previously [5]. All HE stained sections were examined by microscope to identify squamous cell groups of at least 25 cells, which was considered the minimal number of cells needed to be able to assess a MIB1 LI. No squamous cell groups of 25 cells or more were found in sections of 30 of the 108 patients (28%), and as a result the MIB1 LI could not be measured in these patients.

Squamous cell groups of 25 cells or more were subsequently identified in the adjacent MIB1 stained section of 78 patients. The MIB1 LI of small squamous cell groups was scored as the percentage MIB1 stained nuclei of the total number of nuclei within the group. For large squamous cell groups a non-selective line was used. To define a non-selective line, we used a 36 points Merz graticule (type GM1, Leica) [13], placed in one of the ocular tubes of the microscope. The graticule has 6 curved lines, each consisting of 6 semi-circles, disposed in a square lattice. The graticule was projected over each large squamous cell group and all

MIB1 stained and non-stained nuclei of the cell group hitting the lines in the graticule were separately counted. The MIB1 LI was scored as the percentage MIB1 stained nuclei of all nuclei hitting the graticule lines.

SPF_{10} LiPA PCR HPV detection.

HPV detection was performed on the next adjacent paraffin section of the AgarCyto cell block using a highly sensitive short fragment polymerase chain reaction (SPF_{10} PCR) assay [14-16]. In cases with a positive PCR result the PCR product was reverse hybridized on a line probe assay (LiPA) for the simultaneous detection of 25 HPV genotypes. HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68 were regarded as high-risk, and HPV genotypes 6, 11, 34, 40, 42, 43, 44, 54, 70, and 74 were regarded as low-risk [14-16].

Evaluation and analysis.

All 108 patients were analyzed, as well as subgroups of patients. Group 1 consists of the patients who underwent LLETZ during the initial colposcopy (n=49), and group 2 consists of the patients that underwent LLETZ during follow-up colposcopy (n=59). The group of 78 patients with squamous cell groups $\geq$ 25 cells (thus with an assessable MIB1 LI) was analyzed separately. Data were analyzed using the SPSS (version 11) statistical software package. Linear- and logistic- regression analysis and receiver operating characteristics (ROC) curves were used to assess the relation between the detection of CIN 3 and the MIB1 LI and hr-HPV detection. Independent samples t-tests were performed to assess differences in the mean MIB1 LI between patients with and without CIN 3. All values of $p < 0.05$ were considered significant.

The sensitivity and specificity of the MIB1 LI and hr-HPV detection for the identification of patients with CIN 3 were calculated, including the 95% confidence intervals (95% CI). In order to establish a cut-off for the MIB1 LI, data on the mean MIB1 LI for CIN 3 on histology, as described by Bulten et al. were used [5]. The mean MIB1 LI for patients with CIN 3, minus 3 standard deviations ($0.39 - (3 \times 0.06) = 0.21$) was chosen, because theoretically, a sensitivity of > 99% for the detection of CIN 3 can be achieved with this cut-off.

RESULTS.

Detection of CIN 3.

The histopathological diagnoses of the LLETZ specimens of the 108 patients are presented in table 1. CIN 3 was diagnosed in 55 patients (51%), 29 patients were diagnosed at the initial colposcopy, and 26 at follow-up colposcopy. Of the 55 patients with CIN 3, one (2%) was referred with ASC-US, 14 (25%) with CIN 1, and 40 (73%) with CIN 2 scrapes (Figure 1C).

Table 1.

The mean MIB1 LI and standard deviation (SD) for the different subgroups of patients in relation with the histopathological diagnosis.

	Group 1		Group 2			
	Initial LLETZ*	**n=49**	**Follow-up LLETZ****	**n=59**	**All Patients****	**n=108**
No CIN	0.24 (0.34)	2	0.04 (0.07)	5	0.10 (0.18)	7
CIN 1	0.08 (0.13)	8	0.19 (0.20)	6	0.13 (0.17)	14
CIN 2	0.26 (0.19)	10	0.17 (0.19)	22	0.20 (0.19)	32
CIN 3	0.43# (0.25)	29	0.38# (0.21)	26	0.40# (0.24)	55

** p=0.001, and ** p<0.001, significant relation with linear regression analysis of the MIB1 LI with the degree of CIN.*

 # p<0.001, significantly higher MIB1 LI of patients with CIN 3 than of patients without CIN 3 with the independent samples t-test.

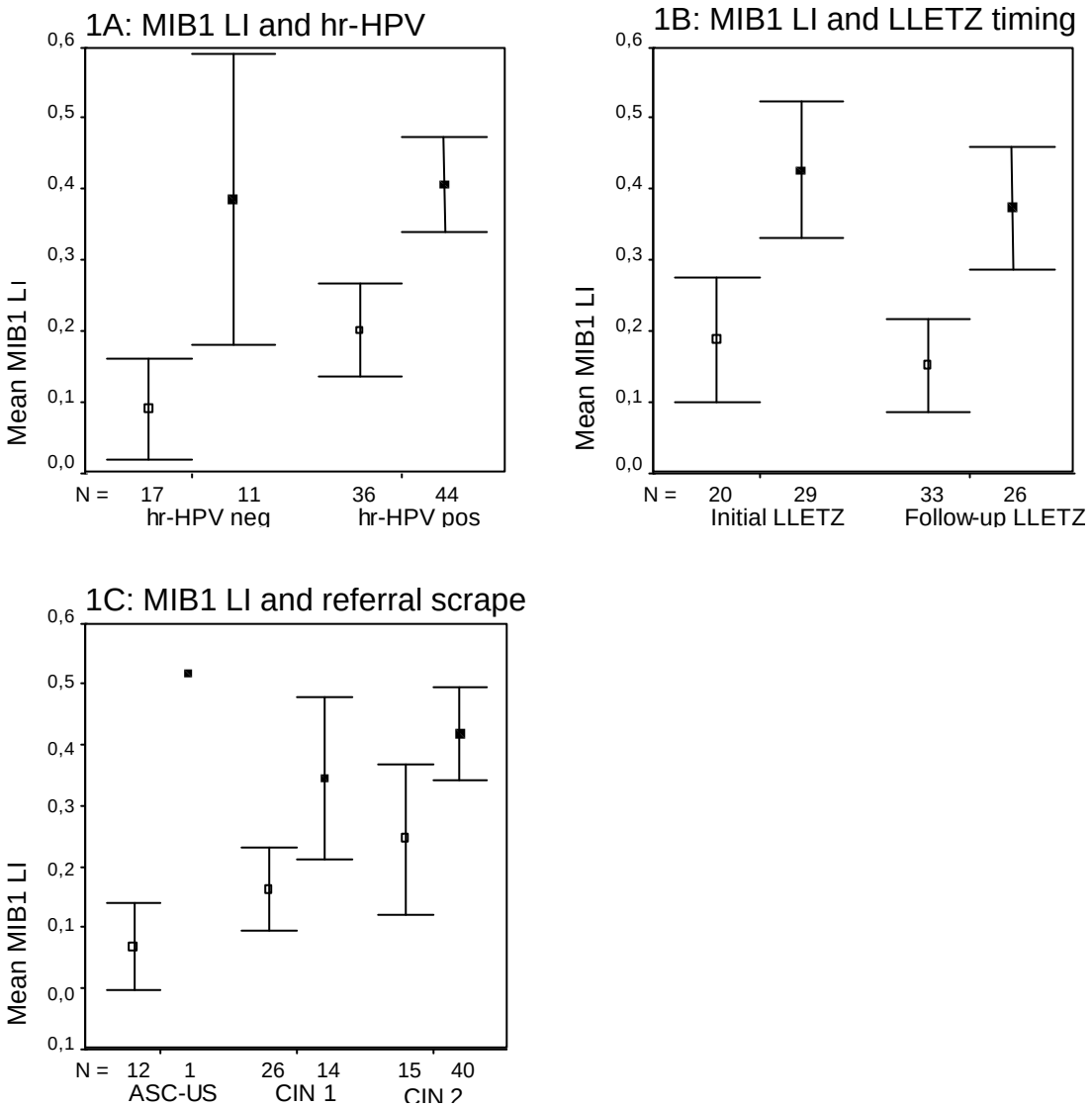
The MIB1 LI and hr-HPV detection.

Table 1 shows a significantly higher mean MIB1 LI ($p<0.001$) of patients with CIN 3 than of patients without CIN 3 in both subgroups. Additionally, linear regression analysis showed a significant increase of the MIB1 LI ($p\leq0.001$) from no CIN towards CIN 3 (Table 1).

Stepwise logistic regression analysis showed a highly significant relation of the MIB1 LI ($p<0.001$) with the presence of CIN 3, while no significant relation was observed between hr-HPV detection and the presence of CIN 3 ($p=0.15$).

Figure 1.

The mean MIB1 LI and the detection of CIN 3 in relation with hr-HPV detection (1A), timing of LLETZ (1B), and the referral cervical scrape (1C). (Bars represent two standard errors of the mean).



1A: Significant difference in the mean MIB1 LI between patients with (¦) and without (?) CIN 3, either hr-HPV-positive (p<0.001), or hr-HPV-negative (p=0.01).

1B: Significant difference in the mean MIB1 LI between patients with (¦) and without (?) CIN 3, either at the initial LLETZ (p<0.001), or at follow-up LLETZ (p<0.001).

*1C: Significant difference in the mean MIB1 LI between patients with **(¦)** and without (?**)** CIN 3 referred with ASC-US (p=0.003), CIN 1 scrapes (p=0.017), and CIN 2 scrapes (p=0.019).*

Figure 1 shows that the MIB1 LI of patients with CIN 3 was significantly higher than of patients without CIN 3, irrespective of the detection of hr-HPV (Figure 1A), irrespective of the fact whether LLETZ was performed initially or during follow-up (Figure 1B), and irrespective of the referral cervical scrape result (Figure 1C).

Figure 2 shows that the area under the ROC-curve (AUC) of the MIB1 LI (0.77 $\pm$ 0.05) is significantly higher than of hr-HPV detection (0.56 $\pm$ 0.06). The AUC of hr-HPV detection was not significantly different from 0.50, indicating that the addition of hr-HPV detection to the MIB1 LI will not improve the test results.

Table 2 shows that 45 of the 55 patients with CIN 3 (82%) and 20 of the 53 patients without CIN 3 (38%) had a MIB1 LI of $\geq$ 0.21. Hr-HPV was detected in respectively 44 (80%) and 30 patients (57%).

Table 3 shows that the MIB1 LI and hr-HPV detection have a similar sensitivity (respectively, 82% and 80%) and a specificity of respectively 62%, and 48%, but the overlap in 95% CI indicates a similar specificity as well.

Analysis of only the 78 patients with cell groups of $\geq$ 25 cells in the AgarCyto cell block showed that CIN 3 was detected in 47 of these patients. A MIB1 LI $\geq$ 0.21 was found in 45 patients with CIN 3 and 21 patients without CIN 3, and hr-HPV was detected in respectively 39, and 23 patients. Table 3 shows a sensitivity of 96% for the MIB1 LI, and 83% of hr-HPV detection in this group, with a specificity of respectively 59%, and 51%.

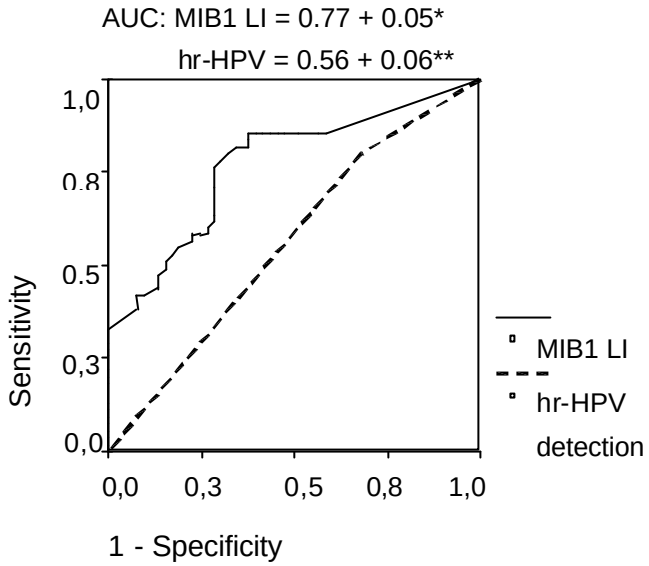
Table 2.

The number and percentage of women diagnosed with CIN 3 in the different subgroups in relation with a MIB1 LI $\geq$ 0.21 and detection of hr-HPV.

Group	Markers	No CIN 3	CIN 3	Total
Group 1	n=	20	29	49
Initial	MIB1 LI $\geq$0.21	9 (45%)	24 (83%)	33 (67%)
LLETZ	Hr-HPV pos.	12 (60%)	23 (79%)	35 (71%)
Group 2	n=	33	26	59
Follow-up	MIB1 LI $\geq$0.21	11 (33%)	21 (81%)	32 (54%)
LLETZ	Hr-HPV pos.	16 (49%)	21 (81%)	37 (62%)
All	n=	53	55	108
Patients	MIB1 LI $\geq$0.21	20 (38%)	45 (82%)	65 (60%)
	Hr-HPV pos.	28 (52%)	44 (80%)	72 (67%)

Figure 2.

Receiver operating characteristics (ROC) curves of the MIB1 LI and hr-HPV detection for the detection of CIN 3 (n=108).



*AUC = Area under the curve, * = Significant difference (p<0.001), and ** = No significant difference (p=0.28) with 0-hypothesis of AUC = 0.5*

Table 3.

The sensitivity and specificity of a MIB1 LI ≥ 0.21 and hr-HPV detection, for the identification patients with CIN 3 (95% CI).

	MIB1 LI ≥ 0.21		**Hr-HPV pos.**	
	Sensitivity	Specificity	Sensitivity	Specificity
All Patients n=108	82% (72-92%)	62% (48-76%)	80% (70-90%)	48% (34-62%)
Group 1, initial LLETZ n=49	83% (69-97%)	55% (33-77%)	79% (64-94%)	40% (18-62%)
Group 2, follow-up LLETZ n=59	81% (66-96%)	67% (51-83%)	81% (66-96%)	51% (33-69%)
Group 3, patients with an assessable MIB1 LI n=78	96% (90-100%)	59% (50-68%)	83% (72-94%)	51% (42-60%)

DISCUSSION.

The appropriate management of patients referred from a screening program for cervical cancer prevention with cervical scrapes classified as ASC-US, suspect for CIN 1, or CIN 2 is still a matter of debate. The MIB1 LI in AgarCyto cell block sections of cervical scrapes in this study showed a highly significant relation with the degree of CIN, and the MIB1 LI of patients with CIN 3, was significantly higher than of patients without CIN 3. In normal epithelium, MIB1 reactivity is only observed in the parabasal and basal cells of the epithelium. MIB1 reactivity in higher epithelial levels is found in patients with CIN, and the MIB1 LI can differentiate well between the different CIN grades on histopathological specimens [5-8, 17-21]. The significant increase in the mean MIB1 LI with higher grades of CIN on histological samples has been described previously [5-8]. This is the first study that shows that an identical significant increase of the MIB1 LI with the degree of CIN can be found in cervical scrapes. This is probably due to the used AgarCyto cell block technique, which largely preserves the histological architecture of the squamous cell groups [12].

In conventional and in monolayer liquid based cervical scrapes, the use of MIB1 LI is limited because cell group architecture is less well preserved in these scrapes, making assessment of a labeling index practically impossible [10,21]. Only a few studies have used MIB1 in conventional cervical scrapes as a marker for high-grade CIN [11,23]. In one study, MIB1 positive cells were identified in the scrapes and if they were dysplastic, they showed a high sensitivity (89%) and specificity (65%) for the detection of high-grade CIN [11]. However, in that study, MIB1 was used for identification only, while cyto-morphometric parameters remained diagnostic. So, MIB1 was used as a facilitator for the detection of the dysplastic cells, which seems of limited additional benefit to conventional cytological assessment. Still, other studies have stressed that MIB1 is useful as an adjunct and complimentary tool to conventional cytology, especially in scrapes that are difficult to assess, but these studies did not investigate MIB1 as a primary marker for CIN 3 [9, 10, 24]. One other study used four or more MIB1 positive cells per conventional scrape as a cut-off for the detection of high-grade CIN, and did show a 71% correlation between the MIB1 reactivity and the follow-up results of 49 patients [23]. We used the AgarCyto cell block method, that has the advantage of a more objective assessment of the MIB1 LI. Additionally, assessment of the MIB1 LI in AgarCyto cell blocks resembles that in histopathological specimens, and due to this resemblance, a similar sensitivity in identifying patients with CIN 3 was expected.

The sensitivity of the MIB1 LI for the detection of CIN 3 was 82% in this study, and a similar sensitivity of hr-HPV detection was found. The specificity of the MIB1 LI and hr-HPV

detection was also similar and rather low. Different factors may cause a false positive MIB1 LI (MIB1 LI $\geq$ 0.21) in the patients without CIN 3. Firstly, it may be the result of patients with CIN 2 in this group, as they have a mean MIB1 LI that is little lower than the used cut-off. Secondly, it may be the result of tangentially cut squamous cell groups in certain sections, as has been reported for tissue sections [22]. This leads to an over-representation of the MIB1 positive basal and parabasal layers and results in a higher MIB1 LI in these cell groups.

It has been suggested in the literature that the MIB1 LI (Ki-67 index) is an excellent measure of a neoplasm's proliferation, and that MIB1 immunoreactivity may provide information about what effect HPV is having on particular cells [20,21]. A hr-HPV infection causing CIN lesions by hyperproliferation leads to full thickness immunoreactivity of MIB1 staining [25]. With this in mind, we expected a higher specificity of the MIB1 LI than of hr-HPV detection for the identification of patients with CIN 3, but due to the small numbers, the 95% CI showed overlap. The absence of detectable hr-HPV in 11 of the 55 patients with CIN 3 may be the result of a sampling error. However, hyperproliferation (MIB1 LI of 0.28-0.83) was diagnosed in 9 of these 11 hr-HPV-negative patients with CIN 3, and this is supposed to be the result of an hr-HPV infection [8]. Alternatively it is possible that certain CIN lesions have developed in the absence of hr-HPV, as has been described recently [26].

The MIB1 LI in the 78 patients with cell groups of $\geq$ 25 cells in the AgarCyto cell block showed a higher sensitivity (96%) for the identification of patients with CIN 3 than hr-HPV detection (83%). The specificity in this subgroup was the same as in the total group. This indicates that when the MIB1 LI can be assessed, it may be used as a triage tool to identify patients with CIN 3. However, in a large number of patients (28%) the MIB1 LI could not be assessed. The lack of sufficiently large squamous cell groups in the in the Agar Cyto cell block sections of these 30 patients may be due to a sampling error of the cervical scrape itself and/or due to the Agar Cyto cell block procedure. This fact, together with the rather extensive laboratory procedure that is needed to prepare an AgarCyto cell block section of a cervical scrape, makes this technique unsuitable for clinical practice.

In conclusion, the MIB1 LI in AgarCyto cell blocks of cervical scrapes in a subgroup of patients with an assessable MIB1 LI shows a high sensitivity for the identification of patients with CIN 3 among patients referred with ASC-US, CIN 1, or CIN 2 scrapes. However, the large number of patients in whom the MIB1 LI was not assessable in combination with the rather low sensitivity and specificity of the MIB1 LI or hr-HPV detection in AgarCyto cell block sections of the total group make this technique unsuitable for clinical practice.

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THE ROLE OF GENOTYPE SPECIFIC HUMAN PAPILLOMAVIRUS DETECTION IN DIAGNOSING RESIDUAL CERVICAL INTRAEPITHELIAL NEOPLASIA

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ABSTRACT

Objectives: We assessed prospectively whether residual cervical intraepithelial neoplasia (CIN) after treatment for high-grade CIN can be predicted by genotype-specific high-risk HPV (hr-HPV) detection in follow-up cervical scrapes.

Methods: A broad spectrum, highly sensitive SPF_{10}-LiPA-PCR HPV detection technique was used on cervical scrapes before Large Loop Excision of the Transformation Zone (LLETZ), on the LLETZ biopsy, and on follow-up scrapes of 90 patients treated for high-grade CIN.

Results: Hr-HPV was detected in the biopsy of 93% (n=84) of the patients and in the follow-up scrapes of 48% (n=43) of the patients. In 12 patients, genotype-specific hr-HPV persistence was detected in both follow-up scrapes. In 10 patients, residual CIN was detected. In 5 of these patients (including all patients with residual CIN 3), the follow-up scrapes showed genotype specific hr-HPV persistence. In 2 patients, a different hr-HPV was detected, and 3 patients had hr-HPV-negative follow-up scrapes. Conventional cytological follow-up was abnormal in 13 patients including all 10 patients with residual CIN. The negative predictive value (NPV) of hr-HPV detection on follow-up scrapes was high (94%). Repeat detection of genotype-specific hr-HPV showed a lower sensitivity and NPV than repeat detection of any hr-HPV, but its specificity was higher. Repeat conventional cytological follow-up showed the highest sensitivity and NPV.

Conclusions: The presence of hr-HPV in cervical scrapes after LLETZ for high-grade CIN is a risk factor for the presence of residual CIN. Hr-HPV genotype-specific persistence is specifically present in patients with residual CIN 3. However, hr-HPV detection cannot predict or exclude the presence of residual CIN in the individual patient and additional procedures remain necessary.

INTRODUCTION.

High-risk genotypes of human papillomaviruses (hr-HPV) are etiologically related to the development of cervical intraepithelial neoplasia (CIN) and cervical cancer. Hr-HPV has been detected in more than 99% of all squamous cell carcinomas of the uterine cervix [1]. Infections with hr-HPV, especially genotype-specific persistence of cervical hr-HPV infections, have been related to a 100-300-fold risk of developing CIN 3 lesions [2,3].

Screening programs for cervical cancer, in combination with treatment of (pre)-malignant lesions, have reduced both the incidence and mortality of cervical cancer [4]. Large loop excision of the transformation zone (LLETZ) has been shown to be highly effective in treatment of CIN lesions [5,6]. However, residual CIN can be detected in 2-16% of patients after treatment of CIN [7-10]. Due to false negative follow-up cervical scrapes, several sequential scrapes are necessary to assure the absence of residual CIN. Therefore, research is focused on identifying new markers to detect residual CIN. Detection of hr-HPV in follow-up cervical scrapes of patients treated for CIN may prove to be such a marker as it has a high negative predictive value [7-9,11]. However, a large number of patients with hr-HPV-positive follow-up scrapes did not have residual CIN [7-9]. All these patients would need additional procedures to exclude or diagnose residual CIN. Therefore, it remains uncertain whether hr-HPV detection can be of benefit in the follow-up after treatment for CIN.

Our study investigated whether genotype-specific persistence of hr-HPV in follow-up cervical scrapes after 3 and 6 months, detected with an ultra sensitive hr-HPV detection method, can identify patients with residual CIN after treatment for high-grade CIN.

MATERIAL AND METHODS.

Prospectively, all patients (n=90) treated between April 1997 and November 1999 with LLETZ for CIN 2 or 3 at the colposcopy clinic of the University Medical Center (UMC) Nijmegen were included. All patients were first time referrals from the cervical cancer-screening program.

One liquid based cervical scrape preceding LLETZ by less than 1 month, and 2 follow-up cervical scrapes, 3 and 6 months after LLETZ, were taken of each patient. All cervical scrapes were taken using the Cervex brush® (Rovers Medical Devices B.V., Oss, the Netherlands) and were processed into AgarCyto cell blocks, allowing for multiple analysis as previously described [12]. In addition to the liquid-based scrapes, conventional cervical scrapes were made during each visit for diagnosis and follow-up. HPV detection was

performed on all liquid-based cervical scrapes and on the biopsies using a broad-spectrum short fragment polymerase chain reaction (SPF_{10} PCR). Subsequent HPV genotyping was performed via a reverse hybridization line probe assay (LiPA), allowing for simultaneous typing of 25 genotypes of HPV, including hr-HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 54, 56, 58, 59, and 68. This HPV detection test is highly sensitive, specific and reproducible and has been clinically validated [13-15].

All patients included in this study underwent a repeat colposcopy 6 months after LLETZ. A repeat LLETZ was performed in 13 patients in whom residual CIN was suspected on the basis of a conventional follow-up cervical scrape interpreted as CIN. In 12 of these patients, a lesion suspect for CIN was present at colposcopy. Histopathological examination confirmed the presence of residual CIN in 10 of these 13 patients and these patients were regarded as having residual CIN. In three patients no CIN was detected in the second biopsy, and they had normal follow-up cervical scrapes there after. The other 77 patients had a median conventional cytologic follow-up of 32 months (24-47 months). Follow-up scrapes were taken, either 12, 24, and 36 months after LLETZ in patients with normal follow-up scrapes, or every six months in patients with minimally abnormal scrapes followed by normal cervical scrapes. None of these patients was diagnosed with recurrent or residual CIN during further follow-up.

A patient was considered to have genotype-specific hr-HPV persistence when at least 1 hr-HPV genotype detected either before LLETZ, or in the LLETZ biopsy was also detected in a follow-up cervical scrape.

The long-term (median 32 months) conventional cytologic follow-up in combination with the repeat colposcopy six months after LLETZ was used as the gold standard for the detection of residual CIN in this study. The sensitivity, specificity, negative and positive predictive values for detection of residual CIN by (genotype-specific) HR-HPV testing at 3 and 6 months after LLETZ and by conventional cytology at 3 and 6 months after LLETZ were calculated using the defined gold standard. The performance of HR-HPV detection in the 2nd (6-month) follow-up scrape was reported on separately, since in most colposcopy clinics the first follow-up appointment is scheduled 6 months after LLETZ. Regarding the small number of patients with residual CIN in our study, 95% confidence intervals (CI) were calculated as well.

RESULTS.

Histopathological examination of the LLETZ biopsies revealed CIN 2 in 18 patients and CIN 3 in 72 patients. Hr-HPV genotypes were detected in the cervical scrapes preceding LLETZ of 71 patients (79%, CI 71-87%), and in the biopsy specimen of 84 patients (93%, CI 88-98%). All patients with hr-HPV genotypes in the cervical scrape before LLETZ did have hr-HPV genotypes in the LLETZ biopsy, and in 69 patients (82%, CI 74-90%) the scrape and the biopsy contained at least 1 identical hr-HPV genotype.

Table 1.

Hr-HPV detection in the LLETZ biopsy and in the follow-up cervical scrapes.

hr-HPV detection	hr-HPV positive	Multiple hr-HPV genotypes	Identical hr-HPV genotypes	Different hr-HPV genotypes
LLETZ Biopsy	84 (93%)	47 (52%)	n.a.	n.a.
1st Follow-up scrape	34 (38%)	5 (14%)#	23* (68%)#	11* (32%)#
2nd Follow-up scrape	27 (30%)	6 (21%)#	15* (56%)#	12* (44%)#
Both Follow-up scrapes	18 (20%)	2 (11%)#	12* (67%)#	6* (33%)#
Either one Follow-up scrape	43 (48%)	12 (27%)#	28* (65%)#	15* (35%)#

*n.a. = not applicable. - * Compared with hr-HPV detection in the LLETZ biopsy. - # % of the number of hr-HPV positive cervical scrapes.*

Additional hr-HPV genotypes were detected in the biopsy specimen of 30 patients (33%) when compared with the cervical scrape before LLETZ, while in 7 patients (8%) an hr-HPV genotype detected in the cervical scrape before LLETZ was not detected in the biopsy.

Multiple hr-HPV genotypes were detected in the biopsies of 47 patients (52%, CI 47-57%), ranging from 2 to 5 different genotypes. There were no significant differences in the prevalence of (multiple) hr-HPV genotypes between patients with CIN 2 and CIN 3.

Table 1 displays the detection of hr-HPV in the LLETZ biopsy and in the follow-up cervical scrapes. Hr-HPV was detected in at least one follow-up cervical scrape of 43 patients (48%, CI 43-53%). In 18 patients (20%, CI 12-28%) both follow-up cervical scrapes contained hr-HPV. In 12 of these 18 patients, both follow-up scrapes contained identical hr-HPV genotypes as detected in the biopsy, and in 5 patients residual CIN was detected.

The first follow-up cervical scrape of 34 patients (38%, CI 33-43%) contained hr-HPV (Table 2). The hr-HPV genotype was identical to the hr-HPV genotype in the LLETZ biopsy in 23 of these hr-HPV-positive patients (68%), while in the other 11 patients (32%) different hr-HPV genotypes were detected. hr-HPV was detected in the second follow-up cervical scrape of 27 patients (30%, CI 21-40%). This follow-up scrape contained an hr-HPV genotype identical to the genotype(s) in the LLETZ biopsy in 15 patients (56%), and a different hr-HPV genotype in the other 12 patients (44%).

HPV 16, 18 and 31 were the most prevalent HPV genotypes detected. HPV 18 and 54 showed a relatively higher frequency after than before LLETZ.

At least 1 of the 2 follow-up cervical scrapes was suspect for CIN in 13 patients. A repeat LLETZ was done in all 13 patients, and residual CIN was detected in ten patients (11%). The second LLETZ biopsy revealed CIN 1 in 3 patients, CIN 2 in 4 patients, CIN 3 in 3 patients, and repair reactions or atypical metaplasia in 3 patients.

Hr-HPV genotype-specific persistence was detected in 5 patients with residual CIN, 3 with residual CIN 3 and 2 with residual CIN 2. A different hr-HPV genotype (compared with the first biopsy) was detected in the follow-up scrapes of 2 of the 3 patients with residual CIN 1. In 1 patient with residual CIN 1 and in 2 patients with residual CIN 2, no hr-HPV was detected in the follow-up cervical scrapes (Table 2). The long-term conventional cytologic follow-up of the other patients with genotype-specific hr-HPV persistence showed normal cervical scrapes in all patients. None of the other 77 patients was diagnosed with residual or recurrent CIN, or had recurrent abnormal cervical scrapes during the 32 months of further cytological follow-up.

Table 2 *Hr-HPV detection in patients with residual CIN (patient 1-10) and patients with genotype specific Hr-HPV persistence in both cervical scrapes with or without residual CIN (patient 7-18).*

Patient	Original lesion	hr-HPV genotype detected in				Residual lesion
		Scrape before LLETZ	LLETZ biopsy	1st FU scrape	2nd FU scrape	
1	CIN 2	52	52	16	NEG	CIN 1
2	CIN 2	NEG	NEG	NEG	NEG	CIN 2
3	CIN 2	52	52,58	NEG	NEG	CIN 2
4	CIN 3	16,53	16,52,53	39	39	CIN 1
5	CIN 3	51,56	51,56	NEG	NEG	CIN 1
6	CIN 3	31,51	31,58	58	NEG	CIN 2
7	CIN 2	56	56	56	56	CIN 2
8	CIN 3	16	16	16	16	CIN 3
9	CIN 3	16,18	18	18	18	CIN 3
10	CIN 3	16	16	16	16	CIN 3
11	CIN 2	31	31,44,54	31,52	31,52	no 2nd biopsy
12	CIN 3	16	16	16	16	no 2nd biopsy
13	CIN 3	31,33	31,33	33	16,33	no 2nd biopsy
14	CIN 3	31	31,44,54	54	54	no 2nd biopsy
15	CIN 3	NEG	31	31	31	no 2nd biopsy
16	CIN 3	16	16,54	18,42,54	18,42,54	no 2nd biopsy
17	CIN 3	16	16,51	51	16	no 2nd biopsy
18	CIN 3	16,51,58	16,58	16,51	58	no 2nd biopsy

Table 3 displays the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for residual CIN by hr-HPV detection in either one of the follow-up cervical scrapes, by hr-HPV detection in the 6-month (2nd) follow-up cervical scrape and by conventional cytologic follow-up scrapes. hr-HPV detection in either one of the follow-up scrapes showed a NPV of 94%, but its PPV was low (16%), indicating a high number of false positives. Genotype-specific hr-HPV persistence had a lower sensitivity and NPV than hr-HPV detection in general, while its PPV was comparable and its specificity higher. (Genotype-specific) hr-HPV detection in the 6-month follow-up scrape showed a higher PPV and specificity but a lower sensitivity and NPV than (genotype-specific) hr-HPV detection in either one of the follow-up scrapes. Conventional cytologic follow-up scrapes showed the highest sensitivity (100%) and PPV (77%). All patients with residual CIN 3 were detected with genotype-specific hr-HPV persistence as well as with conventional cytology.

Table 3.

Sensitivity, specificity, PPV, and NPV for the detection of residual CIN (n=10) by hr-HPV testing in either the 1st or 2nd follow-up scrape, by hr-HPV testing in the 2nd follow-up scrape only, and by conventional cytologic follow-up scrapes. (95% CI)

	Sensitivity	Specificity	PPV	NPV
Either one FU scrape with hr-HPV	70% (43-97%)	55% (44-66%)	16% (6-26%)	94% (91-98%)
Either one FU scrape with genotype-specific hr-HPV persistence	50% (19-81%)	71% (61-81%)	14% (2-26%)	92% (85-99%)
Conventional follow-up cytology indicating CIN	100% (94-100%)	96% (92-100%)	77% (54-100%)	96% (92-100%)
The 2nd (6-month) FU scrape with hr-HPV	50% (19-81%)	73% (63-83%)	19% (4-34%)	92% (85-99%)
The 2nd FU scrape with genotype-specific hr-HPV persistence	40% (11-69%)	86% (80-92%)	27% (5-49%)	81% (72-90%)

DISCUSSION.

This study confirms that (genotype-specific) hr-HPV detection in the follow-up of patients treated for high-grade CIN with LLETZ has a high NPV. This high NPV has been reported to be useful in the exclusion of patients at risk for residual CIN [7-9]. However, due to the low prevalence of residual CIN after LLETZ in this study (11%), as well as in the literature [5,6,9], most tests will have a high NPV. To assess whether HR-HPV detection is useful in excluding or diagnosing residual CIN, its sensitivity and specificity have to be evaluated.

The sensitivity of hr-HPV detection in diagnosing residual CIN in this study was lower than in other studies [7-9]. This is probably caused by the inclusion of patients with a hr-HPV-negative cervical scrape at study entrance. These patients were excluded in other studies [7-9]. After exclusion of all entered hr-HPV-negative patients in this study, still 2 patients with residual CIN would not have been identified with hr-HPV detection alone. This is comparable with 2 of 29 patients in an other study [9].

Hr-HPV genotype-specific persistence showed a lower sensitivity regarding the presence of residual CIN than hr-HPV detection in general. This is caused by the presence of hr-HPV genotypes in the follow-up scrapes, which were not detected before LLETZ. In other studies, a comparable discrepancy between the hr-HPV genotypes detected in the original CIN lesion and in the follow-up cervical scrapes was found [7,9]. Genotype-specific hr-HPV persistence identified all patients with residual CIN 3 in this study, while different hr-HPV genotypes were detected in 2 patients with residual CIN 1. These different hr-HPV genotypes may be considered newly acquired causing a (new) CIN lesion [7,10]. On the other hand, the 6 months between the first and second biopsy seem rather short to develop a new CIN 1 lesion. A coexisting CIN 1 lesion (containing a different hr-HPV) next to a high-grade CIN lesion, which is removed by LLETZ, has been described before and may provide a more likely explanation [17].

The specificity of hr-HPV detection in general, and of genotype specific hr-HPV persistence varied from 55-91% and is lower than in other studies [7,9]. The high number of patients with hr-HPV-positive follow-up scrapes, in whom no residual CIN was detected, may have caused the rather low specificity and PPV of hr-HPV detection. The higher specificity of genotype-specific hr-HPV persistence compared with hr-HPV detection in general is caused by a lower number of patients with false-positive follow-up scrapes. This did not result in a higher PPV because of the lower sensitivity.

The higher number of patients with hr-HPV-positive follow-up scrapes in our study compared with other studies [7,9] may be caused by the higher sensitivity of the used SPF_{10} LiPA PCR

HPV detection method that was used. This detection method was validated before and was considered ultra-sensitive especially for the detection of multiple hr-HPV genotypes [13-15,19,20]. Indeed, we detected multiple hr-HPV genotypes in almost half of the patients. Despite performing hr-HPV detection on a mixture of 4 samples of the biopsy and the high sensitivity of the test, 6 patients (7%) remained hr-HPV-negative on all occasions. This indicates that some patients may develop high-grade CIN without any detectable hr-HPV [16], and that these hr-HPV-negative patients (like 1 patient with residual CIN in our study) may cause false-negative results when hr-HPV detection is used in follow-up after treatment of high-grade CIN.

As was found by others, the number of patients with hr-HPV-positive follow-up scrapes declines with time after LLETZ [9,18], as does the number of patients with hr-HPV genotype-specific persistence. The PPV and specificity of hr-HPV detection in the 6-month follow-up scrape were indeed higher than of hr-HPV detection in both the 3- and 6-month follow-up scrapes because a longer follow-up is related to less false-positive tests. But in 2 patients with residual CIN in our study, only the first follow-up scrape was hr-HPV-positive, resulting in a lower sensitivity and NPV of the 6-month follow-up scrape. Another study did not find such a decline in sensitivity with a longer follow-up [9]. However, the patient and the gynecologist may prefer to exclude residual CIN early in the follow-up period and may not choose to wait.

Conventional cytology showed the highest sensitivity (100%) and specificity (96%) in diagnosing residual CIN in our study. The use of a cervex brush in combination with an endocervical brush for the follow-up scrapes may have contributed to this fact. In the literature, different sensitivities of cytologic follow-up have been reported. One study described a 100% sensitivity of conventional cytology [7], while another study described a sensitivity of only 60% [9]. We used colposcopy with long-term conventional cytologic follow-up as the gold standard in detecting residual CIN. This fact makes us cautious in interpreting the performance of conventional cytology by itself. Theoretically, a small residual lesion may be missed with conventional cytology and/or colposcopy, while a persistent hr-HPV infection is detected. This fact would decrease the sensitivity of cytology and increase the sensitivity of hr-HPV detection. Another reason to be cautious in interpreting the performance of conventional cytology is the overlap in 95%-confidence intervals of cytology and hr-HPV detection regarding the sensitivity and the NPV in our study. However, the differences in specificity and PPV without overlap of the 95% confidence intervals seem to favor cytology, despite the small numbers.

In conclusion, a positive hr-HPV test after LLETZ should be considered a risk factor for residual CIN. The high number of hr-HPV positive follow-up scrapes without the presence of residual CIN limits its use during the early follow-up. Genotype-specific hr-HPV detection especially identifies patients with residual CIN 3, has less false-positive results and may be useful in the early follow-up. However, additional procedures like conventional cytology and/or colposcopy remain necessary to diagnose or exclude residual CIN.

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SUMMARY AND FINAL CONSIDERATIONS

Chapter 1.

Prevention of cervical cancer by detection and treatment of pre-malignant stages (i.e. cervical intraepithelial neoplasia (CIN)) is practiced worldwide, by population based screening programs. These screening programs predominantly use exfoliative cytology and/or colposcopy to detect and subsequently treat CIN lesions in order to prevent progression towards cervical cancer.

Chapter 1 gives an overview of the relationship between high-risk human papillomavirus (hr-HPV) infections, CIN, and cervical cancer. Epidemiological and clinical studies have demonstrated that hr-HPV infections play a major role in the development of CIN lesions and cervical cancer. Infections with hr-HPV may initiate a cascade of cell cycle disturbances, leading via multiple repetitive clonal events to the development of cervical cancer. This thesis focuses mainly on the clinical value of hr-HPV detection in the management of patients with CIN.

Chapter 2.

Screening for cervical cancer causes anxiety in the screened population. Patients referred for colposcopy have extremely high levels of anxiety on the Spielberger State and Trait Anxiety Inventory. Chapter 2 shows that these high levels of anxiety are mainly caused by a fear for cancer and/or the colposcopy, but the level of anxiety is not related to the severity of the abnormality in the scrape (i.e. the risk of cervical cancer). Patients who lack the support of a partner, who experience long waiting times, who receive insufficient information, and who experience anxiety, sadness and/or anger show the highest anxiety levels. Colposcopy clinics, in cooperation with referring primary care centres need to evaluate the referral system in order to: (1) provide adequate information already at the screening level (i.e. the general practitioner), (2) reduce colposcopy clinic waiting times, and (3) identify and support patients with high anxiety levels. Additionally, cytological markers that can differentiate, already before referral for colposcopy, between patients with or without a risk for CIN 3 or cervical cancer, may reduce the number of referrals and thus prevent anxiety in those patients.

Chapter 3.

The current Dutch guidelines advise a colposcopy in all patients with two consecutive ASC-US scrapes, or a single scrape indicating CIN. The 'see and treat' policy practiced in many colposcopy clinics often results in unnecessary loop excisions. Chapter 3 shows that an aggressive management of patients referred with ASC-US, treating all colposcopic abnormalities, leads to a faster normalization of the follow-up cervical scrapes than an expectative management, treating only colposcopically suspected CIN 3 lesions. However, no long-term benefit of an aggressive management was found, but the number of loop excisions in the aggressive management group was almost 5-fold.

Detection of hr-HPV has been advocated as a triage tool in patients with ASC-US scrapes, referring only hr-HPV-positive patients for colposcopy. This chapter also shows that triage with hr-HPV results in referral of all patients with CIN 3, but not of all patients with CIN 1 and 2. This means that as long as it has not been proven that hr-HPV-negative CIN 1 and 2 lesions always regress, all hr-HPV-negative patients need follow-up as well. Additionally, the low positive predictive value (PPV) and specificity of hr-HPV detection indicates that still many patients without CIN are being referred for colposcopy. These facts make the odds for introduction of hr-HPV detection in patients with two consecutive ASC-US scrapes in the Netherlands not yet favourable.

Chapter 4.

Little is known about the distribution of HPV genotypes within the uterine cervix, especially when multiple hr-HPV genotypes are detected. In Chapter 4 is shown that different hr-HPV genotypes can be detected in different dysplastic lesions within a cervix as well as within single lesions in that cervix, especially in patients with severe dysplasia, while in more severe lesions only a single hr-HPV genotype can be detected throughout the cervix, even in normal epithelium. It seems that the severity of the lesion may have a relation with the distribution of the HPV genotypes. The presence of multiple hr-HPV genotypes within a single lesion may have a serious impact on the outcome of future (HPV-triage based) screening and vaccination programs. Screening that includes hr-HPV detection needs to be done with an ultra-sensitive test that can detect multiple hr-HPV genotypes in order to detect a persistent hr-HPV genotype among temporary genotypes. Additionally, vaccination against a single or a few hr-HPV genotypes may not prevent the development of CIN 3/cervical cancer under the influence of other hr-HPV genotypes.

Chapter 5.

Adenocarcinomas in situ (ACIS) can be regarded as the glandular equivalent of squamous CIN 3 lesions. Chapter 5 explores which hr-HPV genotypes can be found in patients with ACIS in relation with coexisting CIN lesions and makes a comparison with CIN 2/3 lesions without ACIS. The frequency of specific hr-HPV genotypes is similar in patients with ACIS without coexisting CIN and in patients with ACIS with coexisting CIN, but is significantly different from patients with CIN 2/3 without ACIS. These results indicate that only certain hr-HPV genotypes cause glandular lesions, and the results also suggest that squamous lesions, coexisting with high-grade glandular lesions, may etiologically be different from squamous lesions without coexisting glandular lesions, a fact that needs further study.

Chapter 6.

The long-term use of oral contraceptives or exogenous estrogen suppletion has been identified as independent risk factors for the development of HPV mediated cervical cancer. A relation between the degree of CIN and estrogen receptor (ER), or progesteron receptor (PR) expression has been described previously, but the results of several studies contradict each other. Chapter 6 shows that the ER expression is progressively down regulated with increasing degree of CIN, while the PR expression remains unchanged. In normal epithelium adjacent to CIN lesions, no significant down regulation was observed, indicating that down regulation of the ER expression takes place during the development of CIN in women infected with hr-HPV. The significant decrease in the ER/MIB1 ratio in more severe CIN lesions indicates a loss of normal growth control by sex steroid hormones, which is not observed in normal epithelium adjacent to CIN lesions.

Chapter 7.

CIN lesions that develop under the influence of hr-HPV infections are characterized by hyperproliferation of the epithelium. The proliferation marker MIB1 can differentiate between different degrees of CIN in histopathological specimens. Chapter 7 shows that if the MIB1 LI in AgarCyto cell blocks of cervical scrapes of patients referred with ASC-US, CIN 1, or CIN 2 scrapes can be assessed, it has a higher sensitivity (96%) for the identification of patients with CIN 3, than hr-HPV detection (83%). However, the large number of patients (28%) in whom the MIB1 LI could not be assessed in combination with the rather low sensitivity (80-82%) and specificity (48-62%) of the MIB1 LI and hr-HPV detection in AgarCyto cell block

sections of all patients referred with ASC-US, CIN 1, or CIN 2 scrapes make this technique unsuitable for clinical practice.

Chapter 8.

Hr-HPV often becomes undetectable after adequate treatment of CIN lesions, and hr-HPV detection may play a role in the detection of residual CIN. Chapter 8 shows that hr-HPV detection has a low PPV (3 months after treatment) for the presence of residual CIN, and some patients with residual CIN are hr-HPV-negative (false negative hr-HPV result). Genotype specific hr-HPV persistence after treatment (specifically present in patients with residual CIN 3) showed a higher PPV, but its sensitivity for all residual CIN was less than of hr-HPV detection in general. The presence of hr-HPV in cervical scrapes after treatment for high-grade CIN is a risk factor for the presence of residual CIN. However, hr-HPV detection cannot predict or exclude the presence of residual CIN in the individual patient and additional procedures in both hr-HPV positive and hr-HPV-negative patients remain necessary.

FINAL CONSIDERATIONS.

At the onset of the studies presented in this thesis (end of the 20th century), the expectations of the clinical value of hr-HPV detection were high, especially in primary screening for cervical cancer, in the management of patients with abnormal cervical scrapes, and in follow-up of patients treated for CIN. Studies in this thesis showed that single point hr-HPV detection has only little additional clinical value in the management of CIN.

Why were these high expectations not fullfilled in this thesis, especially since there is little doubt that hr-HPV infections are a necessary factor in cervical carcinogenesis? (1) Genital hr-HPV infections occur in at least 80% of sexually active women, but less than 1% will develop cervical cancer. This low risk results in a low specificity of single point hr-HPV detection. (2) Recently, high cummulative prevalences of hr-HPV DNA have been detected in the cervix of cytologically normal women. The clinical consequences of the detection of hr-HPV in these women are uncertain. Different HPV detection methods with different sensitivities are presently being used, and it remains to be determined which detection method/sensitivity is preferable in clinical practice. (3) The clinical consequences of a hr-HPV negative test in women with CIN also remain to be elucidated. The majority of clinicians will presently treat or at least closely monitor patients with CIN, even if they are hr-HPV-negative. Whether hr-HPV-negative CIN lesions always regress without treatment needs to be studied, but ethical considerations may make such a study difficult to carry out. All these issues indicate that we still know too little about HPV infection, transmission, persistence and clearance, and that as a result single point hr-HPV detection is of limited value in clinical practice.

Are there other options for hr-HPV detection in the management of CIN? Serial hr-HPV detection (in time), and/or quantitative measurement of hr-HPV (viral load) are presently investigated as alternatives for single point hr-HPV detection. Serial detection of a specific hr-HPV genotype indicates the presence of a persistent hr-HPV infection. This means that hr-HPV has not been cleared from the cervix, or it has not become latent. It has been shown that serial detection of a hr-HPV genotype poses a much higher relative risk for high-grade CIN/cervical cancer development than the single point detection of a hr-HPV genotype. However, serial hr-HPV detection will require more samples (in time), will (initially) be more expensive, and its clinical feasability remains to be elucidated. Viral load measurements estimate the number of viral particles present in the cervix and a high viral load indicates that

replication of hr-HPV is taking place. Reliable viral load measurement methods that also measure multiple hr-HPV genotypes are still being developed. The clinical value of viral load measurements remains to be assessed, especially since sampling issues are of major importance with this technique.

Is hr-HPV detection acceptable for women as an alternative for, or addition to conventional cytology? The success of screening programs depends mainly on the participation of the population to be screened. The participation in the Netherlands is presently about 60-65%. Furthermore, screening itself causes anxiety in the screened population, and women with abnormal cervical scrapes have high anxiety levels. HPV infections are sexually transmitted and potentially oncogenic. These facts may worsen the anxiety caused by screening, may introduce feelings of guilt, and may have a negative impact on the screening participation. Whether the possible additional clinical value of hr-HPV detection is nullified by a negative influence of these factors on the participation of women in population based screening programs needs to be studied, before hr-HPV detection can be introduced in the screening program.

Are there other biomarkers that may be alternatives for hr-HPV detection? Surrogate biomarkers that are up or down regulated as a result of HPV mediated cervical carcinogenesis may be indicative of the carcinogenic effect HPV is having on the epithelium. MIB1 for instance has been shown to be an excellent marker for CIN on histology, but did not show an equal sensitivity in AgarCyto cellblocks of cervical scrapes in this thesis. Other biomarkers like $p14^{ARF}$ and $p16^{INK4A}$ are presently investigated as they are upregulated in hr-HPV cervical carcinogenesis. These markers may possibly prove to be better cytological markers than MIB1, because they are exclusively upregulated in dysplastic cells and not in normal cells, but its clinical value remains to be assessed.

Will HPV vaccination make all the abovenamed efforts redundant? HPV vaccinations are being developed and have been shown to be able to prevent genotype specific hr-HPV infections. However, it will take many years before all issues surrounding HPV vaccination (duration of respons, non-responders, polyvalent vaccin development) have been solved, and worldwide vaccination can be introduced. In the mean time efforts are needed to improve the present screening for- and management of- patients with CIN/cervical cancer.

SAMENVATTING EN SLOT BESCHOUWING

Hoofdstuk 1.

Preventie van baarmoederhalskanker is mogelijk door het opsporen en behandelen van (nog) niet kwaadaardige voorstadia van baarmoederhalskanker (i.e. cervicale intraepitheliale neoplasie (CIN)). Dit wordt wereldwijd toegepast via bevolkingsonderzoek programma's (screening). Hierbij wordt voornamelijk gebruik gemaakt van uitstrijkjes en/of colposcopie.
Epidemiologische en klinische studies hebben laten zien dat infecties met bepaalde (hoog risico) types Humaan Papillomavirus (hr-HPV) een grote rol spelen in de ontwikkeling van CIN en baarmoederhalskanker. Hoofdstuk 1 geeft een overzicht van de relatie tussen hr-HPV infecties, CIN en baarmoederhalskanker. Infecties met hr-HPV kunnen leiden tot opeenvolgende verstoringen in de cel cyclus die uiteindelijk kunnen leiden tot het ontstaan van baarmoederhalskanker. Dit proefschrift richt zich voornamelijk op de klinische waarde van hr-HPV detectie in de behandeling van patiënten met CIN.

Hoofdstuk 2.

Het maken van uitstrijkjes ter preventie van baarmoederhalskanker veroorzaakt angst in de onderzochte populatie. Patiënten die worden verwezen voor colposcopie hebben een hoge angst score op de Spielberger State en Trait Anxiety Inventory. Hoofdstuk 2 laat zien dat deze hoge angst scores worden veroorzaakt door angst voor kanker en voor de colposcopie. De ernst van het afwijkende uitstrijkje (i.e. de kans op kanker) heeft echter geen relatie met de angst. Patiënten met de hoogste angstscores bleken vaak: (1) de ondersteuning van een partner te missen, (2) de wachttijd als lang te ervaren, (3) onvoldoende geinformeerd te zijn, en (4) gevoelens van angst, woede en/of verdriet te ervaren. Colposcopie klinieken dienen, in samenwerking met de verwijzende primaire zorgcentra (huisartsen), het verwijzings systeem te evalueren zodat: (1) adequate informatie reeds bij de huisarts wordt verstrekt, (2) de wachttijd voor colposcopie wordt beperkt, en (3) patiënten met hoge angst scores worden geïdentificeerd en ondersteund. Door het toepassen van merkstoffen op uitstrijkjes kan mogelijk een beter onderscheid gemaakt worden tussen patiënten met en zonder risico op baarmoederhalskanker. Hierdoor zou het aantal verwijzingen in deze laatste groep kunnen worden beperkt, waardoor de angst bij een deel van de patiënten kan worden voorkomen.

Hoofdstuk 3.

De huidige Nederlandse richtlijn adviseert een colposcopie bij alle patiënten met twee opeenvolgende Pap 2, of een éénmalig pap 3A uitstrijkje. De 'see and treat' methode die in veel klinieken tijdens colposcopie wordt toegepast, leidt vaak tot onnodige lis excisies. Hoofdstuk 3 laat zien dat een aggressief beleid bij patiënten verwezen met Pap 2 uitstrijkjes, waarbij alle colposcopische afwijkingen worden behandeld, leidt tot een snellere normalisatie van vervolguitstrijkjes dan een expectatief beleid, waarbij alleen colposcopische afwijkingen verdacht voor CIN 3 worden behandeld. Er werd echter geen lange termijn voordeel van de aggressieve benadering gevonden. Dit terwijl het aantal lis excisies in deze groep bijna 5 keer zo hoog was.

Detectie van hr-HPV wordt aanbevolen als een triage instrument voor patiënten met twee opeenvolgende Pap 2 uitstrijkjes, waarbij alleen hr-HPV-positieve patiënten verwezen worden voor colposcopie. Dit hoofdstuk laat ook zien dat triage met hr-HPV detectie resulteert in de verwijzing van alle patiënten met CIN 3, maar niet van alle patiënten met CIN 1 en CIN 2. Omdat er vooralsnog geen bewijs is dat hr-HPV-negatieve CIN 1 en 2 laesies altijd in regressie gaan, dienen ook alle hr-HPV-negatieve patiënten vervolgd te worden. Daarnaast worden door de lage positief voorspellende waarde (PVW) en lage specificiteit van hr-HPV detectie nog steeds veel patiënten onnodig verwezen voor colposcopie. Deze factoren maken dat invoering van hr-HPV detectie bij patienten met twee opeenvolgende Pap 2 uitstrijkjes in Nederland vooralsnog niet kan worden aanbevolen.

Hoofdstuk 4.

Er is nog weinig bekend over de verdeling van HPV genotypen in de baarmoederhals, met name als meerdere hr-HPV genotypen gedetecteerd worden. Hoofdstuk 4 laat zien dat verschillende hr-HPV genotypen kunnen worden gedetecteerd, zowel in verschillende CIN laesies in één cervix, als ook binnen één CIN laesie. Met name als er sprake is van ernstige dysplasie. In ernstigere afwijkingen werd slechts één hr-HPV genotype gedetecteerd, verspreid door de hele baarmoederhals en zelfs in normaal weefsel. Het lijkt alsof de ernst van de afwijking mogelijk een relatie heeft met de distributie van de HPV genotypen. Deze resultaten kunnen vérstrekkende gevolgen hebben voor toekomstige (op HPV-triage gebaseerde) screening en vaccinatie programma's. Screening met hr-HPV detectie zal moeten plaatsvinden met een ultra-sensitieve test. Deze test moet ook multiple hr-HPV genotypen kunnen detecteren, zodat een persisterend hr-HPV genotype kan worden onderscheiden van de voorbijgaande genotypen. Vaccinatie programma's die slechts tegen

één of enkele hr-HPV genotypen beschermen zullen mogelijk de ontwikkeling van een CIN 3 laesie en/of baarmoederhalskanker door andere hr-HPV genotypen niet geheel kunnen voorkomen.

Hoofdstuk 5.

Het adenocarcinoma in situ (ACIS) kan worden beschouwd als de glandulaire equivalent van de planocellulaire CIN 3 laesie. In hoofdstuk 5 wordt onderzocht welke hr-HPV genotypen in patiënten met ACIS gedetecteerd kunnen worden. De resultaten werden ook gerelateerd aan het al of niet aanwezig zijn van een co-existente CIN laesie. De bevindingen worden vergeleken met de frequentie van specifieke hr-HPV genotypen in patiënten met uitsluitend CIN 2/3 laesies. Het blijkt dat de frequentie van specifieke hr-HPV genotypen gelijk is tussen patiënten met ACIS met of zonder co-existente CIN. Echter, de frequentie verschilt significant van patiënten met uitsluitend CIN 2/3. Deze resultaten impliceren dat slechts een beperkt aantal hr-HPV genotypen in de meerderheid van de glandulaire laesies gevonden worden. Daarnaast suggereren deze bevindingen dat een CIN laesie naast een glandulaire laesie mogelijk een andere etiologie heeft dan een solitaire CIN laesie, een feit dat verder onderzoek behoeft.

Hoofdstuk 6.

Het langdurig gebruik van orale anticonceptiva of exogene oestrogenen is onlangs geïdentificeerd als een onafhankelijke risico factor voor het ontwikkelen van HPV geïnduceerd baarmoederhalskanker. De relatie tussen de ernst van de CIN laesie en de expressie van de oestrogeen receptor (ER) en progesteron receptor (PR) werd eerder beschreven. De resultaten van die studies spreken elkaar echter tegen. Hoofdstuk 6 laat een progressieve afname zien van de ER expressie met toenemende ernst van de CIN laesie, terwijl de PR expressie onveranderd blijft. De ER expressie neemt niet af in normaal epitheel direct naast de CIN laesie. Dit betekent dat afname van de ER expressie plaatsvindt tijdens de ontwikkeling van CIN in vrouwen die geinfecteerd zijn met hr-HPV. De significante daling in de ER/MIB1 ratio met toename van de ernst van de CIN laesie impliceert dat de proliferatie zich onttrekt aan de normale controle door sex-steroid hormonen. Dit wordt niet waargenomen in normaal epitheel direct naast CIN laesies.

Hoofdstuk 7.

CIN laesies die zich ontwikkelen onder invloed van hr-HPV infecties worden gekenmerkt door hyperproliferatie van het epitheel. De proliferatie marker MIB1 kan op weefsel (histologie) differentïeren tussen de verschillende graden van CIN. Hoofdstuk 7 laat zien dat in een subgroep van patiënten verwezen met een Pap 2 of 3A ,de MIB1 labelling index (LI) in AgarCyto cel blokjes van uitstrijkjes een hogere sensitiviteit (96%) heeft voor de identificatie van patiënten met CIN 3 dan hr-HPV detectie (83%). Echter, bij een groot aantal patiënten kon geen MIB1 LI worden bepaald (28%). Daarnaast hadden de MIB1 LI en hr-HPV detectie voor de identificatie van patiënten met CIN 3 in de totale groep patiënten verwezen met een Pap 2 of 3A uitstrijkje een relatief lage sensitiviteit (80-82%) en specificiteit (48-62%). Deze factoren maken dat deze techniek in de klinische praktijk niet bruikbaar is.

Hoofdstuk 8.

Hr-HPV is vaak niet meer detecteerbaar na adequate behandeling van CIN laesies. Hr-HPV detectie kan dus mogelijk een rol spelen in de detectie van residu CIN. Hoofdstuk 8 laat zien dat hr-HPV detectie in het vervolg uitstrijkje 3 maanden na behandeling een lage positief voorspellende waarde (PVW) heeft voor de aanwezigheid van een residu CIN, en dat sommige patienten met residu CIN hr-HPV-negatief zijn (vals negatieve HPV test). Genotype specifiek persisterend hr-HPV na behandeling van CIN had een hogere PVW voor residu CIN. De sensitiviteit was echter lager dan van hr-HPV detectie in het algemeen. De detectie van hr-HPV in de cervix na behandeling voor CIN 2/3 is een risco factor voor het aanwezig zijn van residu CIN. Echter, hr-HPV detectie kan een residu CIN niet aantonen noch uitsluiten waardoor in zowel hr-HPV-negatieve als hr-HPV-positieve patiënten andere procedures noodzakelijk blijven.

SLOT BESCHOUWING.

Ten tijde van het opzetten van de studies die in dit proefschrift beschreven zijn (eind 20e eeuw), bestonden er hoge verwachtingen van de klinische waarde van hr-HPV detectie in de screening voor baarmoederhalskanker, in de behandeling van patiënten met een afwijkend uitstrijkje, en in het vervolgen van patiënten na behandeling van CIN. Studies in dit proefschrift hebben laten zien dat éénmalige hr-HPV detectie slechts weinig toegevoegde klinische waarde heeft in de behandeling van CIN.

Waarom zijn de hoge verwachtingen niet bevestigd in dit proefschrift? Dit gezien het feit dat er maar weinig twijfel bestaat over hr-HPV infecties als een noodzakelijke factor in de ontwikkeling van baarmoederhalskanker.

(1) Genitale hr-HPV infecties treden op bij tenminste 80% van de sexueel actieve vrouwen, terwijl minder dan 1% van hen baarmoederhalskanker ontwikkelt. Dit lage risico resulteert in een lage specificiteit van éénmalige hr-HPV detectie. (2) Recent zijn hoge cummulatieve prevalenties van hr-HPV gevonden bij vrouwen met een normaal uitstrijkje. De klinische consequentie van het gevonden hr-HPV bij deze vrouwen is onduidelijk. Verschillende HPV detectie methoden met ieder een verschillende sensitiviteit worden momenteel gebruikt. Het is nog onduidelijk welke detectie methode/sensitiviteit te prefereren is in de klinische praktijk. (3) De klinische consequenties van een negatieve hr-HPV test bij vrouwen met CIN laesies zijn nog niet opgehelderd. De meerderheid van de gynaecologen zal vrouwen met CIN laesies momenteel behandelen, danwel nauwgezet vervolgen, zelfs al zijn deze vrouwen hr-HPV-negatief. Of hr-HPV-negatieve CIN laesies altijd in regressie gaan moet nog worden bewezen, maar ethische overwegingen zouden een dergelijk onderzoek wel eens moeilijk uitvoerbaar kunnen maken. Al deze punten wijzen er op dat wij nog te weinig weten over HPV transmissie, infectie, persistentie, en klaring. Dit heeft tot gevolg dat éénmalige hr-HPV detectie slechts een beperkte toegevoegde waarde heeft in de klinische praktijk.

Zijn er andere opties voor hr-HPV detectie in de behandeling van CIN?

Seriële hr-HPV detectie (in tijd) en/of quantitatieve hr-HPV detectie ('viral load') worden momenteel onderzocht als alternatieven voor éénmalige hr-HPV detectie. Seriële detectie van een specifiek hr-HPV genotype impliceert de aanwezigheid van een persisterende hr-HPV infectie. Dit betekent dat hr-HPV niet is geklaard van de baarmoederhals, of niet latent is geworden in de baarmoederhals. Het is reeds aangetoond dat seriële detectie van een specifiek hr-HPV genotype geassocieerd is met een veel hoger relatief risico op hoog-

gradige CIN laesies en/of baarmoederhalskanker dan éénmalige hr-HPV detectie. Echter, seriële hr-HPV detectie zal meerdere monsters vereisen (in tijd), zal (initieel) duurder zijn, en de klinische waarde daarvan moet nog vastgesteld worden. Viral load detectie schat het aantal virus deeltjes dat in de baarmoederhals aanwezig is. Een hoge viral load impliceert dat virus replicatie in de cervix plaatsvindt. Betrouwbare viral load detectie methoden die tegelijkertijd multiple hr-HPV genotypen kunnen meten zijn nog in ontwikkelling. De klinische waarde van viral load detectie dient nog te worden bepaald, met name omdat monster afname (het uitstrijkje) van grote invloed kan zijn bij deze techniek.

Is hr-HPV detectie acceptabel voor vrouwen als alternatief en/of aanvulling op uitstrijkjes?

Het succes van een screenings programma hangt vooral af van de participatie graad van de te screenen populatie. De participatie graad is in Nederland momenteel ongeveer 60-65%. Screening op zichzelf veroorzaakt reeds gevoelens van angst, en vrouwen met een afwijkend uitstrijkje hebben zeer hoge angst scores. HPV infecties zijn sexueel overdraagbaar en potentieel oncogeen. Deze factoren zouden de reeds door screening veroorzaakte angst kunnen verhogen en schuld gevoelens kunnen uitlokken. Dit zou op die manier een negatief effect op de participatie graad kunnen hebben. Of de eventuele toegevoegde klinische waarde van hr-HPV detectie teniet wordt gedaan door een afname in de participatie graad aan screening voor baarmoederhalskanker dient nog onderzocht te worden. Pas daarna kan hr-HPV detectie ingevoerd worden in het screenings programma.

Zijn er ander merkstoffen die als alternatief voor hr-HPV detectie gebruikt kunnen worden?

Surrogaat merkstoffen waarvan de expressie wordt verhoogd of verlaagd tijdens HPV geïnduceerde ontwikkeling van baarmoederhalskanker vormen een indicatie van het carcinogene effect van HPV op het epitheel. MIB1 is bijvoorbeeld een zeer goede merkstof voor CIN op weefsel, maar wij konden niet een zelfde sensitiviteit in uitstrijkjes vast stellen. Andere merkstoffen, zoals $p14^{ARF}$ en $p16^{INK4A}$ worden momenteel onderzocht omdat deze tot verhoogde expressie komen in HPV geïnduceerde carcinogenese. Deze merkstoffen zijn mogelijk beter dan MIB1 op uitstrijkjes, omdat een verhoogde expressie van deze merkstoffen uitsluitend in dysplastische cellen wordt gevonden en niet in normale cellen.

Zal HPV vaccinatie alle bovengenoemde inspanningen overbodig maken?

HPV vaccins worden momenteel ontwikkeld en hebben reeds laten zien dat ze een genotype specifieke hr-HPV infectie kunnen voorkomen. Het zal echter nog jaren duren voordat alle kwesties rondom HPV vaccinatie (duur van het beschermende effect, het aantal non-responders, polyvalent vaccin ontwikkeling) zijn opgelost. Pas daarna kan tot wereldwijde vaccinatie worden overgegaan. In de tussentijd blijven nieuwe inspanningen nodig om de huidige screening op, en behandeling van vrouwen met CIN laesies en/of baarmoederhalskanker te verbeteren.

CURRICULUM VITAE

Ruud Bekkers werd als vierde in een gezin van 5 kinderen op 22 oktober 1964 geboren in Schayk. Na de lagere school en het Atheneum B, studeerde hij geneeskunde (en 1 jaar psychologie) aan de Katholieke Universiteit Nijmegen. Tijdens zijn studie ontmoette hij zijn huidige vrouw, Lotte. Beiden ontwikkelden een liefde voor de tropengeneeskunde. Na diverse stages in Burkina Faso, India en Indonesie, en een co-schap in Tanzania, behaalde Ruud in 1991 de artsenbul.

In 1991 en 1992 volgde hij als arts-assistent chirurgie (Zeist) en later gynaecologie/obstetrie (Oss) de tropenopleiding. In 1993 vertrok hij met Lotte (die net haar artsenbul had gehaald) naar Kwazulu, één van de niet-officiele thuislanden van Zuid-Afrika. In Ceza werkten hij en Lotte 4 ½ jaar als tropenarts in een klein afgelegen ziekenhuis, waar Ruud de laatste 2 jaar Medical Superintendent was. In 1997 is Willemijn in Ceza geboren en 8 weken later keerden zij terug naar Nederland. Ruud begon direct na terugkeer met de opleiding tot gynaecoloog op de afdeling gynaecologie/obstetrie van het UMC St Radboud in Nijmegen. Reeds in Ceza en later ook in Nijmegen werd Ruud geboeid door de oncologie. Eind 1999 begon hij, naast zijn klinische opleiding, met promotie onderzoek dat tot dit proefschrift heeft geleid. In 2000 en 2001 zijn Anneloes en Bernadet geboren. In 2003 rondde Ruud zijn klinische opleiding af en werd hij fellow gynaecologische oncologie in het UMC St Radboud. Vanaf November 2003 wordt dit vervolgd met een 2-jarig klinisch fellowship gynaecologische oncologie, gesponsored door het Koningin Wilhelmina Fonds.

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Leef vandaag naar beste weten en vermogen
zodat morgen vandaag een gisteren is vol tevredenheid
en morgen een dag die vol hoop en vertrouwen
op geluk en gezondheid geleefd kan worden

Walk on !

Pa Jan
(†2002)