HUMAN PAPILLOMAVIRUS AND NATURAL HISTORY OF CERVICAL INTRAEPITHELIAL NEOPLASIA: CLINICAL CONSEQUENCES

The work presented in this thesis was performed at the Departments of Pathology and Obstetrics and Gynaecology, Vrije Universiteit Medical Center, Amsterdam, and the Department of Obstetrics and Gynaecology, University Hospital Rotterdam, The Netherlands.

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Humaan Papillomavirus en het natuurlijk beloop van Cervicale Intraepitheliale Neoplasie: klinische consequenties

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1. General introduction

Cervical cancer is the second most common cancer in women world-wide after breast cancer. Each year, there are approximately 437,000 new cases of invasive cancer of the cervix diagnosed and more than 200,000 women die from the disease, 79% of which occur in developing countries. [1] In the Netherlands approximately 715 new cases of cervical cancer are diagnosed each year with an age-standardised incidence rate of 8,6 new cases per 100,000 women. The age-standardised mortality rate for the Netherlands has been estimated at 2,4 deaths per 100,000 women with a total of 234 deaths reported in 1995. [2] The average age-standardised mortality rate of developing countries is 2,5 times that of industrialised areas. [1]

Cervical cancer is considered a preventable disease. Its development through premalignant stages detectable by cervical cytology years before cervical cancer appears has resulted in the organisation of population based cervical cancer screening programs. There is strong evidence that these organised screening programs are an effective approach which has led to a substantial reduction in morbidity and mortality of cervical cancer. [3-6] However, there are some important drawbacks including low attendance and limited sensitivity of cytological screening, resulting in high numbers of false-negative cases. [7-9] The sensitivity of cytology is limited by sampling-error, in which the abnormal cells do not get placed on the slide, and reading-error, where a few abnormal cells are not identified among the multitude of normal cells present on the slide. Sensitivity rates for cytology of only 40% to 80% for high grade Cervical Intraepithelial Neoplasia (CIN 2 and 3) have been reported. [8,10-11] In addition, cytology has a rather low specificity. In screening, this results in a high number of women diagnosed with borderline and mildly dyskaryotic smears among women without high grade cervical lesions, resulting in an unnecessary costly follow-up and anxiety among women concerned. [9,12] Thus, there is a need for a more accurate screening tool.

Several epidemiological studies have established a strong relation between infection with high-risk human papillomavirus (HPV) and the development of cervical cancer and its precursors. [13-21] High-risk HPV DNA can be identified in nearly all cervical carcinomas [15,22] and in case-control studies around the world a strong association between cervical carcinomas and the presence of high-risk HPV types (odds ratios up to 200) was found. [20-21,23-26] Out of the 15 high-risk HPV types identified at present, HPV type 16 accounts for the highest proportion of cervical cancer, followed by HPV type 18. In follow-up studies of women with normal cervical smears a high-risk HPV positive test indicated a more than 100 times higher risk to develop Cervical Intraepithelial Neoplasia grade 3 (CIN 3; severe cervical

dysplasia) than women without high-risk HPV. [27-28] In women with abnormal cervical smears and a high-risk HPV positive test this risk was also increased. [29-32]

It is assumed that about 80% of women acquire high-risk HPV ever, and in most of these cases the infection is transient. [33-36] Only a small proportion of women infected with high-risk HPV will develop CIN 3, in most women with premalignant cervical lesions the lesion regresses spontaneously. [37-38] Relatively very few women develop cervical cancer.

A review, summarising natural history studies on premalignant cervical lesions over the past 40 years, concluded a high regression of CIN 1 (mild cervical dysplasia), i.e. 57%, with a persistence of 32%, a progression to CIN 3 (severe dysplasia) of 11% and a progression to invasive cervical cancer of 1%. The corresponding figures for CIN 2 (moderate dysplasia) were regression of 43%, persistence of 35%, progression to CIN 3 of 22% and progression to invasive cervical cancer of 5%. In CIN 3, regression of 32% was found, with an estimated progression rate to invasive cervical cancer of more than 12%. [39] So, the more severe the lesion, the lower the chance of regression. The natural history data of cervical premalignant lesions have important implications in treatment policy of lesions, i.e. whether to treat or follow-up, and therefore there is a strong need for better and additional progression markers to predict the clinical outcome of an individual woman. Over-treatment of these women can be prevented by selecting only those women with premalignant cervical lesions at risk for progression.

The use of high-risk HPV testing, next to cervical cytology, in primary and secondary screening of cervical cancer has been proposed. But before a possible implementation can be initiated several important questions concerning the natural course of CIN disease in relation to HPV status have to be answered. Whether high-risk HPV clearance is related to regression of cervical lesions has been assumed, but has never been documented. Progression of CIN lesions is associated with a persistent infection with high-risk HPV [40] but the exact definition of persistence and the risk on the development of CIN 3, the stage before cervical cancer develops, is unknown. The best way to study the natural history of this process is in a prospective study with a long-term follow-up without taking biopsies, because it is known that these will interfere with the natural course of CIN, [41-42] and a well-defined study endpoint to avoid development of cervical cancer.

The aim of this thesis is to investigate the role of high-risk HPV in the natural course of CIN. Better insight into the relation between human papillomavirus and the natural history of CIN lesions may lead to more efficient cervical cancer screening strategies and treatment policies once women are referred to gynaecologist.

2. Cervical cancer and its precursors

2.1 Pathology

The uterine cervix is covered with squamous and columnar epithelium. The outer surface of the cervix (ecto-cervix) and the vagina contain non-keratinizing squamous epithelium. The inner part of the cervix (endo-cervix) and the endocervical canal contain mucus-secreting columnar epithelium. On the so-called transformation zone, metaplastic transformation of columnar epithelium into squamous epithelium occurs. This is a physiological process and arises from the subcolumnar "reserve cells". These cells mount up and differentiate to normal squamous epithelium. It remains unclear whether columnar cells derive from these reserve cells. The border between the transformation zone and the columnar epithelium is called the squamous-columnar junction (SCJ). Morphogenetically, there are two different squamocolumnar junctions (see figure 1). The first is termed the *original* squamocolumnar junction and is the site at which the native squamous covering of the ectocervix touches the endocervical columnar epithelium at time of birth. During puberty the columnar epithelium rolls outward onto the portio ("eversion") and over time is replaced by squamous epithelium. This is the so-called *neo* squamocolumnar junction. The region between the neonatal original squamocolumnar junction and the postpubertal functional squamocolumnar junction is termed the transformation zone. [43] This transformation zone is localised on the ecto-cervix in the majority of women aged between 20-40 years, but is withdrawn into the endocervical canal during life. Due to the high turnover of cells, the transformation zone is assumed to be more susceptible to oncogenic influences, because the majority of squamous intraepithelial and invasive lesions develop in that particular zone. Cervical lesions on the transformation zone can be identified by colposcopy and are diagnosed by histological examination.

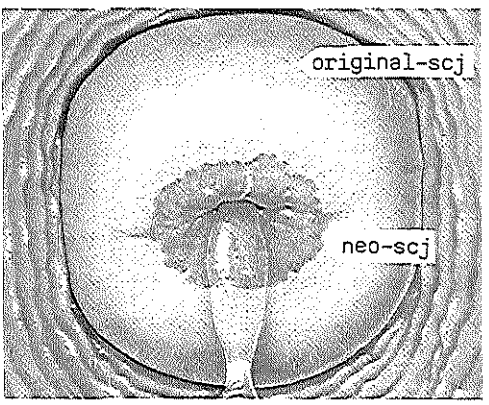


Figure 1:
The original and neo squamouscolumnar junction on the cervix. [44]

The fact that invasive squamous cell carcinoma of the cervix is preceded by recognisable precursor lesions has been known for some time. Broders (1932) was probably the first to recognise carcinoma *in situ* as a precursor of invasive cancer [45] and Papanicolaou together with Traut (1941) demonstrated that the condition could be diagnosed by exfoliated cytology. [46] Later, the existence of dysplasias as precursors of carcinoma *in situ* was recognised. [47] Since that time, successive nomenclatures for the sequence of pathological conditions which are considered premalignant, or preinvasive, have been introduced, ostensibly for purposes of clarity or simplification. Microscopically, this sequence is characterised by progressive dedifferentiation or atypia of epithelial cells, and progressive involvement of the full thickness of the epithelium, from the basal layer in milder lesions, to the increasingly mature squamous cells on the mucosal surface. Initially, this progression was described in terms of increasing degree of dysplasia (i.e. mild, moderate, severe) and carcinoma *in situ*, where the full thickness of the epithelium is involved by undifferentiated cells. Different degrees of dysplasia may coexist at different sites within the same cervix. To elucidate that the two terms, i.e. dysplasia and carcinoma *in situ*, were part of a continuous process, the terminology of cervical intraepithelial neoplasia (CIN) was introduced and came progressively into use in the early 1980s. [48] This classification emphasised that each dysplastic cervical lesion is potentially malignant and evolves as a result of a continuum of intraepithelial changes that start with minor atypia, progressing through increasing degrees of intraepithelial abnormalities to invasive squamous cell carcinoma. [49] CIN lesions are classified into three groups according to the thickness of epithelial layer involved in neoplastic changes. CIN grade 1 represents less than one third of the thickness of epithelium involved, while in grade 2 one third to two third, and in grade 3 two third to full thickness of the epithelial layer is involved. [50] CIN 3 combines severe dysplasia and carcinoma *in situ.* In 1988, the Bethesda classification system was introduced, aiming at the distinction between lesions with a presumable low and high risk of progression to cancer, respectively. In the Bethesda system CIN 1 is classified as low grade squamous intraepithelial lesion (low SIL) while CIN 2 and 3 are classified as high grade SIL (high SIL). [51] However, this classification system is not widely used in Europe since follow up data showed that a substantial number of CIN 1 lesions have progressive and CIN 2 lesions regressive potential which argues against the Bethesda classification. [38-39,46,52-53] Invasive cervical carcinoma is histologically differentiated into squamous cell carcinoma, which represents the most common type of cervical cancer, adenosquamous carcinoma and adenocarcinoma. [54]

The Pap test is based on cytomorphological examination of exfoliated cells from the transformation zone, squamous epithelial of the ectocervix and from the endocervical epithe-

Table 1: KOPAC-B (in English CISOC-A) classification system used in the Netherlands for cytomorphological examination of Pap-stained cervical smears.

	C (K)	I (O)	S (P)	O (A)	C (C)
Score	**Composition**	**Inflammation**	**Squamous epithelium**	**Other, and endometrium**	**Columnar epithelium endocervix**
0	Inadequate	Not applicable	Not applicable	Not applicable	Not applicable
1	Endocervical epithelium	Viral infection	Normal	No other abnormalities	Normal
2	Squamous metaplastic cells	Trichomonas vaginalis	Abnormal squamous epithelial cells	Epithelial atrophy	No endocervical epithelium present
3	Endometrium	Bacterial infection	Atypical squamous metaplasia	Atypical repair reaction	Some atypical endocervical cells
4	Endocervical epithelium and squamous metaplastic cells	Candida (monilia) albicans	Mild dyskaryosis	Mild atypical endometrium	Mild atypical endocervical epithelium
5	Endocervical epithelium and endometrium	Haemophilus (gardnerella) vaginalis	Moderate dyskaryosis	Moderate atypical endometrium	Moderate atypical endocervical epithelium
6	Squamous metaplastic cells and endometrium	no inflammation	Severe dyskaryosis	Severe atypical endometrium	Severe atypical endocervical epithelium
7	Endocervical epithelium, squamous metaplastic cells and endometrium	Actinomyces	Carcinoma *in situ*	Adenocarcinoma endometrium	Adenocarcinoma *in situ* endocervical epithelium
8	Only squamous epithelium	Chlamydia	Microinvasive carcinoma	Metastasis malignant tumor	Not applicable
9	Not applicable	Non-specific inflammation	Invasive squamous cell carcinoma	Not applicable	Adenocarcinoma endocervix

The smears are examined for 5 different items (C-I-S-O-C; columns 2-6) by the cytopathologist and depending on the results for each of the 5 items (C-I-S-O-C) a score (column 1) is assigned leading to a 5 digit code. Adequacy (A): A1: adequate; A2: adequate, but suboptimal (specify); A3: inadequate.

lium. [7] In the Netherlands the KOPAC-B (in English CISOC-A) coding system is used. [55-56] In this system (see table 1), five items are scored and assigned values from 0 to 9. C stands for composition, I for inflammation, S for squamous epithelium, O for other abnormalities and endometrium and C for columnar epithelial of the endocervix. A stands for adequacy of the smear. This leads to a 5 digit CISOC code which is translated into the Pap classification. Only the SOC values influences the Pap class. In figure 2, the translation from the CISOC classification to the Pap classification is depicted. Pap 1 indicates normal cytology, Pap 2 very mild dyskaryosis, Pap 3a1 mild dyskaryosis; Pap 3a2 moderate dyskaryosis, Pap 3b severe dyskaryosis, Pap 4 suspected of carcinoma *in situ*, and Pap 5 suspected of at least microinvasive carcinoma. In figure 3 the relationship between the histological CIN and SIL classification and the cytomorphological Pap classification is shown.

Figure 2: Translation of the KOPAC-B (in English CISCO-A) coding system to Pap classification as used in the Netherlands for cytomorphological examination of Pap-stained cervical smears. On the left the CISOC score is depicted for the different items examined. The SOC values influences the Pap class cited on the right.

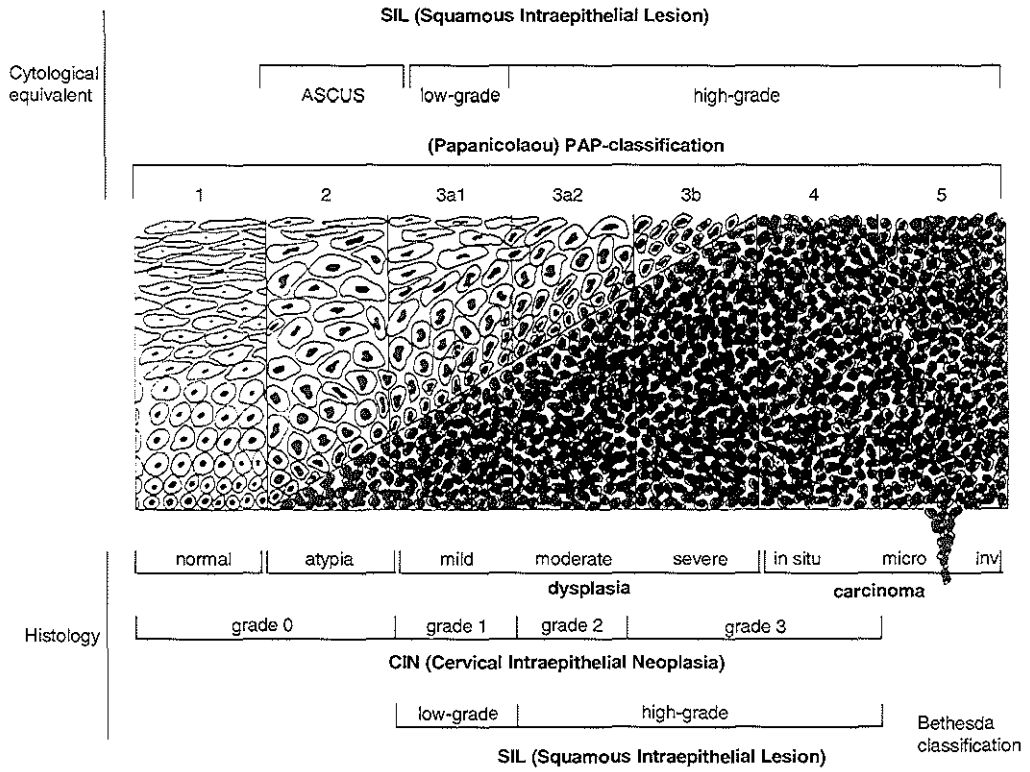
C	I	S	O	C	A		Pap	Description
0	0	0	0	0	3	→	0	Inadequate
		1	1-2	1		→	1	Normal
		2-3	3	2-3		→	2	Very mild dyskaryosis
		4	4	4		→	3a1	Mild dyskaryosis
		5	5	5		→	3a2	Moderate dyskaryosis
		6	6	6		→	3b	Severe dyskaryosis
		7	-	7		→	4	Carcinoma *in situ*
		8-9	7-9	9		→	5	Carcinoma

2.2 Epidemiology

The theory of cervical cancer being related to sexual activity has been postulated for the first time in 1842, based on the relatively low incidence of cervical cancer observed in virgins and nuns compared to high frequencies in prostitutes. [57] Several candidates like Neisseria gonorrhoe, Mycoplasma, Trichomonas and Treponema species, Chlamydia and genital herpes passed in review. Zur Hausen was the first to suggest that a wart virus may be involved since cervical cancer shows a similar epidemiological pattern as papillomavirus

induced condylomata acuminata. [58] Support came from cytopathologists who observed similar morphological changes, koilocytes, in mild dysplastic cervical lesions. Until then, koilocytes had only been observed in HPV induced genital warts. An important breakthrough

Figure 3: Relationship between histological CIN and SIL classification and cytomorphological Pap classification.



was made when zur Hausen and Gissmann were able by recombinant DNA technology to clone and characterise isolated HPV DNA from genital warts and papillomas. Less than 50% homology between the cloned HPV type and HPV DNA from skin warts was shown. Subsequently, they could isolate potentially oncogenic HPV types from cervical cancers and can-

cer derived cell lines that differ from those associated with genital warts. [59-61] Numerous studies have been performed after this discovery to demonstrate the relation between cervical cancer and HPV. Results from case-control studies performed by WHO/IARC showed a very high risk of oncogenic HPV infection for the development of cervical cancer. [20-21,23-26] Recent data have shown that high-risk HPV is present in more than 99% of cervical cancers, both in squamous cell carcinoma and in adenocarcinoma. [22] Risk estimates performed in the WHO/IARC studies of the association between a high-risk HPV positive test and CIN 3 or cervical cancer were far stronger than the ones usually reported for smoking and lung cancer or for Hepatitis B virus and liver cancer. In conclusion, it is now generally accepted that HPV is the most important causative agent for the development of cervical cancer.

Additional risk factors related to sexual behaviour like age at first sexual intercourse, life-time number of sexual partners, other sexual transmitted diseases, use of oral contraceptives, sexual behaviour of the male partner, and parity have been suggested, although in most studies the risk of high grade cervical lesions and cervical cancer associated with these factors have been inconsistent after adjusting for HPV infection. [16,18-19,62-66] An independent risk has also been found between smoking and the development of cervical cancer. Evidence for this dose-dependent effect has been published by several groups. [67-70] Cigarette smoke contains non-organ specific carcinogens and smoke metabolites have been detected in the cervical mucosa. [71] Moreover, smoking cessation improves the possibilities for reversal of CIN. [72] However, the fact that in some populations smoking is associated with a lifestyle with increased risk for HPV infection has suggested that the association seen with smoking may be due to residual confounding from HPV. Indeed, in some studies no association between smoking and cervical cancer could be found after adjustment for HPV. [13,24,73-74]

3. Human Papilloma Virus (HPV)

3.1 Introduction

Human papillomaviruses are nonenveloped, double-stranded DNA viruses of approximately 8000 base pairs. The viral genome can be divided into the early and late regions containing open reading frames (ORFs) coding for viral proteins. About 15% of the viral genome does not code for proteins, but plays a role in regulation of transcription and viral DNA replication (long-control-region, LCR). The "early' region (E) encodes proteins involved in control of transcription and viral (DNA) replication, and cell transformation. The "late" region (L) contains the genes which encode the viral capsid proteins (L1 and L2). The definition of a new HPV type is less than 90% homology in the E6, E7, and L1 ORF with all other HPV

types. [75-77] HPV is classified as a subtype when these regions display between 90% and 98% homology with known types, whereas HPV variants display less than 2% DNA sequence variation in the E6, E7, and L1 region. In this way more than 100 types of HPV have been recognised. [78-79] Generally, HPV types can be divided into mucosotropic types which are mainly found on the mucous epithelium of the oropharynx and anogenital tract and cutaneous types which predominantly infect the skin. [80-81] Both types can be grouped in high-risk or oncogenic and low-risk or non-oncogenic types. By definition, in cervical cancer and CIN 3 mostly high-risk HPV types are found, whereas in CIN 1 and CIN 2 mostly low-risk HPV types are identified. [82] Accordingly, the cancer-associated group of genital HPV types is defined as those found with appreciable prevalence in invasive cervical cancer. [15] The most prevalent type found in cervical carcinomas is HPV type 16 (53%), followed by HPV type 18 (15%), HPV type 45 (9%), HPV type 31 (6%), and HPV type 33 (3%). [83]

3.2 Prevalence

In women with normal cervical cytology the prevalence of HPV (both high- and low-risk types) is age-related, decreasing from 20% in women aged between 20 and 25 years to 6 % in women over 30 years. High-risk HPV types decrease from 10% in women between 20 and 35 years to 4 % in women older than 35 years. This indicates that HPV infections are very common among young women and frequently resolve spontaneously. [36,84-87] The decreasing HPV prevalence with increasing age might be explained by a cohort effect of an increased HPV prevalence in the younger generations due to a different sexual behaviour. More likely, the influence of age reflects acquired immunity to various HPV types, leading to clearance of most primary HPV infections in young asymptomatic women. The lower prevalence of HPV infection in older women compared with younger women is found to be independent of sexual behaviour. [64] Recent studies on HPV DNA prevalence have shown that a second peak in HPV prevalence is seen in women after 55 years of age. [83,88-89] The explanations for this second peak were: 1) A cohort effect: A. the older women have been exposed to the virus more intensively; or B. due to less protection conferred by the fact that the older women do not attend to the screening programs as much as the younger women; 2) reactivation of a virus; and 3) a possible increase in the detection of HPV as atrophic changes occur in the postmenopausal cervix. More studies are needed to identify the real reasons.

In women with abnormal cervical smears the prevalence of high-risk HPV is increased. Numerous studies have investigated this prevalence and, due to the different detection methods and the populations, with highly variable outcomes. In a recent systematic review by Cuzick and co-workers [90] of the available evidence on the role of HPV testing in cervical

cancer, the relevant data on the prevalence of the virus in different disease groups have been documented. When taking only the sensitive amplification methods like PCR and Hybrid Capture II into account, a wide range of high- risk HPV positivity was found in CIN 1 from 30 to 65%. There was a greater consistency regarding sensitivity for detecting CIN 3. These rates were in the 60 to 90% range, with somewhat lower values for CIN 2 of 40 to 70%.

3.3. HPV detection methods

Tests which detect all known high-risk HPV types are sufficient since it is known that the risk of developing cervical cancer is not different for the different high-risk HPV types. [20-21,23-24] Individual HPV typing is only necessary in more detailed epidemiological studies or in studies evaluating the effectiveness of a certain preventive or therapeutic vaccine. In principal, two kinds of tests are available and frequently used for high-risk HPV testing. One is the recently introduced Hybrid Capture II (HC II) system which is based on direct HPV DNA detection by using signal amplification and detects 13 high- and 5 low-risk HPV types. [91] The other is based on detection of HPV DNA after amplification by the polymerase chain reaction (PCR) using HPV general or consensus primers enabling the detection of a broad spectrum of mucosotropic HPV types. Two primer systems are most frequently used, i.e. the MY 09/11 and the GP 5+/6+ primer system. The first uses degenerated bases to account for heterogeneity between various HPV types. As such a mixture of 25 primers is used to detect a wide range of HPV types. [92] In contrast, the GP 5+/6+ primer system uses only two primers, one forward and one reverse, that are designed to be complementary to a region of high homology between various HPV types, allowing the amplification of all mucosotropic HPV types by mismatch acceptance. A high-risk probe cocktail is available to identify in one assay the most prevalent 14 high-risk HPV types (i.e. HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). In addition, by using a low-risk probe cocktail other low-risk HPV types can be identified (for example HPV type 6, 11, 13, 30, 32, 34, 40, 43, 54, and 55). [93-95] By using single HPV specific probes HPV types can easily be identified. Besides the general or consensus PCRs, a wide range of type-specific primers are developed. These can only amplify one type of HPV but might be combined into one PCR by multiplexing. They can be used for confirmation of HPV positivity and in some instances for typing HPV samples.

In conclusion, HC II and GP 5+/6+ PCR are useful for screening purposes to detect high-risk HPV types in a simple, reliable and non-radioactive format. HC II is a commercially available, robust test. GP 5+/6+ PCR is relatively simple and has been clinically validated. [95-96] Although

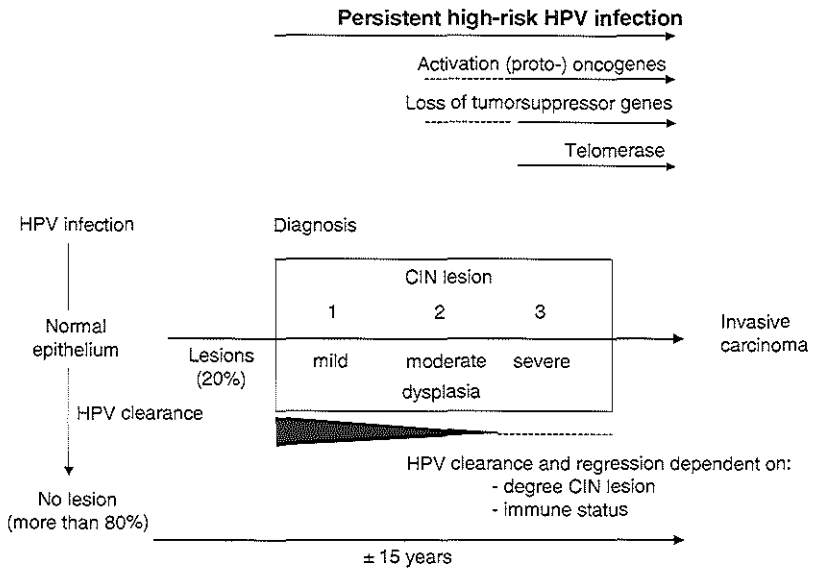
PCR has a higher analytic sensitivity as compared with HC II, both tests have a high sensitivity and negative predictive value for severe cervical lesions.

4. Natural history

The model of cervical carcinogenesis

The fact that a high number of women is infected with high-risk HPV during lifetime but only a small proportion of them develops cervical cancer indicates that cervical cancer is a rare complication of an infection with HPV. From the above cited studies, it is clear that high-risk HPV infection precedes the occurrence of CIN lesions. Together with other data this has resulted in the following model of the relationship between HPV infection and development of cervical cancer (figure 4).

Figure 4: Model explaining the relation between HPV infection and cervical cancer.



When women start sexual activity they may acquire an infection with a high-risk HPV type. The life-time risk to acquire HPV is about 80%. [33-34,97] Especially young women (< 30 years) have a transient infection. [98-101] More than 80% will clear this infection. [102] There are indications that the presence of neutralising antibodies results in a transient infection without

CIN [103] and that persistence and consequently HPV-mediated carcinogenesis is related to a failure of the immunosurveillance since a high incidence of high grade cervical lesions and cervical cancer is seen in immunocompromised patients, such as AIDS patients and transplant recipients. [16,104-105] Pregnancy is also believed to alter the immune-response in women. [106-107] Higher prevalence rates of high-risk HPV have been found in pregnant women, [108-111] whereas others found no difference in HPV detection between pregnant and non-pregnant women, [84,112-113] indicating that the influence of pregnancy on the natural course of an infection with high-risk HPV types is not yet known.

Approximately in 20% of women infected with high-risk HPV a CIN lesion will develop within 2-4 years after acquisition of the virus. [13,27,40] This may lead to maintenance of CIN or progression from CIN 1 to CIN 3. Invasive cervical cancer ultimately manifests in a small subset of cases. Infection with low-risk HPV types may also result in CIN 1 and some in CIN 2. [82] However, these lesions never or extremely rarely progress to CIN 3 and cervical cancer, as indicated by the fact that low-risk HPV types have never been found as single infections (without high-risk HPV types) in CIN 3 and cervical cancer. [15,82] Most of the premalignant lesions, especially CIN 1 and CIN 2 will regress spontaneously. Regression rates varying from 30% to 62% in women with CIN 1 and 17% to 54% in CIN 2 are reported. [29,38-39,52] Even a certain number of CIN 3 lesions will regress. [39] Several studies have reported that women with high-risk HPV positive premalignant lesions were more likely to progress than women with high-risk HPV negative lesions [30-32,53,91,114-115] and therefore, progression of CIN lesions is assumed to be associated with a persistent high-risk HPV infection. [13,40,96] This was also shown in case-control studies in which HPV DNA could be detected persistently in cytological normal archival smears of women years before cervical cancer subsequently developed. [116-118]

The major problem in the development of cervical cancer through the different premalignant stages is the difficulty in predicting which lesion will progress or regress. The clinical usable definition of a persistent infection has not yet been defined and the value of methods of detecting lesions with a high likelihood of progression are not yet available. It has been proposed that high-risk HPV clearance results in regression of CIN, but this process has never been studied properly. Moreover, it is necessary to know if clearance depends on the severity of the underlying lesions.

The interval between the first manifestation of CIN 1 and the development of cervical cancer is estimated to be about 13 years. [119] This long period indicates that, in addition to an oncogenic HPV infection and most probably immunological factors, changes in the cellular genome are mandatory. This theory is substantiated by the fact that the viral oncogene products E6 and E7 bind the gene products of the human tumour suppressor genes p53 and Rb. [120-122] This interaction in proliferating cells leads to dysregulation of cell cycle control and shortcomings in DNA repair, which might result in genetic instability and an increased risk of obtaining changes and mutations (activation of oncogenes, loss of tumor suppressor genes, activation of telomerase) essential for progression to a malignant phenotype. [123] Thus, HPV is capable of initiating cancer through the disruption of multiple tumour-suppressor pathways but alone is not sufficient for the development of the fully transformed cancer phenotype.

5. Prevention of cervical cancer

5.1. Cytology

The development of cervical cancer through premalignant stages detectable by cervical cytology years before cervical cancer appears has resulted in the organisation of population based cervical cancer screening programs. In the Netherlands screening for cervical cancer was started as a trial in 1975 in three regions. [124-125] Nowadays all women between 30 and 60 years are invited by mail to participate in the screening program. Cervical smears are performed by the general practitioner. According to the currently applied screening protocol women with no abnormalities will be contacted again after a period of 5 years. Women presenting with Pap 3a2, moderate dyskaryotic smears, or worse or with successively Pap 2 and/or Pap 3a1, very mild and mild dyskaryotic smears after a 6 months interval are referred for colposcopy. [126] Colposcopy involves observation of the cervical and genital squamous epithelium through a microscope. It allows the gynaecologist to assess the size, location and severity of a cervical lesion, and if necessary a directed biopsy specimen for histological verification can be taken. [127]

The introduction of population-based cervical cancer screening programs has led to a drastically reduced incidence of cervical cancer in developed countries. [3-6,128] However, there are some major drawbacks like compliance, low performance of cytology, and overtreatment of women with CIN.

The majority of women invited to the screening program do participate. In the Netherlands the participation rate is approximately 65%. [129-130] Since it is known that 50% of the cases with invasive cervical cancer arise in women who are not adequately screened an increase of

this participation is desirable. [3,131] Self-sampling is regarded as a possible tool to facilitate screening of women who refuse to participate in cervical cancer screening programs. [132-133] A self-sampling method performed by the woman herself, without intervention by a doctor, could lower the threshold and increase the participation to screening.

The sensitivity and specificity of cervical screening leave much to be desired. Sensitivity rates for cytology of only 40% to 80% for high grade cervical lesions (CIN 2/3) have been reported. [8,10-11] In attempting to increase the sensitivity of the Pap smear the specificity reduced to as low as 70%, [10-11,134-135] and a major problem facing developing countries now is the large number of women whose smears have not been regarded as normal. Most of these women are not at risk of developing cervical cancer and do not have premalignant lesions. Valuable sources, however, are used in investigating those women, probably unnecessarily, resulting in increasing health costs and emotional stress. This represents the negative side of screening programs and indicates that additional tests other than cytology will be necessary to help distinguish those women who are truly at risk.

5.2 Human Papillomavirus

Numerous studies investigating additional testing for high-risk HPV in cervical cancer screening have been published. The main aim of these studies was to improve the sensitivity and specificity of conventional cytology. There is a wide spread of results, depending partly on the population under study, the assay used, and the quality of the study. In studies involving women with low grade cytological abnormalities the sensitivity of HPV testing to detect high grade cervical lesions ranged between 70% and 85%. When HPV DNA testing was used as an adjunct to cytology, detection rates of histologically confirmed high grade lesions rose from 93% to 100%. [11,134,136-137] In another study additional HPV testing identified 41% of the patients with moderate or severe dysplasia who would have been missed by screening using only cytology. [8] A major problem of additional HPV testing is that it does not improve the low specificity from cytological screening alone, especially when HPV testing is performed in young women who have a high percentage of transient infections with high-risk HPV without underlying cervical lesions. [138-139]

Furthermore, it has been suggested that HPV testing could provide quality control of cervical cytology. [140] Rescreening of high-risk HPV positive, cytomorphologically normal smears could reduce the number of cytomorphologically false negative smears in 5 to 7%. [141]

The development of liquid-based cytologic methods in which cell samples are suspended in a collection tube offers the potential of using residual cellular material for HPV DNA testing (also referred to as reflex-cytology). This could eliminate a return visit to obtain a sample for

HPV DNA testing in women who have been found cytologically with low grade cervical abnormalities, resulting in cost-saving. [142]

5.3 Treatment of CIN

According to the Dutch guidelines women with morphologically confirmed CIN 2 or CIN 3 should be treated to prevent the development of cervical cancer. [126] This treatment consists of removal of the transformation zone, the area of the cervix on which most lesions are sited, by LLETZ (Large Looped Excision of the Transformation Zone), laser evaporation, cryocoagulation or cone biopsy. Which treatment is used depends mostly on the expertise in the different clinics. After treatment women are monitored by cervical cytology and in case of an abnormal smear colposcopic examination is indicated. However, there is no 100% cure-rate. After treatment failure rates of 5 to 15% have been observed despite close cytological follow-up, [143-144] and moreover, it appears that cytology after treatment has a low specificity. Many women present with abnormal cytology after treatment but in only 40-60% of them an underlying CIN lesion can be found. [145-146] Colposcopic examination is often inadequate because of the difficulty in interpreting features of the post-treatment cervix, resulting in unnecessary diagnostic procedures. [145] The prediction of residual lesions, i.e. the lesion that is not completely removed, or recurrent lesions beforehand could result in a more effective follow-up.

Removal of the transformation zone enhances the elimination of HPV in women. Effective treatment for CIN results in the eradication of high-risk HPV present before treatment. [147] In residual or recurrent lesions high-risk HPV is often present. [117] This could indicate that persistence of high-risk HPV after treatment predicts a residual lesion or the development of a recurrent lesion. Many women are anxious to hear whether the treatment for their lesion was successful. Additional testing for high-risk HPV during follow-up could improve the risk-assessment for post-treatment CIN (i.e. residual or recurrent lesions). Whether HPV should be used next to, or instead of cervical cytology during follow-up has to be investigated.

6. Outline of this thesis

As discussed in the previous paragraphs, compelling evidence exists for the necessity of infection with high-risk HPV for the development of cervical cancer and its precursor lesions. The use of high-risk HPV testing, as an adjunct to cervical cytology, in cervical cancer screening programs has been proposed but before implementation of the HPV test can be

performed, some questions considering the role of high-risk HPV in the natural course of CIN disease have to be answered.

From 1990 to 1996 a prospective non intervention cohort study was conducted in women referred to the colposcopy clinic because of an abnormal cervical smear in a collaboration between the departments of Pathology and Obstetrics and Gynaecology of the University Hospital Vrije Universiteit in Amsterdam. A total of 353 women were included in the study with abnormal cervical cytology (Pap 3a or 3b; mild to severe dyskaryosis) without having prior cervical pathology. The aim of this study was to establish the relationship between infection with high-risk HPV types and the natural development of CIN lesions. During follow-up no cervical biopsies were performed to avoid interference with the natural course of the disease. Patients were under tight clinical surveillance and closely monitored by cytological and colposcopic evaluation every 3-4 months. HPV genotyping was performed on cellular material from cervical smears by PCR using the primers GP5+/6+. Clinicians and laboratory staff were unaware of HPV test results and clinical findings, respectively. At each colposcopy, serial colpophotographs were taken on colour slides and these were evaluated by three expert colposcopists. The consensus colposcopic impression was recorded as the prediction for the histopathological diagnosis of the most serious lesion: no CIN, CIN 1, CIN 2, or CIN 3 and the size of the lesion measured in cervical quadrants. Since no biopsies were performed, a well-defined endpoint was necessary to avoid development of cervical cancer. Follow-up was ended when women reached *clinical progression*, which was defined as a colposcopic impression of CIN 3 covering three or more cervical quadrants or a cervical smear showing suspected microinvasive carcinoma, or at the end of the study in December, 1996. At the last visit, colposcopically directed biopsies were taken for histological verification of suspected lesions (=*end histology*). If necessary, women were treated according to standard protocol. This unique, non-intervention follow-up study gave us the opportunity to answer the following questions in chapters 2-4 of this thesis:

Chapter 2: *What is the natural course of high-risk HPV on the development of clinical progression and end histology CIN 3 in women with abnormal cervical cytology?*

The influence of high-risk HPV infection status, i.e. persistent infection, acquisition and clearance or continuously negative for infection was studied. The data could be used to assess the diagnostic value of a second high-risk HPV test and cervical smear for end histology CIN 3 after different time points and propose new guidelines for cervical cancer

screening programs. Although risk-assessment of progressive CIN disease is an important part in the set-up of new screenings strategies, identification of those women who will regress is essential to avoid overtreatment. This issue was addressed in the next chapter:

Chapter 3: *Is there a relationship between clearance of high-risk HPV and regression of cervical lesions?*

Cytological regression was related to HPV status and severity of the cervical lesion. In addition, the course of high-risk HPV clearance was related to cervical cytology status. In order to obtain insight in the sequence of high-risk HPV clearance and cytological regression the follow-up of women who reached both events was studied.

During follow-up 91 women were pregnant and we became interested in the influence of pregnancy on the natural course of an HPV infection, moreover since the literature did not come up with a one-sided conclusion on this point. The regular visits of the women included in the study enabled us to answer the next question:

Chapter 4: *Is there a difference in prevalence and clearance of high-risk HPV in pregnant and non-pregnant women?*

To study the effect of pregnancy on the natural course of an HPV infection clearance of high-risk HPV was calculated in pregnant and non-pregnant women.

The previous chapters describe the role of high-risk HPV testing in primary screening for cervical cancer. After treatment of cervical dysplasia a considerable number of women will present with residual or recurrent lesions despite close cytological follow-up. In spite of national guidelines the follow-up policies vary from centre to centre which indicates that there is a need for evaluation and better implementation. Since high-risk HPV is often present in post-treatment CIN the usage of high-risk HPV testing in the follow-up after initial treatment for CIN has been suggested. In a prospective, observational study including 184 women treated for high grade CIN the main question was:

Chapter 5: *Does addition of high-risk HPV testing contribute to a better risk assessment of post-treatment CIN?*

In this study women were monitored by cytology and testing for high-risk HPV DNA at different time-points after initial treatment. The performance of both tests in predicting post-treatment CIN was compared.

Not only the design of the cervical cancer screening programs is important, the effect on morbidity and mortality of cervical cancer also depends on the number of women who participate. Self-sampling by women, without intervention by a doctor, is regarded as a possible tool to facilitate screening in women who refuse to participate because it may lower the threshold and increase the attendance. In the next chapter we describe a study in which the use of a cervicovaginal lavage self-sampling device was evaluated. The main questions we had were:

Chapter 6: *Is cervicovaginal lavage performed by women useful as an alternative screening tool for cervical cytology and HPV detection and is it accepted ?*

A cervicovaginal self-sampling device was tested by 71 women at home. The performance of HPV DNA testing and cervical cytology results in the Pap smear and the lavage in detecting high grade cervical lesions was compared.

The results presented in the previous chapters are evaluated in **Chapter 7**. Clinical consequences are discussed.

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Chapter 2

Relation of Human Papillomavirus status to cervical lesions and consequences for cervical cancer screening: A prospective study

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Abstract

Background A relation has been established between infection with high-risk types of human papillomavirus (HPV) and development of cervical cancer. We investigated a role for testing for HPV as part of cervical cancer screening.

Methods We monitored by cytology, colposcopy, and testing for high-risk HPV 353 women referred to gynaecologists with mild to moderate and severe dyskaryosis. The median follow-up time was 33 months. At the last visit we took biopsy samples. Our primary endpoint was clinical progression, defined as cervical intraepithelial neoplasia (CIN) 3, covering three or more cervical quadrants on colposcopy, or a cervical smear result of suspected cervical cancer.

Results 33 women reached clinical progression. All had persistent infection with high-risk HPV. The cumulative 6-year incidence of clinical progression among these women was 40% (95% CI 21-59). In women with end histology CIN 3, 98 (95%) of 103 had persistent infection with high-risk HPV from baseline. Among women with mild to moderate dyskaryosis at baseline, a second test for high-risk HPV at 6 months predicted end histology CIN 3 better than a second cervical smear.

Conclusion Persistent infection with high-risk HPV is necessary for development and maintenance of CIN 3. All women with severe dyskaryosis should be referred to gynaecologists, whereas women with mild to moderate dyskaryosis should be referred only after a second positive test for high-risk HPV at 6 months.

INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide, and its development through premalignant stages has been the subject of several studies. [1] Nationwide screening programmes have been set up that have led to decreases in the incidence of cervical cancer. [2] Important drawbacks of screening programmes, such as too many screening rounds, overreading of slides, and considerable overtreatment of cervical intraepithelial neoplasia (CIN), underscore the need for changes to the rationale for organised cervical cancer screening programmes. [3] Several epidemiological studies have established a strong relationship between infection with high-risk types of Human Papillomavirus (HPV) and the development of cervical cancer and its precursors. [4-12]

Insight into the relation between HPV and the natural history of CIN lesions can lead to more efficient cervical cancer screening strategies by combining cervical smear with testing for HPV. [13-16] We prospectively studied the development, persistence, and progression of CIN lesions in relation to HPV status in women referred to gynaecologists because of abnormal cervical smear results.

METHODS

Patients

405 women referred to the colposcopy clinic of the Free University Hospital, Amsterdam, from June, 1990, to December, 1992, were eligible for the study and were willing to participate. The inclusion criteria were: an abnormal cervical smear (i.e., mild to moderate or severe dyskaryosis); age 18-55 years; no medical history of cervical pathology, prenatal diethylstilbestrol exposure, or concomitant cancer; sufficient Dutch or English language skills.

At baseline we asked women to complete a questionnaire on smoking, number of lifetime sexual partners, and age at first sexual intercourse. We monitored women every 3-4 months by testing for HPV, cytology and colposcopy. [17] Clinicians and the laboratory staff were unaware of HPV test results and the clinical findings, respectively. Cervical smears were classified according to the KOPAC classification, the standard classification in the Netherlands. [18] Smears are cytomorphologically classified as Pap 1, Pap 2 (very mild dyskaryosis), Pap 3a (mild to moderate dyskaryosis), Pap 3b (severe dyskaryosis), Pap 4 (suspected of carcinoma in situ) and Pap 5 (suspected of at least micro-invasive carcinoma). Standard colposcopic assessment with acetic acid and iodine solutions was done by the colposcopist. Serial colpophotographs were taken on colour slides. Three expert colposcopists assessed the colpophotographs after each patient's visit, according to international standards. [19]

We recorded the consensus colposcopic impression as the prediction for the histopathological

diagnosis of the most serious lesion- no CIN, CIN 1, CIN 2, or CIN 3- and the size of the lesion measured in cervical quadrants. Colposcopic criteria was validated in previous studies. [20,21] We did not take biopsies during follow-up for verification of the colposcopic impression to avoid any interference with the natural course of the disease.

353 women met the inclusion criteria. We ended follow-up if women reached the primary endpoint of clinical progression, defined as CIN 3, or at the end of follow-up in December, 1996. At the last visit, colposcopically directed biopsy samples were taken. In women who had normal colposcopy, random biopsies were taken. Women with CIN 2 or CIN 3 were treated according to standard protocols by large-looped excision of the transformation zone (LLETZ) or conization. LLETZ or conization were also done in women with no CIN or CIN 1, but for whom the last cervical smear result showed severe dyskaryosis or worse, or when lesions were suspected on colposcopy as being as severe as CIN 2 or 3, or both. For end histology, we classified women according to the highest CIN grade found in the biopsy sample or on LLETZ or conization. All histology samples, including cervical biopsy samples, LLETZ, and conizations, were checked by an experienced pathologist who was masked to clinical information, and were classified as no CIN or CIN 1, 2 or 3, according to international criteria. [22-23] In 29 women, biopsy samples were not taken. These 29 women had normal cytology at their last visits and were lost to follow-up or refused biopsy, and were taken in analyses to have no CIN.

The study protocol was approved by the ethics review board of the hospital, and all women voluntarily signed informed consent before enrolment.

Human papillomavirus testing

Testing for HPV was done by EIA PCR, which used HPV general-primer-mediated PCR with the general primers $GP5^{+}/6^{+}$.[24-25] This test has been clinically validated. [10,11,17] We used one assay for all 14 high-risk types of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). In addition, we analysed the PCR amplification products for individual high-risk types.

We defined women as having newly acquired HPV if they had a negative result at baseline but a positive result at a later visit. Women were clear of infection when no type of HPV from the previous visit was detected at the next visit, otherwise we took infection to be persistent.

Statistical analysis

We took the timepoints of acquisition and clearance to be at the midpoint between positive and negative results for HPV. We used Kaplan-Meier analysis to estimate the clearance of infection among women with positive results for high-risk HPV at baseline and among those who acquired high-risk types of HPV during follow-up. Infection status throughout the study, persistent infection, acquisition and clearance of infection, and continuously negative for infection with high-risk HPV,

was related to clinical progression and end histology. We defined clinical progression (the primary endpoint) as reaching a colposcopic impression of CIN 3 covering three or more cervical quadrants or a cervical smear showing suspected microinvasive carcinoma. Women who reached the primary endpoint were withdrawn from the study because of the high risk of progression to cervical cancer. Since the time to clinical progression was known, we used Kaplan-Meier analysis to estimate cumulative incidence. We repeated the analysis with censoring at the time of clearance of women with a positive result at baseline.

End histology CIN 3 was our secondary endpoint and was related to baseline status and types of high-risk HPV. Since we did not take biopsy samples during follow-up, the time to first occurrence of CIN 3 was not known. We therefore omitted time from the analysis and used logistic regression to identify additional risk factors.

For women with mild to moderate dyskaryosis at baseline, we used two-by-two tables to assess the diagnostic value for end histology CIN 3 of a second test for HPV and a second cervical smear at 6 months, 12 months, and 18 months after baseline. For these analyses, the last observations were carried forward for women who had reached the primary endpoint and were withdrawn from the study before 18 months of follow-up. In addition, we used the McNemar test to assess significance of clearance of high-risk HPV and return to normal cytology from baseline to 6 months, from 6 months to 12 months, and from 12 months to 18 months.

RESULTS

After the initial visit, 52 of 405 women were excluded because: the primary endpoint had been reached (25); the squamocolumnar junction was not visible at colposcopy (17); the classification of colposcopic impression was impossible because of erosion (five); and unintended protocol violations at baseline (a biopsy sample was taken accidentally in five). Of these 52 women, 34 (65%) women had severe and 18 (35%) mild to moderate dyskaryosis. Among the women with severe dyskaryosis, histology showed two had microinvasive cancers, 30 CIN 3, one CIN 2, and one CIN 1. Of the women with mild to moderate dyskaryosis, four had CIN 3, two CIN 2, one CIN 1, and eight no cervical intraepithelial neoplasia, and for three women no biopsy sample was taken because of no suspicion on colposcopic impression.

The 353 women enrolled attended a total of 3070 visits. The median number of visits per woman was nine (rang two to 16). The mean age was 32 years (range 18-55) and median follow-up time was 33 months (rang three to 74). Of these women, 56 (16%) had severe dyskaryosis, and 297 (84%) mild to moderate dyskaryosis at baseline, and 233 women (66%) had positive results for high-risk HPV (table 1). 13 different high-risk HPV genotypes were identified and 31 multiple infections detected.

Table 1: Baseline characteristics.

Baseline characteristics*	n	Clinical progression by end histology					
		Yes		No			
		CIN 3 (n=32)	CIN 2 (n=1)	CIN 3 (n=71)	CIN 2 (n=29)	CIN 1 (n=64)	No CIN (n=156)
Severe dyskaryosis hr HPV positive (n=51)†							
Lesion ≥ 3 quadrants	22	..	..	..	..	..	..
CIN 2	18	9‡	0	4	1	1	3
CIN 1	4	1	0	2	0	0	1
Lesion < 3 quadrants	29	..	..	..	..	..	..
CIN 3	4	1	0	2	0	0	1
CIN 2	9	1	0	2	0	3	3
CIN 1	13	2	0	5	0	2	4
CIN 0	3	0	0	2	0	0	1
Severe dyskaryosis hr HPV negative (n=5)							
Lesion ≥ 3 quadrants	0	..	..	..	..	..	..
Lesion < 3 quadrants	5	..	..	..	..	..	..
CIN 2	2	0	0	0	0	1	1
CIN 1	2	0	0	0	1	0	1
CIN 0	1	0	0	0	0	1	0
Mild to moderate dyskaryosis hr HPV positive (n=182)†							
Lesion ≥ 3 quadrants	63	..	..	..	..	..	..
CIN 2	35	8	1	13	5	4	4
CIN 1	28	4	0	7	6	2	9
Lesion < 3 quadrants	119	..	..	..	..	..	..
CIN 3	2	0	0	1	0	1	0
CIN 2	25	4	0	10	1	4	6
CIN 1	68	2	0	16	4	11	35
CIN 0	24	0	0	4	1	4	15
Mild to moderate dyskaryosis hr HPV negative (n=115)							
Lesion ≥ 3 quadrants	19	..	..	..	..	..	..
CIN 2	14	0	0	2	1	7	4
CIN 1	5	0	0	0	0	2	3
Lesion < 3 quadrants	96	..	..	..	..	..	..
CIN 2	13	0	0	0	1	5	7
CIN 1	43	0	0	1	7	7	28
CIN 0	40	0	0	0	1	9	30

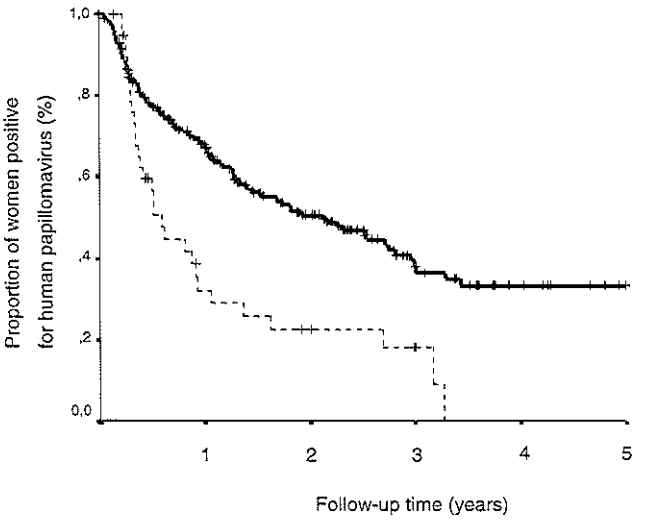
CIN=cervical intraepithelial neoplasia. hr HPV=high-risk human papillomavirus. * Cytology (severe or mild to moderate dyskaryosis) results; total size of lesion, which can consist of more and less parts, measured in cervical quadrants; and CIN grading according to most severe part of colposcopic lesion. † Human papillomavirus distribution= severe 26x16, 5x18, 4x31, 2x33, 4x35, 3x52, 2x58, 1x56, 4x multiple; mild to moderate 76x16, 12x18, 15x31, 9x33, 6x35, 2x39, 1x45, 8x51, 7x52, 4x56, 11x58, 2x59, 2x66, 27x multiple. ‡ One woman reached clinical progression because of cervical smear showing suspected microinvasive carcinoma.

148 women did not complete follow-up because of: refusal to participate further (104); discordance between cytomorphological result and colposcopic impression (18); non-classification of colposcopic impression (17); repeated suspected carcinoma in situ (five); and protocol violations (four). These women were equally distributed during follow-up and were censored at the time of the last visit.

Among the 233 women with positive results for high-risk HPV at baseline, the median time to clearance of infection was 25 months (95% CI 17-34; figure 1). No difference in clearance was found for women with severe or mild to moderate dyskaryosis at baseline. 122 women had

persistent infection with high-risk HPV (from baseline to last visit). Of 120 women with a negative results for high-risk HPV at baseline, 39 acquired new high-risk infections (figure 1). For these women, the median clearance time for HPV was 6 months (range 4-8 months; figure 1). 27 of these 39 women had normal cytology (19) or very mild dyskaryosis (eight) at the time of acquisi-

Figure 1: Clearance of high-risk human papillomavirus in women with cytomorphologically abnormal smears and positive results for high-risk human papillomavirus at baseline.



tion. 33 women reached clinical progression (the primary endpoint), 14 of whom had severe dyskaryosis at baseline and 19 mild to moderate dyskaryosis at baseline. 30 had colposcopically defined CIN 3 covering three or more cervical quadrants, and in three others, the cervical smear was read as suspected microinvasive carcinoma. All of these 33 women had persistent infection with high-risk HPV. The mean age was 32·2 years (range 22-45), which did not differ significantly from the mean age of the remaining women (32·4 years).

No clinical progression was seen in women negative for high-risk HPV (figure 2). The cumulative 6-year incidence for women with a positive result for high-risk HPV at baseline was 18% (figure 2). After censoring at the time of clearance, when women were no longer at risk of progression, the cumulative 6-year incidence was 40% (figure 2). In the 33 women with clinical progression, histology showed CIN 3 in 32 and CIN 2 in one. The latter woman was withdrawn from the study after 6 months of follow-up with a colposcopic impression of CIN 3 covering three cervical quadrants, whereas during follow-up two cervical smears were read as moderate dyskaryosis.

End histology CIN 3 was present in 103 women, of whom 31 had severe and 72 mild to moderate dyskaryosis at baseline (table 1). At base-line, 100 (97·1%) had positive results for high-risk HPV and in 98 infection was persistent (table 2).

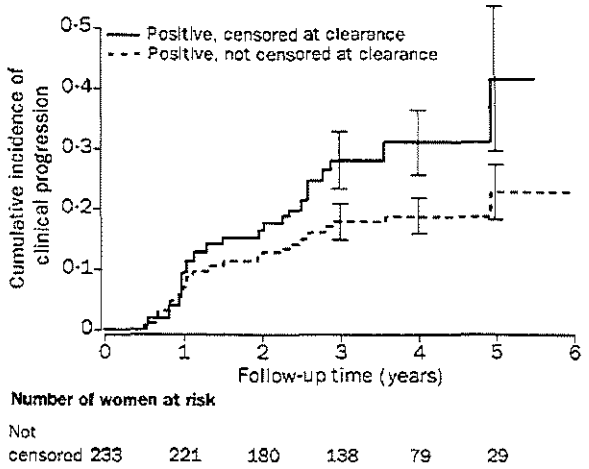


Figure 2: Cumulative incidence of clinical progression among women positive for high-risk human papillomavirus at baseline.

Among women with end histology CIN 3, only two had cleared initial infection with high-risk HPV. One of these two women cleared a double infection with types 51 and 66, and 32 months before diagnosis of CIN 3 acquired type 33. The other woman had HPV type 51 at baseline. After three positive results for HPV type 51 (9 months), six tests were negative (28 months), but five subsequent tests were positive for type 51 again (24 months; table 2).

Among women with a negative results for high-risk HPV at baseline, three had CIN 3. Two of these had newly acquired and subsequently persistent infections (types 51 and 58, respectively) for 3 years before the detection of CIN 3. The other women had two negative results, of which one was negative for high-risk HPV and the other contained no amplifiable DNA (ß-globin negative). Follow-up ended after 4 months, because this woman wanted to withdraw from the study. Women with persistent infection with high-risk HPV from baseline till the last visit were at higher risk of end histology CIN 3 than those who had negative results throughout the study (odds ratio 327 [95% CI 42-2468]; table 2).

The risk for end histology CIN 3 in women who had acquired infection, cleared infection, or both during the study was slightly higher than for women with negative results, but not significantly so (2·9 [0·2-20]). The risk of end histology CIN 3 did not differ between individual types of high-risk HPV.

To study possible bias in the relation between high-risk HPV status and end histology CIN 3 in all women, we also analysed this relation in the 148 women who did not complete follow-up, and compared this value to the group who did complete follow-up (n=205). The percentage of women who reached end histology CIN 3 did not differ significantly for persistent, clearance and acquisition, or absence of infection with high-risk HPV (data not shown).

Table 2: High-risk human papillomavirus status during study and risk for end histology of CIN 3.

HPV status during study*	Number of women	Clinical progression (n=33)						Odds ratio for end histology CIN 3 (95% CI)
		Yes (n=33)		No (n=320)				
		CIN 3 n=32	CIN 2 n=1	CIN 3 n=71	CIN 2 n=29	CIN 1 n=64	CIN 0 n=156	
Persistent	122	32	1	66	7	6	10	327 (42-2468)
Clearance and acquisition	150	..	..	4†	15	35	96	2·9 (0·2-20)
Negative	81	..	..	1	7	23	50	1·0

HPV=human papillomavirus * Stratified according to high-risk human papillomavirus status at base-line and course during the study. † Two women with end histology CIN 3 cleared initial infection and acquired infections that had persisted for 24 and 36 months, respectively, at time of biopsy. The other two women had negative results at baseline and acquired infections that had persisted for 36 months at the time of biopsy.

Apart from high-risk HPV, no additional risk for end histology CIN 3 was found for age in tertile groups, smoking, age at first sexual intercourse, and history of more than two sexual partners (table 3). These risk factors do not, therefore, influence histological outcome after cytology becomes abnormal.

Cervical cytology and testing for HPV at 6 months, 12 months, and 18 months was analysed in the 297 women referred with mild to moderate dyskaryosis, since these women, in contrast to women with severe dyskaryosis, are not generally referred directly to gynaecologists. For women with mild to moderate dyskaryosis, the odds ratio for end histology CIN 3 after a positive result for high-risk HPV test at baseline was 23 (7-75, table 4). The odds ratio for a second high-risk HPV test at 6 months, 12 months and 18 months, increased to 61 (15-250), 72 (17-280) and 89 (21-350), respectively. These odds ratios show wide 95% CIs because of the low number of women with end histology CIN 3 among those negative for high-risk HPV. Clearance among women without end histology CIN 3, however, which is the underlying mechanism for the increase in odds ratio, was significant for each time period of 0-6 months, 6-12 months, and 12-18 months (table 4). For a second abnormal cytomorphological test at 6 months, 12 months and 18 months the odds ratios for end histology CIN 3 are 3·6, 7·9, and 19·3, respectively (table 4). The return to normal cytology among women without end histology CIN 3, which is the underlying mechanism for the increase in odds ratio, was significant (table 4).

Table 3: Baseline risk factors for end histology CIN 3.

Baseline risk factor	CIN 3 / total	Crude odds ratio (95% CI)*	Adjusted odds ratio (95% CI)†
High-risk human papillomavirus			
Positive	100/233	29 (9.2-96.0)	25 (7.9-86.0)‡
Negative	3/120	1.0	1.0
Smoking			
Never	31/120	1.0	1.0
1-10 cigarettes/day and ex-smoker	25/92	1.0 (0.6-2.0)	0.7 (0.3-1.5)
> 10 cigarettes/day	45/132	1.5 (0.9-2.6)	1.1 (0.6-2.1)
Missing data	2/9		
Number of sexual partners			
0-1	21/96	1.0	1.0
2-4	32/98	1.7 (0.9-3.3)	1.5 (0.8-3.3)
5-9	22/70	1.6 (0.8-3.3)	1.6 (0.7-3.7)
>10	21/61	1.8 (0.9-3.8)	1.5 (0.6-3.5)
Missing data	7/28		
Age at first sexual intercourse (years)			
≥ 20	14/52	1.0	1.0
18-19	22/104	0.7 (0.3-1.6)	0.6 (0.2-1.5)
16-17	44/120	1.6 (0.8-3.2)	1.2 (0.5-2.8)
≤15	19/55	1.4 (0.6-3.3)	0.9 (0.3-2.3)
Missing data	4/22		
Age (years)			
≤27	30/110	1.0	1.0
28-34	40/117	1.4 (0.8-2.4)	1.6 (0.8-3.1)
≥35	33/126	0.9 (0.5-1.7)	1.6 (0.7-3.2)

* Crude and adjusted odds ratio were calculated by logistic regression analysis. † Each variable adjusted for all other variables in table. ‡ p<0.0001

To compare the effectiveness of testing for high-risk HPV and repeated cervical smear to identify women with cervical intraepithelial neoplasia 3, we calculated the sensitivity, specificity and positive and negative predictive value. The sensitivity and specificity for end histology CIN 3 among women with mild to moderate dyskaryosis, and a second test for high-risk HPV at 6 months were 97% and 65%, respectively, whereas the positive and negative predictive values were 46% and 99%, respectively (table 4). For a second abnormal cytomorphological test showing mild to moderate dyskaryosis, these values were 70%, 61%, 36% and 87%, respectively (table 5). As a second test at 6 months to identify women with end histology CIN 3, the sensitivity and the negative predictive value of HPV testing were better at a similar degree of specificity and positive predictive value, than those for a second cervical smear.

DISCUSSION

Clinical progression was not seen in women in the absence of high-risk HPV. Persistent infection with high-risk HPV is, therefore, required for development and maintenance of CIN 3.

Factors other than high-risk HPV have been reported to contribute to the development of CIN 3.[8,9,26,27] In our study, however, we found no additional risk factors for end histology CIN 3. Although the number of women with high-risk HPV was low, we found no differences in capacity to induce and maintain CIN 3 between types of high-risk HPV. This finding is in agreement with other studies and shows that testing for groups of types of high-risk HPV is sufficient for clinical usage. [10,11] The yearly incidence of clinical progression, our primary endpoint, was 6% during the first 36 months of follow-up, which is similar to the 5% reported previously. [17] In the other 3 years of follow-up, the yearly incidence was 1% (Figure 2, line B). When women were censored at the time of clearance of HPV, the incidence was constant at 8% per year, which suggests that women are at risk for progression only as long as infection with high-risk HPV persists. Of women with end histology CIN 3, the secondary endpoint, 95% had persistent infection with high-risk HPV from baseline to end of follow-up. In five women with CIN 3 who did not have persistent infection, the samples negative for high-risk HPV were retested with type-specific HPV PCR to ensure that they contained no HPV. [28] Three women had been HPV negative at base-line and two had cleared the initial high-risk HPV type. Four of them acquired a new high-risk infection that subsequently persisted for about 3 years before CIN 3 was diagnosed. In these 5 women with CIN 3, the high-risk HPV negative samples were retested. In all five women, high-risk HPV was identified. Among those, two women acquired high-risk HPV about 3 years before end histology CIN 3 was diagnosed. Therefore, no clinical progression, CIN 3, or both were seen among women without high-risk HPV infections, which is in agreement with other studies. [26,29]

The overall median clearance time of high-risk HPV infections was 25 months. After 5 years of follow-up 67% had cleared infection. The median clearance time of newly acquired HPV was 6 months. This clearance time is similar to that in a study of 365 young women with normal cytology, in whom newly acquired HPV had an overall clearance time of 2 years in 91%, with a median clearance time of 8 months. [26] The long overall clearance time in our study can be explained by high proportion of women with CIN 3 who hardly clear HPV. [30]

Our analyses may have been subject to bias. However, among the women with mild to moderate dyskaryosis excluded from follow-up, the proportion of those with CIN 3 (four [22%] of 18) was similar to that among those included (72 [24%] of 297). In some women with end histology CIN 3, therefore, a CIN 3 lesion had been present from the start of the study. In addition, excluded or included women with mild to moderate dyskaryosis did not differ significantly for positive results for HPV, age, smoking, number of sexual partners, and age of first sexual intercourse.

Based on our results, new guidelines for cervical cancer screening strategies can be developed for cytology in conjunction with testing for high-risk HPV. In accordance with current policies, we advise the referral of women with severe dyskaryosis or worse directly to gynaecologists, irre-

Table 4: Performance of testing for high-risk human papillomavirus at baseline and at time of end histology CIN 3 in women with mild to moderate dyskaryosis at baseline.

Time of test for high-risk human papillomavirus after entry (months)	Total number of women in follow-up	Positive infection plus CIN 3 / total number of positive women*	Negative infection plus CIN 3 / total number of negative women*	Odds ratio for CIN 3 with positive result at time indicated (95% CI)†	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
0	297	69/182	3/115	23 (7-75)	96	50	38	97
6	283	65/140	2/143	61 (15-250)	97	65	46	99
12	262	58/117	2/145	72 (17-280)	97	71	50	99
18	250	54/99	2/151	89 (21-350)	97	77	55	99

PPV=positive predictive value; NPV=negative predictive value. * Change in denominator is because of women who had ended study and cleared or acquired high-risk human papillomavirus at time indicated. † Increase in odds ratio caused by human papillomavirus persistence among women with end histology CIN 3 and clearance among women without end histology CIN 3. Clearance is significant during each interval (McNemar test χ^2: t_0-t_6 months (n=283), t_6-t_{12} months (n=262) and t_{12}-t_{18} months (n=250): 22, 5·0, 8·0, respectively).

Table 5: Performance of cervical smear at end histology CIN 3 in women with mild to moderate dyskaryosis at baseline.

Time of cervical smear after entry (months)	Total number of women in follow-up	Women with mild to moderate dyskaryosis or worse and CIN 3 / total number of women with mild to moderate dyskaryosis or worse	Women with less than mild to moderate dyskaryosis and CIN 3 / total number of women with less than mild to moderate dyskaryosis	Odds ratio for CIN 3 in mild to moderate dyskaryosis or worse at time indicated (95% CI)*	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
0	297	72/297	0	NA	NA	NA	NA	NA
6	283	47/132	20/151	3·6 (2·0-6·5)	70	61	36	87
12	262	44/96	16/166	7·9 (4·1-15)	73	74	46	90
18	250	44/75	12/175	19·3 (9·2-41)	79	84	59	93

PPV=positive predictive value; NPV=negative predictive value. NA=not applicable because mild to moderate dyskaryosis was an inclusion criterium for study. * Increase in odds ratio from t_6 to t_{18} caused by number of women who return to normal cytology among those without end histology CIN 3. Return to normal cytology is significant during each interval (McNemar test χ^2: t_6-t_{12} months (n=262) and t_{12}-t_{18} months (n=250); 11·3 and 4·8, respectively).

spective of testing for HPV, because of the high proportion of women with CIN 3. We propose that all women with mild to moderate dyskaryosis are retested for high-risk HPV after 6 months. Only women who have positive high-risk HPV test results at initial testing and 6 months should be referred to gynaecologists. Women with negative high-risk HPV test results at both visits, or who are positive at baseline and negative at 6 months can remain in the population- based screening programmes. For women who are negative for high-risk HPV at the first visit and positive after 6 months, we recommend repetition of the cervical smear and testing for HPV after an additional 6 months. This approach should substantially decrease overtreatment of women with mild to moderate dyskaryosis. The potential risk of missing women with progression is restricted to the few who have false-negative results for high-risk HPV. In this study, only two of 103 (2% [95% CI 0·5-8]) women with end histology CIN 3 had a false-negative high-risk HPV test. This low risk of false-negative results for high-risk HPV is clearly compensated for by its much higher sensitivity than for a second cervical smear, as is current practice.

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Chapter 3

Cytological regression and high-risk HPV clearance in women referred for colposcopy because of an abnormal cervical smear

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Abstract

Background Persistent high-risk Human Papillomavirus (HPV) infection is necessary for the development and maintenance of premalignant cervical lesions. However, a substantial number of these lesions regress but its relationship with HPV is not established.

Methods We monitored by cytology, colposcopy, and testing for high-risk HPV 353 women referred to the gynaecologist with abnormal cervical cytology (mild to severe dyskaryosis). The median follow-up time was 33 months (range 3-74). Biopsy samples were taken only at the last visit. Kaplan Meier curves were used to estimate cytological regression and clearance of high-risk HPV.

Results Women with a high-risk HPV positive test had a cumulative one-year incidence of cytological regression to normal cytology of 37% (95% CI 25-50), 34% (95% CI 20-47), and 12% (95% CI 3-22) in mild, moderate, and severe dyskaryosis, respectively. In women with a high-risk HPV negative test these incidences were 68% (95% CI 54-84), and 53% (95% CI 16-90) for mild and moderate/severe dyskaryosis, respectively. The cumulative one-year incidence of high-risk HPV clearance in smears with normal cytology, very mild, mild, moderate and severe dyskaryosis was 46% (95% CI 26-66), 50% (95% CI 32-68), 29% (95% CI 18-50), 23% (95% CI 11-35), and 25% (95% CI 12-38), respectively. Overall, high-risk HPV clearance preceded cytological regression with a mean time of 3 months. Six out of eight women who reached cytological regression without HPV clearance had end histology CIN 3, whereas no CIN 3 was seen in women (n=4) who cleared HPV without cytological regression.

Interpretation In women with abnormal smears high-risk HPV testing predicts cytological regression. Even if cytological abnormalities persist no CIN 3 will develop without high-risk HPV.

INTRODUCTION

Several studies have established that an infection with high-risk HPV is the main cause for the development of cervical cancer. In nearly all cervical carcinomas high-risk HPV types can be identified. [1] Women with normal cervical cytology and a high-risk HPV positive test have a more than 100 times higher risk to develop Cervical Intraepithelial Neoplasia grade 3 (CIN 3) than women without high-risk HPV [2,3] Moreover, in women with abnormal cervical cytology a persistent high-risk HPV infection is required for the maintenance and development of cervical lesions. [4-6] This indicates that women with a persistent high-risk HPV infection should be referred for diagnosis and treatment. Many women acquire high-risk HPV during life but most of them clear this infection. [7-10] Only a small proportion of women infected with high-risk HPV will develop CIN 3 or cervical cancer, and in most women with premalignant cervical lesions the lesions regress spontaneously. [11-13] The more severe the CIN lesion, the lower the chance of regression. [12] Over-treatment of these women can be prevented by selecting women with premalignant cervical lesions which will regress. Although a direct relationship between clearance of high-risk HPV and regression of the lesion has been assumed, this has never been documented. Therefore, we prospectively studied clearance of high-risk HPV and cytological regression by following women referred to the gynaecologist because of an abnormal cervical smear. No biopsy samples were taken during the study to avoid any interference with the natural course of the disease.

METHODS

Patients and follow-up

353 women referred to the colposcopy clinic of the University Hospital Vrije Universiteit, Amsterdam, from June, 1990, to December, 1992, were included in the study. Inclusion criteria were: referral because of an abnormal cervical smear (i.e., mild to moderate or severe dyskaryosis); age 18-55 years; no medical history of cervical pathology, prenatal diethylstilbestrol exposure, or concomitant cancer; and sufficient Dutch or English language skills. According to the guidelines of the population-based cervical cancer program in the Netherlands at the time of inclusion, women were referred to the gynaecologist in case of two consecutive cervical smears read as mild or moderate dyskaryosis within 6 months, or one smear read as severe dyskaryosis or worse. The mean age was 32 years (range 18-55) and the median follow-up time was 33 months (range 3-74). The characteristics of this cohort of women have been described previously. [6]

Women were monitored every 3-4 months by cytology, colposcopy, and testing for high-risk HPV.[5,6] Clinicians and laboratory staff were unaware of HPV test results and clinical findings,

respectively. Cervical smears were classified according to the KOPAC classification, the standard classification in the Netherlands. [14] This is a modification of the Pap classification. [15] Cervical smears are cytomorphologically classified as Pap 1, Pap 2 (very mild dyskaryosis), Pap 3a (mild to moderate dyskaryosis), Pap 3b (severe dyskaryosis), Pap 4 (suspected of carcinoma in situ), and Pap 5 (suspected of at least microinvasive carcinoma). The colposcopic assessment by a gynaecologist has been described previously. [16] No biopsy samples were taken during follow-up to avoid any interference with the natural course of the disease. Follow-up was ended if women reached clinical progression (n=33), defined as a colposcopic impression of CIN 3 covering three or more cervical quadrants or a cervical smear suspected of microinvasive carcinoma, or at the end of the study in December, 1996. At the last visit, colposcopically directed biopsy samples were taken for histological verification of suspected lesions (= end histology). In women with normal colposcopy, random samples were taken. If necessary, women were treated according to standard protocols. For end histology, we classified women according to the highest CIN grade found in the biopsy sample or on additional treatment. [6]

The study protocol was approved by the ethics review board of the hospital, and all women voluntarily signed informed consent before enrolment.

Human papillomavirus testing

Testing for HPV was done by EIA PCR, which used HPV general-primer-mediated PCR with the general primers GP 5+/6+ to detect a broad spectrum of mucosotropic HPV types. [17,18] PCR products were used to identify in one assay all 14 high-risk types using EIA (HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). In addition, individual high-risk HPV types were determined. This has been described previously and has been clinically validated. [18,19]

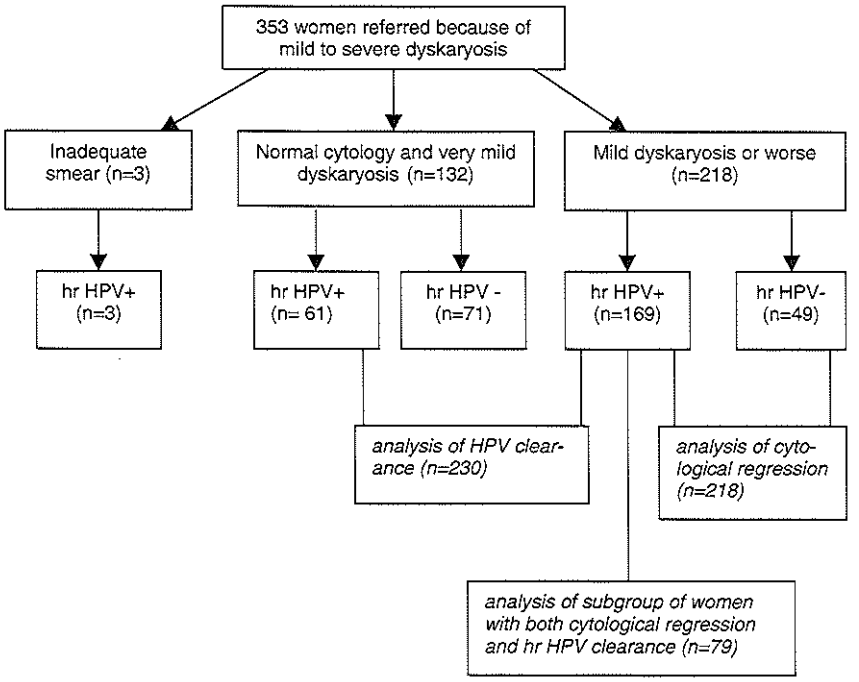
Definitions of HPV clearance and cytological regression

Women had cleared the HPV infection when none of the high-risk HPV types from the previous visit were detected at the next visit. Regression of abnormal cervical cytology was defined as a return to normal cytology (at least two consecutive cervical smears read as normal).

Statistical analysis

The groups used in the analysis are given in figure 1. Kaplan Meier curves were used in the main analyses. The time points of cytological regression and clearance of HPV were taken at

Figure 1: Study population at baseline* and analysis groups



* Time interval between referral and enrolment in study (baseline) is about 2 months.

the midpoint between positive and negative results for cervical cytology and HPV, respectively. The course of cytological regression was assessed among 218 women with abnormal cervical smears (mild dyskaryosis or worse) at baseline and related to age at baseline in tertile groups (≤ 28 years; 28-34 years; and ≥ 34 years), high-risk HPV status (high-risk HPV type positive (n=169) or negative (n=49)) and cervical cytology status (i.e., mild dyskaryosis; moderate dyskaryosis, and severe dyskaryosis) at baseline. Women who were withdrawn from the study because of clinical progression (n=33) were not censored. [6] In the analysis of 49 women with abnormal cervical cytology and a negative high-risk HPV test at baseline two groups of abnormal cytology: i.e., mild dyskaryosis (n=41), and moderate to severe dyskaryosis (n=8) were used because of the small number of women with moderate dyskaryosis or worse who had a high-risk HPV negative test at baseline. We estimated the course of HPV clearance related to cervical cytology status at baseline and age at baseline in tertile groups

among 230 women with a high-risk HPV positive test at baseline. Three women with an inadequate cervical smear sample (Pap 0) at baseline were excluded from the analysis. Kaplan-Meier curves were compared using the log-rank test; for ordered groups the test for linear trend was used. Clearance of high-risk HPV was related to age in 5-year intervals using the chi-square test. Differences between cytological regression or high-risk HPV clearance for a single or multiple high-risk HPV type infection at baseline were analysed by the chi-square test.

In order to obtain insight in the sequence of high-risk HPV clearance and cytological regression the follow-up times until cytological regression and high-risk HPV clearance were compared in women who reached both events (n=79) during follow-up using the Student's t-test. A two-sided p-value of < 0.05 was considered statistically significant.

RESULTS

Cytological regression

Of the 353 women referred with abnormal cytology and included in the study, 218 (61.8%) still had an abnormal cervical smear (i.e., mild dyskaryosis or worse) at baseline (figure 1).

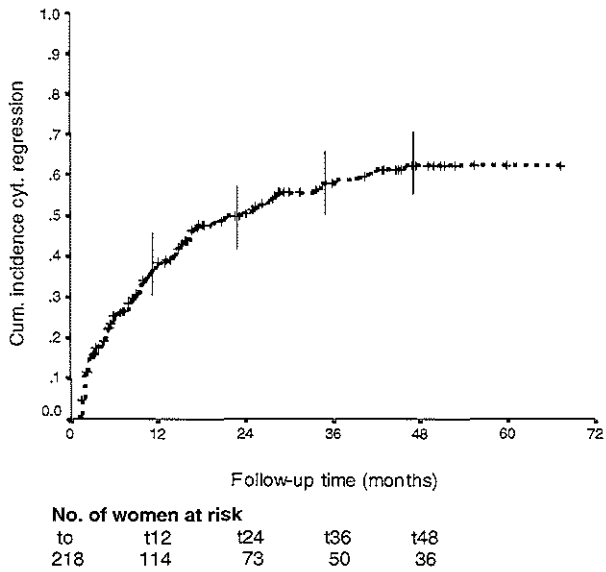


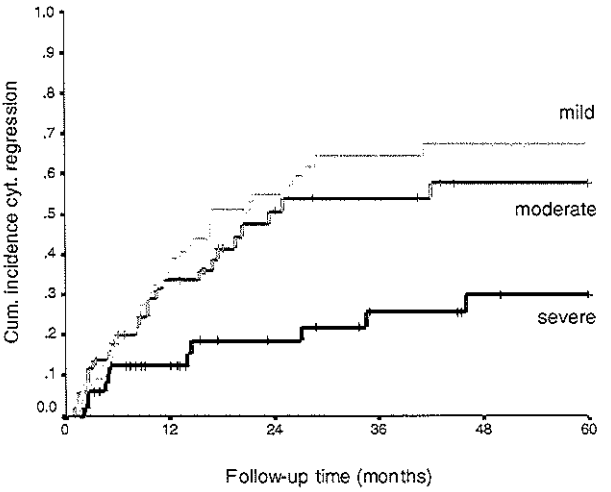
Figure 2: Cytological regression in women with abnormal cervical cytology at baseline. Vertical bars represent 95 percent confidence intervals.

The mean age in this group was 33 years (range 20-55). The cumulative one-year incidence of cytological regression was 38% (95% C.I. 31-45; figure 2). After four years of follow-up

62% (95% C.I. 54-70) of the women had reached normal cervical cytology. No difference in cytological regression was found for age at baseline in tertile groups.
Of the 218 women with abnormal cervical cytology 169 (77.5%) had a high-risk HPV positive test at baseline. In this group, the cumulative one-year incidence of cytological regression in women with either mild dyskaryosis, moderate dyskaryosis, or severe dyskaryosis was 37% (95% C.I. 25-50), 34% (95% C.I. 20-47), and 12% (95% C.I. 3-22), respectively (log-rank for trend p<0.005, figure 3). The median regression time was 17 (95% C.I. 6-27), 24 (95% C.I. 1-50), and > 60 months (95% C.I. >60), respectively.

Figure 3: Cytological regression in high-risk HPV positive women at baseline stratified for mild (n=67), moderate (n=51), and severe dyskaryosis (n=51) at baseline.

Log-rank for trend: p<0.005



Of the remaining 49 (22.5%) women with abnormal cervical cytology and a high-risk HPV negative test at baseline, the one-year cumulative incidence of cytological regression in women with mild dyskaryosis or moderate to severe dyskaryosis was 68% (95% C.I. 54-84), and 53% (95% C.I. 16-90), respectively (log-rank p=0.99, figure 4). The median regression time was 5 (95% C.I. 1-10) and 6 (95% C.I. 1-17) months, respectively.

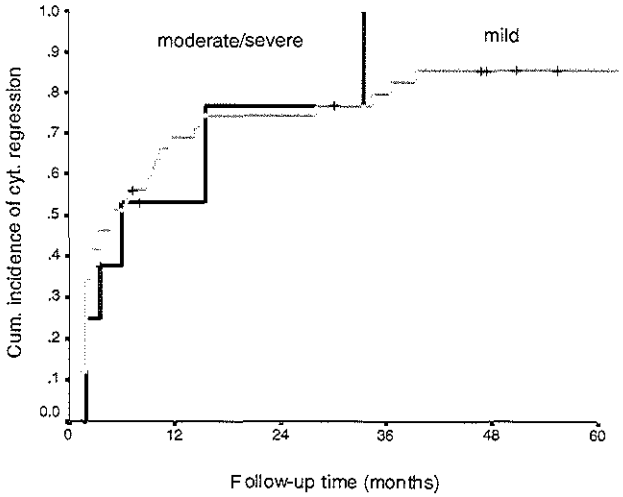
Clearance of high-risk Human Papillomavirus

HPV clearance was analysed among the 230 women with a high-risk HPV positive test and an adequate cervical smear at baseline. The mean age was 32 years (range 18-55). No difference in HPV clearance was seen for age at baseline (5-year interval). Overall, the cumulative one-year incidence of HPV clearance was 35% (95% C.I. 28-42). After four years of

follow-up 66% (95% C.I. 56-75) of the women cleared their high-risk HPV infection (Kaplan Meier curve not shown).

Figure 4: Cytological regression in high-risk HPV negative women at baseline stratified for mild (n=41), moderate/severe dyskaryosis (n=8) at baseline.

Log-rank: p=0.99



After one year of follow-up the cumulative incidence of HPV clearance in women with normal cytology, very mild, mild, moderate, and severe dyskaryosis was 46% (95% C.I. 26-66), 50% (95% C.I. 32-68), 29% (95% C.I. 18-50), 23% (95% C.I. 11-35), and 25% (95% C.I. 12-38), respectively (log-rank for trend p=0.02; figure 5). All women who cleared high-risk HPV did this within 40 months of follow-up. The clearance curves of the women with normal cytology and very mild dyskaryosis at baseline intertwined several times during follow-up, indicating no significant difference. This was also seen in the women with moderate and severe dyskaryosis at baseline.

Table 1 shows the distribution of high-risk HPV types at baseline related to cytological regression and clearance of high-risk HPV. No difference in cytological regression or clearance of high-risk HPV was seen in women with a single or multiple high-risk HPV type infection at baseline (p=0.82 and p=0.39, respectively). We looked exploratory for differences in cytological regression and HPV clearance between the different high-risk HPV types, but the numbers were too small to draw definite conclusions.

Sequence of cytological regression and HPV clearance

Among the 169 women with abnormal cervical cytology and a high-risk HPV positive test at baseline, seventy-nine women reached both cytological regression and high-risk HPV clear-

Table 1: Human Papillomavirus type distribution at baseline related to cytological regression and high-risk HPV clearance.

Baseline HPV type	Cervical smear at baseline							Cytological regression		HPV clearance	
	inadequate smear (n=3)	normal smear (n=27)	very mild dyskaryosis (n=34)	mild dys-karyosis (n=67)	moderate dyskaryosis (n=51)	severe dys-karyosis (n=51)	total (%) (n=233)	Yes (n=76)	No (n=157)	Yes (n=111)	No (n=122)
16	1	8	14	31	25	25	104 (45%)	31	73	41	63
18	-	2	4	4	1	5	16 (7%)	3	13	6	10
31	-	5	2	3	5	4	19 (8%)	3	16	5	14
33	-	2	2	4	1	1	10 (4%)	4	6	5	5
35	-	2	1	3	-	3	9 (4%)	2	7	5	4
39	-	-	1	1	-	-	2 (1%)	1	1	1	1
45	-	-	1	-	1	1	3 (1%)	-	3	-	3
51	-	2	-	3	2	1	8 (4%)	5	3	6	2
52	-	-	3	1	3	3	10 (4%)	4	6	7	3
56	-	-	-	3	1	1	5 (2%)	4	1	5	-
58	1	1	1	4	4	2	13 (6%)	7	6	9	4
59	-	-	2	-	-	1	3 (1%)	-	3	3	-
66	-	-	-	1	1	-	2 (1%)	2	-	2	-
68	-	-	-	-	-	-	-	-	-	-	-
Multiple* infections	1	5	3	9	7	4	29 (12%)	10	19	16	13

* Multiple high-risk HPV type infections: 4x16/31; 1x16/18; 2x18/31; 1x18/33; 1x18/56; 1x31/33; 1x33/58; 1x35/66; 1x35/51; 1x35/54; 1x35/56; 2x39/45; 1x39/58; 1x 45/51; 1x45/52; 1x45/56; 1x51/52; 2x51/66; 1x58/66; 1x33/39/58; 1x35/45/59; 1x39/56/18; 1x31/35/51/66.
(Chi-square: cytological regression related to single or multiple high-risk HPV type infection p= 0.82; high-risk HPV clearance related to single or multiple high-risk HPV type infection p= 0.39).

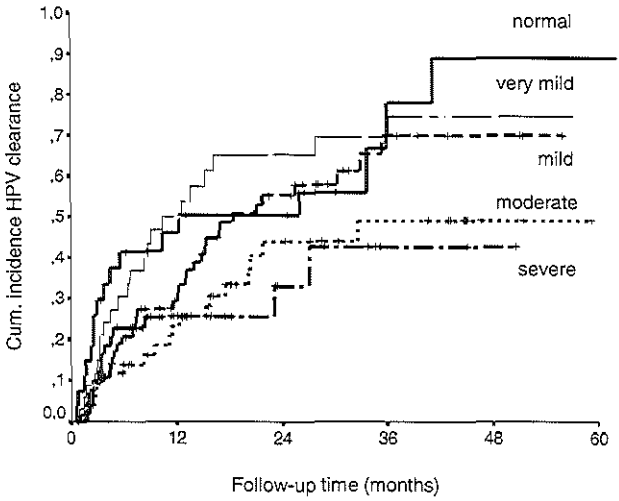


Figure 5: High-risk HPV clearance stratified for normal cytology (n=27), very mild (n=34), mild (n=67), moderate (n=51), and severe dyskaryosis (n=51) at baseline.

Log-rank for trend: p=0.02

ance during follow-up. The mean age at baseline in this group was 33 years (range 21-55 years). These women cleared high-risk HPV significantly earlier (mean 3 months; range -25 to 44 months; symmetrically distributed) than reaching cytological regression (two-sided test $p<0.05$).

Eight women reached cytological regression without HPV clearance. End histology showed CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia) and CIN 3 (severe dysplasia) in one, one, and six women, respectively (follow-up time range 8-56 months, follow-up time until cytological regression range 1-24 months). One woman, with end histology CIN 3, had a multiple high-risk HPV infection with HPV type 16 and 31.

HPV clearance without cytological regression was seen in four women. They all had a single high-risk HPV infection. In this group end histology showed no CIN, CIN 1, and CIN 2, in two, one, and one women, respectively (follow-up time range 5-47 months; follow-up time until HPV clearance range 3-41 months). No end histology CIN 3 was found in women who cleared HPV without cytological regression.

DISCUSSION

Cytological regression was seen more often in women without high-risk HPV than in women with a high-risk HPV infection. This was irrespective of the severity of the lesion; after 4 years all high-risk HPV negative women with mild dyskaryosis and 85% of the women with moderate to severe dyskaryosis reached cytological regression. These results indicate that regression of cervical lesions starts with clearance of high-risk HPV, which depends on the severity

of the lesion, and is followed by cytological regression. This relationship was confirmed since the women in our study cleared their high-risk HPV infection on average 3 months earlier than regression of their smears occurred.

In women with a high-risk HPV positive test cytological regression was seen more often in less severe lesions (figure 3). After 4 years 58% to 68% of the women with mild to moderate dyskaryosis reached cytological regression in comparison with only 30% in women with severe dyskaryosis. This is in agreement with other natural history studies, reported in a review by Östör. [12]

Richart and Barron prospectively followed women with abnormal cervical smears, also without taking cervical biopsies. [20] Spontaneous regression was seen in only 6 % of the women. However, they only included women with at least three consecutive abnormal cervical smears. Our regression rates are supported by others who also studied the natural history of cervical lesions. [12,13,21-23] Rates of regression varying from 30% to 62% in women with mild dysplastic cervical lesions and 17% to 54% in moderate dysplastic lesions were reported. All these studies differ from our study on two points; at first, they made no allowance for high-risk HPV status, and second, we did not define regression on colposcopic or histological criteria, but on cytology in 2 consecutive smears.

No CIN 3 lesions were found in the four women who cleared high-risk HPV without cytological regression. An explanation for the persistence of abnormal cervical smears in these women might be a concomitant infection with a low-risk HPV type but no low-risk HPV types were identified. Another explanation could be over-scoring of cytology. Indeed, revision of the smears yielded better Pap results in three women. Six of the eight women with cytological regression without HPV clearance had end histology CIN 3. This stresses the importance of high-risk HPV detection in the prediction of the presence of CIN 3.[4,6, 24-25]

At baseline 12% of the women had an infection with multiple high-risk HPV types. We considered these infections to be cleared when none the high-risk HPV types from a previous visit could be detected at the next visit. This could result in a lower prevalence of HPV clearance and subsequent regression in women with multiple infections in comparison with women with a single infection. However, we did not find any statistically significant differences in cytological regression or HPV clearance between the groups with multiple and single infections.

Over-treatment in women with abnormal smears may be prevented by implementing a wait-and-see period to allow clearance of HPV and subsequently regression of the lesion. However, the exact time-interval before re-testing is difficult to determine since the chance to regression of the lesion should be traded off with the chance to develop CIN 3 in case

Table 2: Cumulative incidence of cytological regression, end-histology CIN 3, and clinical progression in high-risk HPV positive women with mild (n=67), moderate (n=51), or severe dyskaryosis (n=51) at baseline at different time points during follow-up *.

Follow-up in months	Cytological regression		End histology CIN 3		Clinical progression	
	Cum. incidence %	95% C.I. †	Cum. incidence %	95% C.I. †	Cum. incidence %	95% C.I. †
Mild dyskaryosis						
3	7.6	1.1-14.1	1.5	0.0-4.5	-	-
6	20.6	10.4-30.8	3.0	0.0-7.2	-	-
9	28.8	17.3-40.3	6.3	0.2-12.4	3.3	0.0-7.9
12	37.2	24.8-49.6	11.2	3.2-19.2	6.8	0.3-13.3
18	51.0	38.1-63.9	14.6	5.6-23.6	6.8	0.3-13.3
24	54.9	41.9-67.9	19.8	9.5-30.1	6.8	0.3-13.3
Moderate dyskaryosis						
3	11.8	2.8-20.8	-	-	-	-
6	17.8	7.0-28.6	4.0	0.0-9.6	-	-
9	24.4	12.1-36.7	8.1	0.3-15.9	2.1	0.0-6.3
12	33.6	19.8-47.4	8.1	0.3-15.9	2.1	0.0-6.3
18	41.3	26.5-56.1	22.7	10.6-34.8	6.6	0.0-14.0
24	50.5	34.6-66.4	27.0	14.2-39.8	9.5	0.4-18.6
Severe dyskaryosis						
3	6.0	0.0-12.7	-	-	-	-
6	12.4	2.9-21.9	8.0	0.3-15.7	-	-
9	12.4	2.9-21.9	22.6	10.6-34.6	4.6	0.0-11.0
12	12.4	2.9-21.9	35.2	21.4-49.0	19.9	7.3-32.5
18	18.4	6.3-30.5	54.8	40.2-69.4	31.5	16.2-46.8
24	18.4	6.3-30.5	59.6	45.1-74.1	38.5	21.9-55.1

* The survival analyses were performed in women with a high-risk HPV positive test at baseline for 3 different cervical cytology groups at baseline : 1. women with mild dyskaryosis (n=67); 2. women with moderate dyskaryosis (n=51); and 3. women with severe dyskaryosis (n=51). Only high-risk HPV positive women were at risk to develop end-histology CIN 3 and clinical progression. [6]

† 95% C.I. = 95% confidence interval.

of HPV persistence. This follow-up study enabled us to map both the risk of either regression or progression (i.e., end histology CIN 3 or clinical progression) in women with abnormal cervical smears. The progression data are presented in Table 2 and are partly derived from our previous study. [6] In several European countries cervical cancer screening guidelines allow a wait-and-see period of 6 months in women diagnosed with mild dyskaryosis before referral for colposcopy. [14,23] In these women a risk to develop end histology CIN 3 of 3.0% has been accepted to allow about 20% cytological regression (table 2). These risks were similar in women with moderate dyskaryosis. Since we did not find differences in regression and progression in women with mild or moderate dyskaryosis we believe that they should be seen as one group. Therefore, as opposed to current guidelines, women with moderate dyskaryosis should also be re-tested after 6 months. Because of these results the classification of premalignant cervical lesions in LSIL (mild dyskaryosis) and HSIL (moderate to severe dyskaryosis) should be questioned since this results in over-treatment of women with moderate dyskaryosis. Therefore, we advocate the use of the CIN classification in future risk-assessments with a dividing-line between CIN 2 and CIN 3.

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Chapter 4

Clearance of high-risk Human Papillomavirus during pregnancy: decreased during first trimester with a catch-up postpartum

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Abstract

Background We followed 353 women referred with abnormal cervical cytology in a non-intervention cohort study. We compared high-risk HPV rates in the subsequent trimesters and postpartum in 91 women. High-risk HPV clearance was compared with 179 non-pregnant women. Our main questions were: 1. changes HPV rate during pregnancy?, and 2. is there any difference between HPV clearance in pregnant and non-pregnant women?

Methods Women were monitored 3-4 monthly by cytology, colposcopy, and were tested for high-risk HPV. The median follow-up time was 33 months (range 3-74).

Results Non-pregnant women showed high-risk HPV rates of 61%, 55%, 52%, and 50%, respectively, in four subsequent 3-months periods. High-risk HPV rates in the first, second, and third trimester of pregnancy, and postpartum were significantly lower, i.e. 45%, 41%, 41%, and 30%, respectively. Repeated measurements did not reveal a significant decline of HPV rate during pregnancy. But, high-risk HPV rates were decreased postpartum in contrast with the first trimester with 15% (McNemar, n=52, p=0.02) and with the third trimester with 17% (McNemar, n=48, p=0.02). Although, postpartum, women cleared HPV more effectively than non-pregnant women (Hazard Ratio 4.6; 95% C.I. 1.6-12.8) the 12 months' clearance rate did not differ in both groups.

Conclusion These results suggest a lowered immune-response against HPV during the first trimester of pregnancy with a catch-up postpartum.

INTRODUCTION

Several epidemiological and biological studies have established the important role of infection with high-risk Human Papilloma Virus (HPV) for development of cervical cancer and its precursor lesions. In women with or without abnormal cervical smears a positive high-risk HPV test result indicates an increased risk for development of high grade cervical lesions. [1-5] Moreover, in nearly all cervical cancers high-risk HPV types have been detected. [6]

The increased prevalence of high grade cervical lesions and cervical cancer in immunocompromised patients, such as AIDS patients and transplant recipients, indicates that persistence of high-risk HPV and consequently HPV-mediated carcinogenesis is related to failure of immunosurveillance. [7-9] Pregnancy is believed to alter immune-response in women. [10-11] Some authors concluded pregnancy has no effect on CIN. [12] Others reported higher regression rates of cervical dysplasia in the postpartum period compared with non-pregnant women. [13] In contrast, high prevalence rates of high-risk HPV have been found in pregnant women, [14-17] although in other studies no differences in HPV prevalence between pregnant and non-pregnant women were seen. [18-20] In short, the influence of pregnancy on the natural course of infection with high-risk HPV types is not yet known.

We performed a non-intervention follow-up study in 353 women referred for colposcopy because of abnormal cervical cytology and compared high-risk HPV clearance in pregnant women with that of non-pregnant women. Our main questions were: 1. changes the HPV rate during the trimesters of pregnancy and postpartum?, and 2. is there any difference between HPV clearance in pregnant and non-pregnant women?

METHODS

Women

From June, 1990, to December, 1992, 353 women referred to the colposcopy clinic of the University Hospital Vrije Universiteit, Amsterdam, were followed in a non-intervention study. The characteristics of this cohort have been described previously. [4] Briefly, the inclusion criteria were: referral because of an abnormal cervical smear (i.e. mild to severe dyskaryosis); age 18-55 years; no history of cervical pathology, prenatal diethylstilbestrol exposure, or concomitant cancer; and sufficient Dutch or English language skills. The median follow-up time was 33 months (range 3-74).

91 women were followed during their pregnancies; 72 women once, 16 women twice, and three women three times. These 113 pregnancies were used to compute high-risk HPV prevalence rates. The first recorded pregnancy in each woman was used to analyse HPV clearance. We excluded 4 pregnancies ending in abortion and 4 women who acquired HPV

during follow-up prior to their pregnancy. 179 non-pregnant women with a positive high-risk HPV test at baseline (figure 1; group A) and 38 pregnant women with a positive high-risk HPV test at their first visit during pregnancy (figure 1; groups B and C) were used to study high-risk HPV clearance between pregnant and non-pregnant women.

Women were monitored every 3 to 4 months by cytology, colposcopy and HPV testing. [2,4] Clinicians and laboratory staff were blinded for HPV test results and clinical findings, respectively. No biopsy samples were taken during follow-up to avoid any interference with the natural course of the disease. Follow-up ended if women reached clinical progression (n=33), defined as a colposcopic impression of CIN 3 covering three or more cervical quadrants or a cervical smear suspect for microinvasive carcinoma, or at the end of the study in December, 1996. At the last visit, colposcopically directed biopsy samples were taken for histological verification of suspected lesions (= end histology). In women with normal colposcopy, random samples were taken. If necessary, women were treated according to standard protocols.

The study protocol was approved by the ethics review board of the hospital, and all women voluntarily signed informed consent before enrolment.

Human papillomavirus testing

Testing for HPV was done by EIA PCR, which used HPV general-primer-mediated PCR with the general primers GP 5+/6+ to detect a broad spectrum of mucosotropic HPV types. [21-22] PCR products were used to identify in one assay all 14 high-risk types using EIA (HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). In addition, individual high-risk HPV types were determined. This test has been described previously and has been clinically validated. [22-23]

Definition of HPV clearance

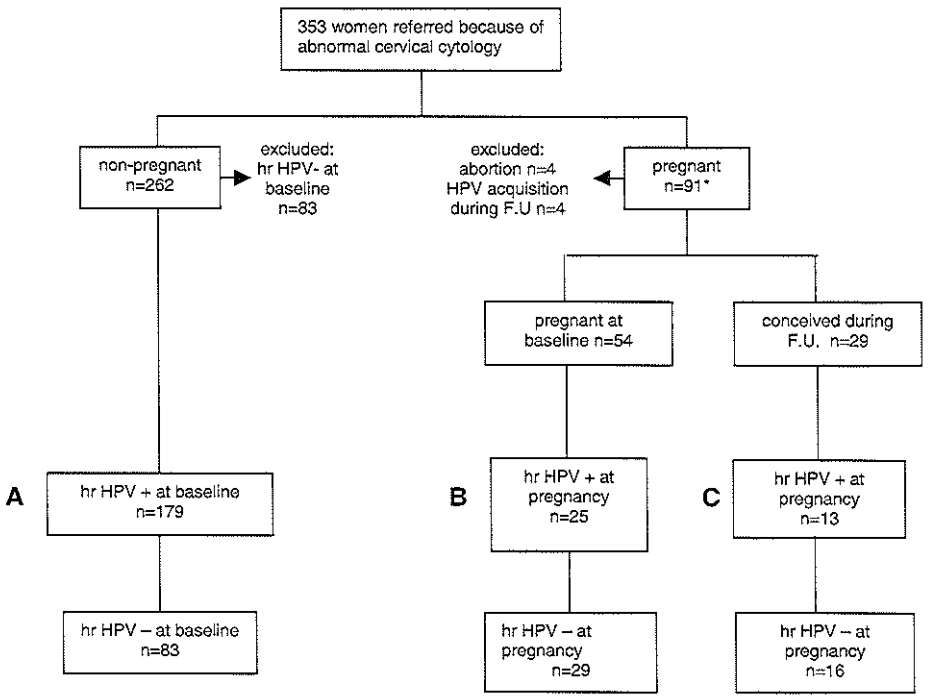
Women were considered to be clear of infection when no high-risk HPV type from the previous visit was detected at the next visit. [4]

Statistical analysis

Since not all women were seen in each trimester of pregnancy, comparisons between first, second, and third trimester, or postpartum HPV samples were made by the McNemar test, only among those with specimens available at both times. Being interested in the effect of pregnancy, only the first postpartum visit within 6 months after delivery was included in the calculation (range 5-25 weeks after pregnancy). Kaplan Meier curves were used to estimate

clearance of high-risk HPV in pregnant and non-pregnant women during a period of 12 months. The time-point of HPV clearance was taken at the mid-point between positive and negative test results for high-risk HPV. [4] We included 179 non-pregnant women with a high-risk HPV positive test at baseline (figure 1; group A) and 38 women with a high-risk HPV positive test at their first visit during pregnancy (figure 1; groups B and C). In pregnant women the baseline starting-point for the analysis was the last menstruation. 25 high-risk positive women (66%) were pregnant at baseline (figure 1; group B) and 13 became pregnant during follow-up (figure 1; group C). Combining different groups in relation to the starting-point of a study can potentially bias the study results. We analysed the influence of this heterogeneity. Kaplan Meier curves did not show significant differences in clearance rates between the pregnancy groups (log-rank p=0.6) or with the non-pregnant reference group (log-rank p=0.4). Adjustment for pregnancy groups in Cox regression analysis (see below) did not significantly effect the computed relative risks. Thus, we did not find any evidence that combining both groups introduces bias.

Figure 1: Characteristics of study groups.



Cox-regression was performed to calculate the relative risks (Hazard Rates) to clear high-risk HPV in pregnant and non-pregnant women in different time periods. Adjustment was made for women who conceived during follow-up. Age distribution at baseline and at time of pregnancy was compared by the Mann-Whitney test. The Fisher-exact test was used to calculate differences in HPV rates and abnormal cervical smears in pregnant and non-pregnant women. Throughout all analyses, a value of $P < 0.05$ was considered significant. We did not correct for multiple testing.

RESULTS

The median age in the pregnant and non-pregnant group of women was 30 years (range 20-44 years) and 32 years (range 19-55 years), respectively (p=0.23). At baseline 68% (179 out of 262) of non-pregnant women had a positive high-risk HPV test result compared to 62% (54 out of 87) of pregnant women (p=0.28). At baseline no significant difference in number of abnormal cervical smears was observed in high-risk HPV positive pregnant and non-pregnant women; 133 out of 179 (74%) non-pregnant women had an abnormal cervical smear compared to 15 out of 25 (60%) pregnant women at baseline (p=0.13). Among women with cervical smears obtained in the first trimester of pregnancy 35% had abnormal cervical cytology (mild dyskaryosis or worse). Abnormal cytology in the second, and third trimester and postpartum was present in 37%, 26%, and 31%, respectively. Comparison of paired cervical smear samples among the different trimesters with the postpartum visit did not show any significant difference (p>0.1).

High-risk HPV rates

In non-pregnant women the HPV rate at the different time periods (i.e. 3, 6, 9, and 12 months of follow-up) was 64% (165 out of 258), 57% (139 out of 244), 53% (114 out of 216), and 50% (103 out of 207 available samples), respectively (table 1).

Among pregnant women whose HPV samples were obtained in the first trimester of pregnancy 45% (28 out of 62) had positive high-risk HPV DNA samples (table 1). The high-risk HPV rate in the second and third trimester, and postpartum was 41% (28 out of 69 available samples), 41% (22 out of 54 samples), and 30% (30 out of 100 samples), respectively. Of the remaining 13 women without a postpartum visit, six women wanted to withdraw from the study and had a cervical biopsy during pregnancy, and seven women had a follow-up visit more than 6 months postpartum. High-risk HPV positive rates in three monthly periods in the non-pregnant groups were significantly higher than during pregnancy trimesters and postpartum (table 1). Among 31 women with paired samples available in the

first and second trimester a high-risk HPV positive test was found in 52% and 45%, respectively (p=0.5). Among 29 women with paired samples available in the first and third trimester a high-risk HPV positive test was seen in 45% on both time points (p=1.0). A statistically significant decline in high-risk HPV positive tests of 46% to 31% was found in the 52 women with paired samples in the first trimester and postpartum (p=0.02). Comparing third trimester results with postpartum results (paired data available from 48 women) also showed a significant decline from 44% to 27% high-risk HPV positive tests (p= 0.02).

High-risk HPV clearance

Among 38 pregnant women with a high-risk HPV positive test at the first visit during pregnancy the cumulative 12-months incidence of high-risk HPV clearance was 42% (95% C.I. 25-59). 179 non-pregnant women with a positive high-risk HPV test at baseline had a cumulative 12-months incidence of high-risk HPV clearance of 31% (95% C.I. 24-38). These rates did not differ (Hazard Ratio: 1.24; 95% C.I. 0.69-2.20).

Table 1: HPV rates and relative risks to clear high-risk HPV in pregnant and non-pregnant women at different time periods.

Time period	HPV rate		Crude hazard ratio (95% C.I.)	Adjusted hazard ratio* (95% C.I.)
0-3 months				
non-pregnant	64%	p=0.017	1.0	1.0
pregnant	45%		NA†	NA†
3-6 months				
non-pregnant	57%	p=0.023	1.0	1.0
pregnant	41%		0.7 (0.2-2.3)	0.35 (0.05-2.6)
6-9 months				
non-pregnant	53%	p=0.021	1.0	1.0
pregnant	41%		3.7 (1.1-12.8)	3.3 (0.8-13.7)
9-12 months				
non-pregnant	50%	p=0.002	1.0	1.0
pregnant	30%		3.4 (1.3-9.1)	4.6 (1.6-12.8)

* adjusted for women who conceived during follow-up. † hazard ratio was not applicable since all observations were censored.

The Kaplan Meier clearance curves (figure 2) cross at 9 months. Trimester dependent hazard ratio's, and if desired adjusted for pregnancy groups (table 1), show the differences between the curves are not related with random variation. At 9 to 12 months of follow-up, i.e. the postpartum period, pregnant women were 4.6 times (95% C.I. 1.6-12.8) more at risk to

clear HPV than non-pregnant women. The hazard ratio's for clearance during the second and third trimester were 0.35 (95% C.I. 0.05-2.6) and 3.3 (95% C.I. 0.8-13.7), respectively. Clearance is delayed during pregnancy with a catch-up postpartum.

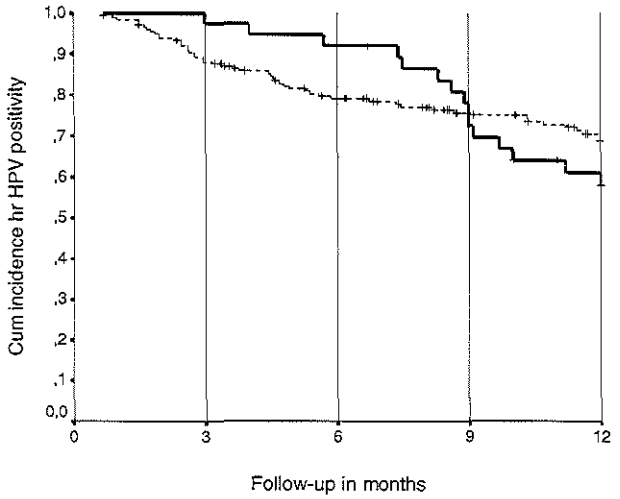
DISCUSSION

Our results show that during pregnancy in a group of women initially referred for colposcopy with an abnormal cervical smear the prevalence of high-risk HPV is higher than in the postpartum period. This effect is also demonstrated by the trimester dependent clearance rates, because during the postpartum period women were more at risk to clear HPV than non-pregnant women.

Figure 2: Clearance of high-risk HPV in 38 pregnant and 179 non-pregnant women.

——— pregnant

····· non-pregnant



Women at risk

pregnant	38	37	34	27	19
non-pregnant	179	156	128	105	89

However, over 12 months of follow-up HPV clearance did not differ between pregnant and non-pregnant women.

The high-risk HPV rate in pregnant women varied between 41% and 45% during the trimesters, and 30% in the postpartum period. Similar figures were found by others. Kemp et al. [19] found HPV prevalence rates of up to 42%, and Fife et al. [17] up to 36% during pregnancy. However, most of these women had normal cervical cytology smears and were

performed in a pregnant population of young women with a high incidence of sexual transmitted diseases.

Like us, several other studies have found, using a variety of HPV detection techniques, a significantly higher prevalence of high-risk HPV during pregnancy in comparison with the postpartum period. [14-15,17] Several reasons for this have been postulated. Since it is suggested that pregnant women tend to have fewer sexual partners than non-pregnant women of the same age, and therefore have a decreased risk to acquire a new HPV infection, [18,24] other pregnancy-related explanations for this change in HPV prevalence have been suggested such as hormonal influences, [25-26] and immunological factors, resulting in the up-regulation of viral replication. [11] Sethi et al. [11] showed that serologic response to HPV type 16 was higher in non-pregnant than in pregnant women suggesting that pregnancy reduces humoral immune response against HPV.

In pregnant women we found a reduced HPV clearance during the first trimester with a catch-up postpartum, indicating that probably during pregnancy an altered immune-response is present. Moreover, women clear their HPV infection at the same rate during pregnancy. Postpartum they clear faster than non-pregnant women after a same period of follow-up. However, the 12 months HPV clearance rate did not differ between pregnant and non-pregnant women.

The downward trend in high-risk HPV prevalence postpartum may be explained by cervical trauma occurring at the time of delivery with additional repair of the cervical epithelium. [13] Indeed, in a study in which postpartum regression rates of cervical lesions were compared in women who delivered vaginally or by caesarean section, a higher postpartum regression was seen in women with vaginal deliveries. [27]

From recent studies we know that HPV clearance is associated with and precedes on average 3 months regression of cervical lesions. [28-29] Thus following clearance an increased regression rate could be expected postpartum. This phenomenon was already shown by Yost et al. [13] However, during a 12-month period, no difference in clearance rates was found between pregnant and non-pregnant women. These results indicate that pregnant women with a high-risk HPV positive test during pregnancy are not at higher risk for progression of underlying lesions than non-pregnant women. Indeed, when we followed them for an additional 6 months period the cumulative incidence of HPV clearance in pregnant and non-pregnant women became similar (data not shown). In addition, no difference in number of women with progressive CIN was seen in the follow-up of pregnant and non-pregnant women with high-risk HPV positive tests, suggesting that both groups are at similar risks (data not shown). In conclusion, although at long term the course of an HPV infection is not affected

we found evidence that pregnancy influences high-risk HPV clearance. Our results suggest that possible inhibiting factors on the immune-response during the first trimester of pregnancy will be overtaken during the postpartum period.

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Chapter 5

Addition of high-risk HPV testing improves the current guidelines on follow-up after treatment for Cervical Intraepithelial Neoplasia

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Abstract

Background We assessed a possible role for high-risk Human Papillomavirus (HPV) testing in the policy after treatment for Cervical Intraepithelial Neoplasia (CIN) 2 or 3 (moderate to severe dysplasia). According to the Dutch guidelines follow-up after treatment consists of cervical cytology at 6, 12, and 24 months. Colposcopy is only performed in case of abnormal cervical cytology.

Methods In this observational study 184 women treated for CIN 2 or 3 were prospectively monitored by cervical cytology and high-risk HPV testing 3, 6, 9, 12, and 24 months after treatment.

Results Post-treatment CIN 2/3 was present in 29 women (15.8 %). A positive high-risk HPV test 6 months after treatment was more predictive for post-treatment CIN 2/3 than abnormal cervical cytology (sensitivity 90% and 62% respectively; with similar specificity). At 6 months the negative predictive value of a high-risk HPV negative, normal smear was 99%. Largely overlapping, partly different groups of women with post-treatment CIN 2/3 were identified by HPV testing and cervical cytology.

Conclusion Based on these results we advocate to include high-risk HPV testing in monitoring women initially treated for CIN 2/3. In case of a high-risk HPV positive test or abnormal cervical cytology colposcopy is indicated. All women should be tested at 6 and 24 months after treatment and only referred to the population based cervical cancer screening program when the tests are negative on both visits.

INTRODUCTION

After treatment for high grade Cervical Intraepithelial Neoplasia (CIN) failure rates of 5 to 15% have been observed. [1-4] One of the drawbacks of close cytological follow-up after treatment is that many women present with abnormal cytology but in only about 40-60% of them an underlying CIN lesion is present, indicating high sensitivity but low specificity for post-treatment CIN. [5-6] Colposcopic examination, as an adjunct to cytology, is often inadequate because of the difficulty in interpreting features of the post-treatment cervix, resulting in unnecessary diagnostic procedures. [5]

According to the Dutch guidelines, as formulated by the Dutch Society of Cervical Pathology and Colposcopy in 1995, follow-up after treatment for CIN 2 or 3 (moderate to severe dysplasia) consists of cytological follow-up at 6, 12, and 24 months after treatment. Only in the case of an abnormal cervical smear is colposcopic examination indicated. [6-7] After three consecutive negative smears women return to the cervical cancer screening program. In some other European countries monitoring also consists of cytological follow-up. [8-10] For instance, in the U.K. a total of six smears within 5 years of follow-up are recommended before routine recall. However, in spite of these national guidelines the follow-up policies still vary from center to center, indicating a need for evaluation and better implementation.

It is assumed that effective treatment for CIN lesions results in the eradication of the high-risk Human Papilloma Virus (HPV) infection present before treatment. [11] Persistent infection with high-risk HPV types is required for the development and progression of primary CIN lesions. [12-14] High-risk HPV is also often present in post-treatment CIN. [9]

In this observational study we evaluated the rationale for our current follow-up policy, and whether addition of high-risk HPV testing contributes to a better risk-assessment of post-treatment CIN.

METHODS

Patients

From 1990 to 1996, 184 women diagnosed with CIN 2 or 3 (moderate and severe dysplasia) at the colposcopy outpatient clinic of the University Hospital Vrije Universiteit in Amsterdam and consecutively treated by cone biopsy or colposcopic guided LLETZ (Large Loop Excision of the Transformation Zone) were included in this study. All fulfilled the following inclusion criteria: 1) an adequate HPV sample (β-globin PCR positive) at initial treatment; 2) at least one adequate HPV sample after treatment; 3) no previous history of cervical pathology; 4) no prenatal DES (diethylstilboestrol) exposure; and 5) no concomitant cancer. The median fol-

low-up time was 24 months (range 3-76 months). The study protocol was approved by the ethics review board of the hospital.

Cervical cytology and HPV testing.

In this prospective, observational study post treatment follow-up was performed by cervical cytology and HPV testing at 3, 6, 9, 12, and 24 months after initial treatment. Since high-risk HPV testing was used for the evaluation of the current follow-up policy, the test results were blinded until the analysis. Cervical scrapes were obtained using a Cervex® brush (Rovers Medical Devices B.V., Oss, The Netherlands). After a smear was made on a glass slide the brush was placed in a buffer solution (PBS) and sent to the laboratory for HPV detection. [15] Cervical smears were classified according to the KOPAC classification, the standard classification in The Netherlands. [16] Smears were cytomorphologically classified as Pap 1 (normal), Pap 2 (very mild dyskaryosis), Pap 3a (mild to moderate dyskaryosis), Pap 3b (severe dyskaryosis), Pap 4 (suspected of carcinoma in situ) and Pap 5 (suspected of at least micro-invasive carcinoma). According to the guidelines colposcopic examination including sampling for histological verification of suspect lesions was only performed in case of a cytomorphologically abnormal smear (≥ Pap 3a, mild dyskaryosis or worse). [6-7,17] All histological samples were reviewed by an expert pathologist who was unaware of the clinical findings.
A ß-globin PCR was performed to ascertain the quality of the target DNA. HPV testing was performed by EIA PCR using HPV-general-primer-mediated PCR with the general primers GP 5+/6+. All 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) were tested for in one assay. In addition, the PCR amplification products were analyzed for individual high-risk HPV types. This test has been described earlier and clinically validated. [12,14,18]

Study endpoint.

The study endpoint was *post-treatment CIN 2/3* defined as a histologically confirmed CIN 2 or 3 lesion after previous treatment. Follow-up ended when patients reached this endpoint. According to the Dutch guidelines women returned to the population based cervical cancer screening program after three consecutive negative cervical smears within 24 months after treatment since these women are considered not to have an elevated risk for post-treatment CIN 2 or 3. [7,17]

Statistical analysis.

We used two-by-two tables to assess the diagnostic value for post-treatment CIN 2/3 of a high-risk HPV test and a cervical smear at 3, 6, 9, 12, and 24 months after initial treatment, respectively. In these analyses women without a suspected cervical lesion on colposcopic examination, or with CIN 0 (no CIN) or CIN 1 (mild dysplasia) in the biopsy were considered as "negative". For these analyses, the last observations were carried forward for women who already reached the endpoint and women who returned to their general practitioner before 24 months of follow-up. Women with repeated negative cervical smears were considered to have a colposcopically normal cervix. The Mc-Nemar test was used to identify a significant difference in HPV testing and cytology for women with post-treatment CIN 2/3 at different time-points.

RESULTS

Characteristics of the study group

The mean age at baseline was 34 years (range 21-70 years). Of the included 184 women 152 were treated by LLETZ and 32 women by cone biopsy (see Table 1). At initial treatment three women (1.6%) with a CIN 3 lesion had negative high-risk HPV tests, both in the cervical smear and biopsy, and remained negative during follow-up after treatment. HPV type 16 was the most prevalent high-risk HPV type at baseline, accounting for 116 of the 181 (64.1%) high-risk HPV positive women. After treatment high-risk HPV remained detected in 48 of the 184 women (26.1%). Post-treatment CIN 2/3 was seen in 29 (15.8%) women with a median time until diagnosis of 6 months (range 3-39 months).

Post-treatment CIN 2/3

The characteristics of the 29 women with post-treatment CIN 2/3 are presented in Table 2. All women with post-treatment CIN 2/3 had CIN 3 at initial treatment and the mean age was 35 years (range 21-58 years). Seventy-two percent (21 of 29) of the cases were diagnosed within 1 year after treatment. Three months after initial treatment the high-risk HPV test was positive in 27 of the 29 cases (93%). The most prevalent high-risk HPV type was HPV type 16, accounting for 81% (22 of 27) of the HPV types. In two women with post-treatment CIN 2/3 no high-risk HPV could be demonstrated in the biopsy or additional treatment tissue. One of them (patient 19) had a high-risk HPV positive test 3 months after treatment and cleared this infection before 6 months of follow-up. In 26 of the 29 (89.7%) women with post-treatment CIN 2/3 the same high-risk HPV type could be detected in the post-treatment lesion as at initial treatment. This could indicate that the treatment did not result in eradication

Table 1: Characteristics of the 184 women included in the study.

Characteristic		Number of patients n	(%)
high-risk HPV test at initial treatment	Positive	181	(98.4)
	Negative	3	(1.6)
Histology at time of initial treatment	CIN 2	9	(4.9)
	CIN 3	175	(95.1)
Treatment	LLETZ	152	(82.6)
	Cone biopsy	32	(17.4)
high-risk HPV test 3 months after treatment	Positive	48	(26.1)
	Negative	136	(73.9)
Cervical smear 3 months after treatment	Abnormal	31	(16.8)
	Normal	153	(83.2)
Follow-up	Posttreatment CIN 2/3	29	(15.8)
	No evidence of disease	155	(84.2)
Histology posttreatment CIN 2/3	CIN 2	9	(31.0)
	CIN 3/cancer*	20	(69.0)

* One woman developed cervical cancer after initial treatment for CIN 3.

of the virus. Only one woman (patient 21) with an initial HPV type 16 infection cleared this type and acquired HPV type 58, 19 months after treatment. Two women, one with CIN 2 (patient 19) and one with CIN 3 (patient 20), had a high-risk HPV negative test at post-treatment CIN 2/3.

In another woman, initially treated for a small CIN 3 lesion by LLETZ, follow-up after treatment ended after 28 months because of a cervical smear read as Pap 4 (suspect for carcinoma in situ). Subsequent colposcopy and biopsy showed cervical carcinoma. The intermittent three cervical smears were read as normal. The four high-risk HPV tests before the diagnosis of cervical cancer were persistently positive for HPV type 16. Histology revealed an undifferentiated small cell carcinoma of the cervix and she underwent radical hysterectomy.

Prediction of post-treatment CIN 2/3

The high-risk HPV test and cervical smear results at different time-points during follow-up of all participating women are shown in Table 3. At the different time points two subgroups of women were compared; i.e. women who reached post-treatment CIN 2/3 during follow-up and the remaining women.

Table 2: Characteristics of patients with post-treatment CIN 2/3 (n=29)

Patient	Age	Initial treatment		Test 3 months after treatment		Follow-up until (months)			Post-treatment CIN	
		Treatment	HPV type	Cytology	HPV type	Abnormal cytology*	hr HPV+*	Post treatment CIN	Histology	HPV type
1	28	LLETZ	16/33	3b	16	pers	pers	4	CIN 3	16
2	35	LLETZ	16	3a	16	pers	pers	6	CIN 3	16
3	33	LLETZ	16/31	3b	16/31	pers	pers	3	CIN 3	16/31
4	29	LLETZ	16	3a	16	pers	pers	6	CIN 3	16
5	28	LLETZ	16	3b	16	pers	pers	4	CIN 3	16
6	31	LLETZ	58	3b	33/58	pers	pers	8	CIN 2	58
7	58	LLETZ	33	3a	33	pers	pers	5	CIN 3	33/35
8	25	LLETZ	16	3a	35	pers	pers	5	CIN 2	16
9	36	LLETZ	16	3b	16	pers	pers	3	CIN 3	16
10	38	LLETZ	33	3b	16	pers	pers	3	CIN 3	33
11	56	Cone biopsy	16	4	33	pers	pers	7	CIN 3	16
12	25	LLETZ	16	3b	16	pers	pers	3	CIN 2	16
13	21	LLETZ	16	4	16	pers	pers	6	CIN 3	16/54
14	51	LLETZ	16	4	16/54	pers	pers	3	CIN 3	16
15	27	LLETZ	16	3a	16	pers	pers	4	CIN 2	16
16	54	LLETZ	16	3a	16	pers	pers	4	CIN 2	16
17	28	LLETZ	16	3b	16	pers	pers	3	CIN 3	16
18	23	Cone biopsy	16	2	16	7	pers	7	CIN 3	16
19	35	LLETZ	16	1	16	19	-	19	CIN 2	-
20	27	LLETZ	16	1	-	6	-	9	CIN 3	-
21	35	LLETZ	16	2	-	19	19	23	CIN 3	58
22	42	LLETZ	16	1	16	20	pers	24	CIN 2	16
23	33	LLETZ	33/35	1	33/35	10	pers	10	CIN 2	33/35
24	51	Cone biopsy	16	1	16	7	pers	7	CIN 3	16
25	33	Cone biopsy	16	2	16	22	pers	22	CIN 2	16
26	38	LLETZ	16	1	16	39	pers	39	CIN 3	16
27	34	LLETZ	16	1	16	28	pers	28	Cancer	16
28	31	LLETZ	16	2	16	15	pers	15	CIN 3	16
29	31	LLETZ	16	2	16	15	pers	15	CIN 3	16

Cytology: Pap 1 = normal dyskaryosis; Pap 2 = very mild dyskaryosis; Pap 3a = mild to moderate dyskaryosis; Pap 3b = severe dyskaryosis; Pap 4 = suspected of carcinoma in situ. * pers = persistent abnormal cervical cytology or high-risk HPV positive after initial treatment (range, time until next visit during follow-up: 2-9 months).

At 3, 6, 9, and 12 months post-treatment more women with post-treatment CIN 2/3 would be identified by high-risk HPV testing than cervical cytology. The sensitivity for post-treatment CIN 2/3 among women with a high-risk HPV positive test or an abnormal cervical smear at 3 months after treatment was 93% vs 58%, respectively (at 6 months 90% vs 62%; at 9 months 90% vs 69%; at 12 months 90% vs 72%; and at 24 months 93% vs 93%, respectively). Only at 3 and 6 months after treatment the sensitivity of a high-risk HPV positive test was significantly higher than that of an abnormal cervical smear (Mc-Nemar test $P < 0.01$, and $P < 0.05$, respectively). In women without post-treatment CIN 2/3 the number of high-risk HPV-positive tests or abnormal cervical smears at the different time-points was comparable.

The specificity of a positive high-risk HPV test or an abnormal cervical smear at 3 months after treatment was 86% vs 91%, respectively (at 6 months 92% vs 91%; at 9 months 96% vs 92%; at 12 months 96% vs 95%; and at 24 months 99% vs 96%, respectively).

All 21 women with a high-risk HPV positive test 3 months after treatment without post-treatment CIN 2/3 cleared the HPV infection during follow-up (median 8 months; range 4-18 months). Among them, 16 women with at least three normal cervical smears, returned to their general practitioner. In the remaining five women a colposcopically directed biopsy was taken because of an abnormal cervical smear. In two women no CIN was present, three had a CIN 1 lesion (mild dysplasia).

The negative predictive value of a high-risk HPV negative, cytomorphologically normal cervical smear was very high. At 3 months after treatment the negative predictive values of a high-risk HPV negative cytomorphologically normal smear, or either a high-risk HPV-negative smear or a cytomorphologically normal smear were 98%, 98%, and 92%, respectively (at 6 months 99%, 98%, and 93%; and at 24 months 100%, 99%, and 99%, respectively).

DISCUSSION

Our results show that at 6 months after treatment for high-grade CIN a positive high-risk HPV test is more predictive for post-treatment CIN 2/3 than abnormal cervical cytology. The negative predictive value of a high-risk HPV-negative cytomorphologically normal cervical smear is very high and the presence of high-risk HPV 24 months after treatment is a risk-factor for post-treatment CIN 2/3. Therefore, we consider high-risk HPV testing valuable in the early detection or prediction of post-treatment CIN 2/3. Three months after treatment only 26% of the women with a high-risk HPV positive test at baseline still had a positive high-risk HPV test, indicating that in most women treatment resulted in eradication of high-risk HPV. Cervical cytology was abnormal in 17% of the women. But it is known that reading cervical smears 3 months after ablative treatment is difficult because of the "repair-effect". [19]

Table 3: High-risk HPV test and cervical cytology results at 3, 6, 9, 12 and 24 months of follow-up in 184 women initially treated for CIN 2 or 3.

	At 3 months follow-up		At 6 months follow-up		At 9 months follow-up		At 12 months follow-up		At 24 months follow-up	
Post-treatment CIN 2/3*	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**
Number	29	155	29	155	29	155	29	155	29	155
mild dyskaryosis or worse	17	14	18	14	20	13	21	8	27	6
normal cytology	12	141	11	141	9	142	8	147	2	149
high-risk HPV positive	27	21	26	13	26	6	26	6	27	2
and mild dyskaryosis or worse	17	6	17	7	19	2	20	3	25	-
and normal cytology	10	15	9	6	7	4	6	3	2	2
high-risk HPV negative	2	134	3	142	3	149	3	149	2	153
and mild dyskaryosis or worse	-	8	1	7	1	11	1	5	2	6
and normal cytology	2	126	2	135	2	138	2	144	-	147

*The last observations are carried forward for women who reached the endpoint and women who returned to their general practitioner before 24 months of follow-up. Mc-Nemar test to identify difference in HPV testing and cervical cytology in predicting post-treatment CIN 2/3 at different time points: t3, t6, t9, t12, and t24 was 8.1 ($p<0.01$), 4.9 ($p<0.05$), 3.1, 2.3, and 0.3, respectively.

The reason why some women present with post-treatment CIN while the majority do not is unclear. Possible explanations include incomplete removal of the CIN lesion, development of a new CIN lesion by reinfection with HPV, and even the revival of so-called dormant or occult HPV infections. [20-21] In 90% (26 of 29) of all cases with post-treatment CIN 2/3 we found the same high-risk HPV type as before the initial treatment. This high number agrees with other studies. [9] Since our HPV assay does not differentiate between HPV type variants we cannot exclude a role for HPV type variants in the genesis of post-treatment CIN 2/3.

At 24 months of follow-up after treatment two out of the 155 (1.3%) women who did not develop post-treatment CIN 2/3 had a positive high-risk HPV test with normal cytology. Since they both had at least three normal cervical smears around the time of acquisition of high-risk HPV they were regarded as having no high grade CIN lesion and were referred to their general practitioner for screening according to the population-based screening program. So far, no recurrent CIN disease has been reported in these women.

The relation between a persistent high-risk HPV infection and the development and maintenance of CIN lesions has already been established. [13-14] Yet, in two women with post-treatment CIN 2/3 no high-risk HPV type could be found in the CIN lesion or corresponding smear (Table 2). HPV negativity was confirmed by type-specific PCR. The occurrence of high-risk HPV negative scrapes in cases with cervical dysplasia is in agreement with an earlier study. [14]

Three facts argue for our view of using high-risk HPV testing, next to cervical cytology, in the follow-up after initial treatment for high-grade CIN lesions: the higher sensitivity of a high-risk HPV-positive test than of an abnormal cervical smear, with similar specificity; the high negative predictive value of a high-risk HPV-negative, cytomorphologically normal cervical smear; and largely overlapping, partly different groups of women with post-treatment CIN 2/3 were identified by HPV testing and cervical cytology. One woman with cervical cancer and another with CIN 3 identified at 28 and 39 months after initial treatment, respectively, had normal cervical smears during follow-up. They would not have been at risk of undue referral to a low-risk group and follow-up procedure if high-risk HPV testing was used to monitor the initial treatment, since all intermittent high-risk HPV tests were positive. In these patients, all cervical smears were revised by an expert panel and were again read as normal.

We advocate to monitor women 6 months after initial treatment both by high-risk HPV testing and cervical cytology. In case of a positive test, colposcopically directed biopsies are indicated. Retesting by both tests should be considered at 24 months after initial treatment to avoid missing cervical carcinomas because of detection problems. Moreover, it is known that acquisition of HPV is increased in women with a history of CIN lesions. [14]

Only when cytological and HPV testing are negative during at least 24 months should women be referred to the population-based cervical cancer screening program. These recommendations will be tested, together with a cost-benefit analysis, in a prospective study involving women treated for high grade CIN.

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Chapter 6

Primary screening for high-risk HPV by home-obtained cervicovaginal lavage is an alternative screening tool for unscreened women

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Abstract

Background Self-sampling is considered an adjuvant tool to facilitate participation of women in cervical cancer screening programs. We evaluated 1. if cervicovaginal lavage is an alternative for the cervical smear in cytology and HPV testing and 2. the acceptance of the self-sampling device in women.

Methods 56 women with abnormal cervical cytology (very mild dyskaryosis or worse) and 15 women with normal cervical cytology obtained a self-collected cervicovaginal lavage at home and filled in a questionnaire on the use of the device. At the colposcopy clinic the gynaecologist performed the same procedure followed by a cervical smear for cytology and HPV DNA testing.

Results The self-sampling device was acceptable to 88% of the women. The concordance between the cytology results in the smear, and the lavage by the doctor and the patient was 54% and 41%, respectively (κ= 0.28 and 0.14). The concordance between high-risk HPV detection in the smear, and the lavage by the doctor and the patient was 93% and 78%, respectively (κ= 0.82 and 0.53). 91% of the women with high-grade CIN had a high-risk HPV positive test in the smear, compared with 91% and 81% in the lavages taken by the doctor and the patient, respectively.

Conclusions HPV DNA testing by home-obtained samples is useful as a screening tool for cervical cancer while cervical cytology by self-sampling is not. Although the sensitivity for high grade CIN by high-risk HPV testing in the lavage by the patient is not significantly lower than that in the cervical smear, self-sampling for HPV DNA is a feasible alternative method in women who decline to participate in population-based cervical cancer screening programs. Participation to the screening program remains the best option.

INTRODUCTION

Cervical cancer is a preventable disease. Its development through premalignant stages, detectable by cervical cytology years before cervical cancer appears, has resulted in the organisation of population based cervical cancer screening programs. These screening programs have contributed to a declined incidence and mortality of cervical cancer. [1,2] However, there are some drawbacks including low attendance and limited sensitivity of cytological screening. [3-5] Moreover, it is known that 50% of the cases with invasive cervical cancer arise in women who are not adequately screened. [6-7]

The screening method used in cervical cancer screening is the classical Papanicolaou smear (Pap smear), directly taken from the cervix. Self-sampling is regarded as a possible tool to facilitate screening of women who refuse to participate in cervical cancer screening programs. [8-9] A sampling method performed by the woman herself, without intervention by a doctor, could lower the threshold and increase the attendance to screening.

Several studies have established that an infection with high-risk HPV is the main cause for the development of cervical cancer. High-risk HPV types can be identified in nearly all cervical carcinomas. [10] Women with normal cervical cytology and a high-risk HPV positive test are more at risk to develop severe cervical dysplasia than women without high-risk HPV [11-12] and, moreover, in women with abnormal cervical cytology a persistent high-risk HPV infection is required for the development and maintenance of severe dysplastic cervical lesions. [13-15] Thus, testing for high-risk HPV, as an adjunct to cervical cytology, has been recommended for screening to determine a high risk group. [15]

The aim of this study was to evaluate testing for HPV DNA and cervical cytology in home-obtained self-collected material by cervicovaginal lavage as an alternative for the Pap smear. A lavage taken by the doctor was included in the study as a control for the lavage taken by the patient. We were also interested in the acceptance of the self-sampling device as an alternative screening tool.

METHODS

From December 1998 until March 2000, 75 women referred to the colposcopy clinic of the University Hospital Rotterdam (n=63) and the University Hospital Vrije Universiteit in Amsterdam (n=12) were asked to participate in the study. Four women with abnormal cervical cytology refused to participate. Of the 71 women enrolled in the study, 56 had abnormal cervical cytology (very mild dyskaryosis or worse) and 15 women had normal cervical cytology. The mean age of the participating women was 35 years (range 20 to 63 years). After an explanation of the study and the use of the self-sampling device by the study co-ordinator, a written

informed consent, approved by the ethics review boards of both participating hospitals, was obtained from each participant.

At intake, women received a cervicovaginal self-sampling device, a form with detailed instructions and a questionnaire on the use of the device. The self-sampling device consisted of an irrigation syringe (50 cc, Bard, Inc. Covington, UK), a disposable female urine catheter (single-use female urine catheter ch.16, Astra Tech Mölndal, Sweden) and a container with 15 cc sterile phosphate-buffered saline (PBS) for irrigation. According to the instructions the catheter had to be attached to the syringe in order to aspirate the irrigation fluid from the container. After aspiration a cervicovaginal specimen was obtained by inserting the tip of the catheter as deep as possible into the vagina and press and release the balloon of the syringe three times, to flush the irrigation fluid in the vagina and back into the syringe. After removal of the catheter the syringe, containing the cervicovaginal specimen, had to be emptied in the container. Women were asked to obtain a cervicovaginal lavage the day before their return visit at the colposcopy clinic. At colposcopy, after introducing a vaginal speculum, the gynaecologist performed a cervicovaginal lavage with a similar device by irrigating the cervix and aspirating the fluid pooled in the posterior vaginal fornix. This was followed by a cervical smear obtained with a Cervex® brush (Rovers Medical Devices B.V., Oss, The Netherlands). After a smear was made on a glass slide the brush was placed in a buffer solution (PBS) and sent to the laboratory for HPV detection. Colposcopic examination followed and biopsy samples were taken for histological verification of suspected lesions. If necessary, women were treated according to standard protocol. When no lesions were seen at colposcopy the cervix was considered to be free of disease (no CIN; Cervical Intraepithelial Neoplasia) and no biopsies were taken. The lavages, the cervical smear and brush for the HPV detection were processed at the Department of Pathology at the University Hospital Vrije Universiteit in Amsterdam. The lavages were vortexed and divided into two specimens. The first was used for cervical cytology reading, the second for HPV DNA testing.

Questionnaire

All participants were asked to fill in a questionnaire on the use of the self-sampling device including the following questions; 1. what is your opinion about the use of the self-sampling device? Answer: easy / difficult, and for what reason? ; and 2. what screening tool would you prefer for your next screening round; i.e., self-sampling or Pap smear? Answer: self-sampling / Pap smear, and for what reason?

Cervical cytology

From each cytology specimen two cytospins were made and Papanicolaou stained. The cytology slides and biopsy samples were read by an expert pathologist who was unaware of the clinical findings. Cervical smears were classified according to the KOPAC classification, the standard classification in the Netherlands. [16] This is a modification of the Pap classification. [17] Cervical smears are cytomorphologically classified as Pap 1 (normal cytology), Pap 2 (very mild dyskaryosis), Pap 3a (mild to moderate dyskaryosis), Pap 3b (severe dyskaryosis), Pap 4 (suspected of carcinoma in situ) and Pap 5 (suspected of at least micro-invasive carcinoma). Histology was classified as CIN (Cervical Intraepithelial Neoplasia) 0 (no dysplasia), 1 (mild dysplasia), 2 (moderate dysplasia), and 3 (severe dysplasia).

High-risk HPV testing

The specimens for HPV testing were centrifuged at 4.000 rpm (2719 x g; Hettich, Rotanta/TR) for 6 minutes to pellet the cells. The supernatant was discarded and the pellet was suspended in 1 ml 0.01 M Tris-HCl (pH 8.3) and stored at -80 ºC till further analysis. A ß-globin PCR was performed to ascertain the quality of the target DNA. Testing for HPV was done by PCR EIA, which used HPV-general-primer-mediated PCR with the general primers GP 5+/6+ to detect a broad spectrum of mucosotropic HPV types. [18-19] PCR products were used to identify in one assay all 14 high-risk HPV types using EIA (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). This has been described previously and has been clinically validated. [19-20]

Statistical analysis

The concordance between the cytology (Pap smear classification) and HPV results from the smear, from the lavage taken by the doctor (lavage-doctor) and from the lavage taken by the patient (lavage-patient) was calculated. The lavage-doctor was included in the study as a control for the lavage-patient, since the first was performed under optimal conditions, i.e., the cervix was visible during irrigation. The kappa (κ) value was computed as a measure of overall agreement beyond chance. A kappa estimate of less than 0.2 indicates poor agreement, a kappa estimate between 0.2 and 0.8 fair to moderate agreement, and a kappa estimate of more than 0.80 good agreement. [21] To compare the performances of HPV testing with cytology results in the smear and the lavages, we calculated for each test the sensitivity, specificity, positive and negative predictive values to detect high grade CIN (CIN 2/3; moderate to severe dysplasia) and tested whether there were differences using the Mc-Nemar test. For each woman, the highest CIN grade in either the diagnostic cervical biopsy or the cervical

tissue obtained at treatment was used as the reference for assessing test performance. Women with unsatisfactory cervical cytology, i.e., no or too few cervical cells, or a ß-globin PCR negative test were not included in these analyses, since we assumed that in a normal situation they would have been asked to repeat the self-sampling or return for a repeat test.

RESULTS

Questionnaire on the use of the self-sampling device

The use of the self-sampling device was considered easy in 49 out of the 56 women (88%) who performed a self-sampling. The remaining seven women concluded that the usage of the device was difficult. They were uncertain about the amount of fluid they had aspirated and questioned the efficacy of the lavage. Three of them had normal cervical cytology.

At the next screening round, 23% (13/56) of the women would prefer the classical Pap smear to the self-sampling. Their reasons were: 1. no problem with gynaecologic examination (n=8), and 2. the self-sampling device is unpractical (n=5). The remaining group favoured the self-sampling. All participating women regularly went to see their doctor for a Pap smear.

Fifteen women, 5 with normal and 10 with abnormal cervical cytology, did not perform the self-sampling. Their reasons were: 1) they forgot to perform it (n=10) and 2) they were too nervous for the colposcopic examination (n=5). Three lavages by the doctor were unsatisfactory for cytological judgement. Four samples (lavage-doctor (n=2) and lavage-patient (n=2) were β-globin PCR negative.

Cytology result	Pap smear		
	Normal	Very mild dyskaryosis	Mild dyskaryosis or worse
Lavage-doctor (n=71)			
Normal	**15**	4	23
Very mild dyskaryosis	-	-	3
Mild dyskaryosis or worse	-	1	**22**
Unsatisfactory*	-	-	3
Lavage-patient (n=71)			
Normal	**11**	4	28
Very mild dyskaryosis	-	-	1
Mild dyskaryosis or worse	-	-	**12**
Not done	4	1	10

* Unsatisfactory indicates no or too few cervical cells detectable. Agreement lavage-doctor and Pap smear: $\kappa = 0.28$; agreement lavage-patient and Pap smear; $\kappa = 0.14$; agreement lavage-doctor and lavage-patient; $\kappa = 0.37$.

Table 1: Cytology results of the lavage taken by the doctor and the lavage by the patient compared with the Pap smear.

Agreement cytology and HPV testing in cervical smear, lavage and self-sampling

There was fair agreement between the cytology results in the Pap smear and the lavage-doctor (κ= 0.28; table 1) with a 54% concordance (37 out of 69 satisfactory slides). The agreement between the cytology results in the Pap smear and the lavage-patient was poor (κ= 0.14), with a concordance of 41% (23 out of 56 slides). A fair agreement was obtained between the cytology results of the lavage-doctor and the lavage-patient (κ=0.37; data not shown), with a 74% concordance between the cytology results performed on the two different samples.

The concordance between the high-risk HPV test results in the smear and the lavage-doctor was 93% (64 out of 69 women with β-globin positive PCR tests), which is a good agreement (κ=0.82) (table 2). An 78% (39 out of 48 women with β-globin positive PCR tests) concordance between the HPV test results in the smear and those in the lavage-patient was seen, indicating moderate agreement (κ= 0.53). There was moderate agreement between the results obtained by HPV testing of the lavage-doctor and the lavage-patient (κ=0.47; data not shown), with a 75% concordance.

	Smear	
Test result	**hr HPV positive**	**hr HPV negative**
Lavage-doctor (n=71)		
hr HPV positive	**48**	2
hr HPV negative	3	**16**
β-globin negative*	1	1
Lavage-patient (n=71)		
hr HPV positive	**30**	-
hr HPV negative	12	**12**
β-globin negative*	-	2
not done	10	5

Table 2: High-risk HPV testing in the lavage taken by the doctor and the lavage by the patient compared with HPV testing in the smear.

* β-globin PCR negative indicates no amplifiable DNA for HPV testing in specimen. Agreement HPV testing in lavage-doctor and smear: κ=0.82; agreement lavage-patient and smear: κ=0.53; agreement lavage-doctor and lavage-patient: κ=0.47.

Detection rate for high grade CIN

High grade CIN was detected in 33 women (46%) (table 3). In two of them the lavages-doctor were unsatisfactory for cytological reading. The cytology results in the lavage-doctor would identify 19 out of the detectable 31 high grade CIN lesions (61%) with a specificity, positive and negative predictive value of 81%, 73% and 71%, respectively. Seven patients with a high grade CIN lesion did not perform the self-sampling. The cytology results in the lavage-patient

would identify 11 out of the 26 eligible patients with high grade CIN (42%) with a specificity, positive and negative predictive value of 93%, 85%, and 65%, respectively, which is not statistically different to the performance of the lavage-doctor (Mc-Nemar χ^2=0.2).

Thirty out of 33 (91%) high grade CIN lesions would be identified when colposcopic examination was performed in case of a positive high-risk HPV test result in the smear. In one woman with a high grade CIN lesion the β-globin PCR was negative in the lavage-doctor. So a high-risk HPV positive test result in the lavage-doctor would identify 29 women out of 32 (91%) women with high grade CIN lesions, whereas a high-risk HPV positive test result in the lavage-patient would identify 21 out of 26 (81%) eligible women with high grade CIN. The performance of HPV testing in the smear and in the lavage-patient was not statistically different (Mc-Nemar χ^2=1.3) The specificity for high grade CIN of high-risk HPV testing in the smear and in the lavage-patient was 42% and 68%, respectively, with positive predictive values of 58% and 70%, respectively, and negative predictive values of 84% and 79%, respectively. No statistically significant difference in the detection of high grade CIN could be found between the HPV test results in the lavage-doctor and the lavage-patient (Mc-Nemar χ^2=1.3).

DISCUSSION

Our results indicate that cytological screening for cervical cancer by self-sampling is no alternative for cytological screening by the classical Pap smear. The agreement between the Pap smear and the lavage by the patient was low, and less women with high grade CIN would be identified by cytology in the lavage than in the Pap smear. In contrast, high-risk HPV testing in self-obtained cervicovaginal lavage is a feasible alternative method. The sensitivity for high grade CIN in women with a high-risk HPV positive test result in the lavage by the patient was lower, although not statistically significant, than in the smear (81% versus 91%). The specificity of the HPV test in self-sampled material was higher than in the smear (68% versus 42%).

We included women with abnormal and normal cervical cytology to evaluate the usage of the self-sampling device in these two groups. The self-sampling device was acceptable to 88% of the participating women. No differences in acceptability was seen in women with or without abnormal cervical cytology. Seventy-seven percent of the participating women would choose self-sampling by vaginal lavage above the classical taken Pap smear as alternative screening tool for their next screening round, on condition that both screening methods obtain equal results. Seven women questioned the efficacy of the self-sampling. Their main problem was the uncertainty about the amount of fluid they aspirated. We only found, however, one β-globin negative sample in these samples, indicating that the perception of the usage in the

women was different than the reality. In future studies we are planning to adjust the instructions on the device.

Table 3: The performance of HPV detection and cervical cytology by Pap smear and lavages taken by the doctor and the patient for the detection of high grade CIN.

Test	Test result	CIN 2/3 Yes	CIN 2/3 No	Sensitivity	Specificity	PPV	NPV
		n	n	%	%	%	%
Cervical cytology							
Pap smear (n=71)	Normal (n=15)	-	15	100*	40	59	100*
	Very mild dyskaryosis (n=5)	3	2				
	Mild dyskaryosis (n=11)	3	8				
	≥ Moderate dyskaryosis (n=46)	27	13				
Lavage doctor (n=71)	Normal (n=42)	12	30	61	81	73	71
	Very mild dyskaryosis (n=3)	1	2				
	Mild dyskaryosis (n=6)	3	3				
	≥ Moderate dyskaryosis (n=17)	15	2				
	Unsatisfactory	2	1				
Lavage patient (n=71)	Normal (n=43)	15	28	42	93	85	65
	Very mild dyskaryosis (n=1)	1	-				
	Mild dyskaryosis (n=1)	1	-				
	≥ Moderate dyskaryosis (n=11)	9	2				
	Unsatisfactory	-	-				
	Not done	7	8				
HPV testing							
Pap smear (n=71)	hr HPV + (n=52)	30	22	91	42	58	84
	hr HPV – (n=19)	3	16				
	β-globin negative	-	-				
Lavage doctor (n=71)	hr HPV + (n=50)	29	21	91	43	58	84
	hr HPV – (n=19)	3	16				
	β-globin negative	1	1				
Lavage patient (n=71)	hr HPV + (n=30)	21	9	81	68	70	79
	hr HPV - (n=24)	5	19				
	β-globin negative	-	2				
	Not done	7	8				

* All women with abnormal cervical cytology underwent colposcopic examination and biopsy samples. Differences in performances of the tests were calculated. Mc-Nemar cytology smear versus lavage-doctor: χ^2=10.1; p< 0.001; cytology lavage-doctor versus lavage-patient: χ^2=0.2; p= n.s.; cytology smear versus lavage-patient: χ^2=13.1; p< 0.001. High-risk HPV testing smear versus lavage-doctor: χ^2=0; p= n.s.; high-risk HPV testing lavage-doctor versus lavage-patient: χ^2=1.3; p= n.s.; high-risk HPV testing smear versus lavage-patient: χ^2=1.3; p= n.s.

We included the lavage taken by the doctor as a control for the lavage taken by the patient. No statistically significant difference in the detection of high grade CIN was found between the test results in the lavage-doctor and lavage-patient, indicating that the conditions of the performances, i.e., irrigating the cervix directly or indirectly, of the two different lavages did not differ. However, the concordance between HPV testing in the lavage-doctor and the Pap smear appeared to be higher than that in the lavage-patient. Fifteen women (21%) did not

perform a home-obtained lavage sample. 10 of them had an abnormal cervical smear. In one-third of the cases emotional stress for the examinations at the colposcopy clinic was the reason for not doing the self-sampling at home.

In our study women were asked to obtain a sample at home. This contrasts with other studies that evaluated self-sampling under optimal conditions, i.e., at the outpatient clinic just after extensive information. [9,22] In our study a more realistic condition was investigated. Our participation rate of 79% was high when compared with other studies evaluating home-obtained self-sampling. A participation of 68% was seen in a study involving 25 women with abnormal cervical cytology who performed a self-administered vaginal lavage at home and returned the sample by mail. [23]

The high performance of high-risk HPV testing in the lavage in our study is in agreement with other studies. Wright et al. found a high-risk HPV positive test in patient-obtained vaginal swabs in 66% of high grade dysplastic cervical lesions or worse in an unscreened population known to have a high incidence of premalignant lesions. [22] Nurse-obtained swabs revealed 84% correlation. They concluded that self-testing was as sensitive as a Pap smear performed by a health care provider, and proposed self testing for HPV DNA in areas where access to care is limited. In other studies a correlation was found of 85% to 93% for high grade CIN in patient-obtained vaginal swabs [9,24] indicating that self-sampling for HPV is also adequate when other techniques are used. In a future study we are planning to compare the acceptability and efficacy of self-sampling by these vaginal swabs with the lavage device.

We found no statistically significant difference in detecting high grade CIN between HPV testing in self-sampled lavage material or physician obtained cervical brush. Moreover, a higher specificity and positive predictive value for HPV testing in self-sampled material was seen. Provided proper instructions are given to the women, self-sampling for HPV DNA testing seems suitable as an alternative screening tool. Although the Pap smear (with additional high-risk HPV testing) remains the best screening tool for cervical cancer and its precursors, the high sensitivity for high grade CIN of high-risk HPV testing in self-sampled material allows us to advise self-sampling in women who decline to participate in such programs because it could largely reduce the risk of cervical cancer associated with not participating in a screening program. [6-7] In women who do participate the Pap smear remains the best option.

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Chapter 7

General Discussion and Conclusions

1. Introduction

Cervical cancer is a preventable disease. Its development through premalignant stages detectable by cervical cytology years before cervical cancer appears has resulted in the organisation of nation-wide cervical cancer screening programs. Although these programs have led to a substantial reduction in mortality and morbidity of cervical cancer [1-4], there are many drawbacks like limited performance of cytology, too many screening rounds, over-reading of slides, and overtreatment of women. [5-7] This indicates that there is a strong need for a more accurate screening tool.

The association between infection with a high-risk HPV type and cervical cancer and its precursor lesions has been established in several studies. [8-12] The use of high-risk HPV testing next to cervical cytology in cervical cancer screening has been proposed but before adequate implementation can be initiated some questions concerning the natural course of CIN disease in relation to HPV status have to be answered.

In the preceding chapters the natural history of HPV infection in relation to CIN has been described and discussed. With the results of these studies the current guidelines on cervical cancer screening in the Netherlands and the follow-up of women treated for high grade CIN can be improved. Modifications in the existing guidelines for certain categories of women with CIN disease will be discussed in the following sections.

2. Testing for high-risk HPV

2.1 Triage of borderline and mild dyskaryosis (Pap 2/3a1; KOPAC: P2-4; A3-4; C2-4)

In the Netherlands about 4% of the cervical smears performed in cervical cancer screening are read as borderline or mild dyskaryosis (BMD). [13] Since 1996, the Dutch guidelines for cervical cancer screening advise women with cervical smears read as BMD to repeat the smear within 6 months. [14-15] If the repeat smear is read as BMD or worse women are referred for colposcopic examination. However, the far majority (i.e. 80-90%) of these women do not have CIN 3, and will regress spontaneously. [16-18] A marker that improves the prediction of high-grade CIN in these women would considerably reduce the number of repeat smears and redundant referral to gynaecologists. Less repeat smears and referrals would not only benefit the efficiency of the cervical cancer screening program, but also decrease the unnecessary anxiety among many women. [19-20]

A review by Cuzick et al. [21] summarised several studies which addressed the issue of HPV testing in triaging women with mildly cytological abnormalities to select women at risk for

high-grade CIN. In a large study involving women with low SIL, testing for HPV DNA was considered not to be a potential triage strategy because 80% of the women had a high-risk HPV positive test. [22] A possible reason for this conclusion may be the young age of the participating women (mean age 25 years) having their primary, and mostly transient, HPV infection. Other study groups found higher sensitivity rates for high grade CIN by combining cervical cytology and high-risk HPV testing in comparison with cervical cytology alone (i.e. about 75% versus more than 90%). [17,23-24] Unfortunately, the specificity of this approach appeared to be as worse as that of cervical cytology alone, which indicates that there still remains a large number of women who will undergo unnecessary colposcopic examinations. [25-27]

How can referral for colposcopy directed biopsies in women with BMD be reduced? According to standard policy, i.e. twice BMD or worse referral to gynaecologists, about 50% of women with an initial cervical smear read as BMD will have reached cytological regression within this 6 months period, and 50% will be referred. [13] Two approaches to additional testing for high-risk HPV in BMD women are possible:

1. Only one high-risk HPV test will be performed, with direct referral in case of a positive test result.

From preliminary results of a randomised trial among 44.000 women currently being conducted, we know that at initial testing 30% of women with BMD have a high-risk HPV positive test. Referral of only those who are high-risk positive is indicated because only they are at risk to develop high grade cervical lesions *(Chapter 2)*. Since no progression to CIN 3 is seen in women with high-risk HPV negative tests they can remain in the population-based screening program *(Chapter 2)* whereas in case of an abnormal smear, subsequent cytological regression can be expected *(Chapter 3)*. However, the most important argument for this approach is the very high negative predictive value of a single high-risk HPV test in these women (99%) *(Chapter 2)*. [17-18] With 70% high-risk HPV negative women with BMD smears, this approach will lead to a profit of 20% less referrals than standard policy (70% minus 50%).

2. Repeat high-risk HPV testing after 6 months to allow for HPV clearance.

During a wait-and-see period of 6 months 20% of the women with an initial high-risk HPV positive BMD smear will clear their infection *(Chapter 3)*. [18] This results in a profit of another 6% less referrals (20% of 30% referred high-risk HPV positive women) and thus, to 26% less unnecessary referrals in comparison with the standard policy.

Whether this additional 6% is cost-effective has to be investigated in cost-benefit analyses. Naturally, additional testing for high-risk HPV will only be successful in women over 30 years of age known to have less transient infections with high-risk HPV. [28]

In conclusion, addition of high-risk HPV testing, next to cervical cytology, can improve the current guidelines on screening in the BMD group. Additional high-risk HPV testing will not only result in a decline of referrals but also in repeat smears. The best policy for additional testing with high-risk HPV in women with BMD; i.e. immediate referral or allowance for HPV clearance, should be tested in cost-benefit analyses. In addition, cost-effectiveness also depends critically on the safety of reducing surveillance in women with BMD who are HPV-negative. This is also an area in need for further research.

Proposed advice high-risk HPV testing in women with borderline or mild dyskaryosis:

Triage by cervical cytology and high-risk HPV testing and re-testing after 6 months in population-based screening:

- high-risk HPV positive at both time points, irrespective of cytology: referral colposcopy
- high-risk HPV negative first, and positive after 6 months, repeat after additional 6 months
- high-risk HPV positive first, and negative after 6 months: remain in screening program
- high-risk HPV negative at both occasions: remain in screening program.

2.2 Should women with moderate dyskaryotic smears (Pap 3a2; KOPAC P5; A5; C5) be referred directly to gynaecologists?

According to the Dutch guidelines, women with a smear read as moderate dyskaryosis are referred for colposcopic examination. However, it is known that most of these referrals are redundant because about 50% of these women will clear high-risk HPV with subsequently spontaneous cytological regression in two years time *(Chapter 3)* and only those women with a persistent infection with high-risk HPV are at risk to develop CIN 3 and worse (odds ratio up to 327; *Chapter 2*).

From the regression study *(Chapter 3)* we learned that the progression and regression rates in women with moderate dyskaryosis are similar to women with mild dyskaryosis. To safe costs it may also be worthwhile to initiate a wait-and-see policy in women with moderate dyskaryotic smears to allow for clearance of HPV. This means that by re-testing after 6

months a 4% risk to develop CIN 3 must be outweighed by 20% of cytological regression in these women (*Chapter 3; Table 2*).

High-risk HPV testing in women with moderate dyskaryosis:
A wait-and-see policy of 6 months will decline the number of women referred to colposcopy with 20%. Whether this approach, in a small group of women, is desirable should be tested in a cost-effective analysis. The acceptability of the risk for CIN 3 in this strategy must be worked out in a prospective study (safety-net).

In women with severe dyskaryosis the current guidelines, i.e., directly referral to colposcopy, should not be changed because of the high progression rate in comparison with a relatively low regression *(Chapter 3, Table 2)*.

1.3 Detection of residual and recurrent CIN disease following treatment for CIN

In the Netherlands follow-up after treatment for CIN 2 or 3 consists of cervical cytology at 6, 12, and 24 months. Colposcopic examination is only indicated in case of an abnormal cervical smear. In case of three consecutive negative smears women return to the population based screening program. [14-15] A role may exist for HPV testing in this post-treatment surveillance to determine more quickly and accurately if treatment has completely eradicated local disease. It is assumed that effective treatment for CIN results in the eradication of high-risk HPV, and in residual or recurrent lesions high-risk HPV is often present. [29-31] This could indicate that persistence of high-risk HPV after treatment predicts post-treatment CIN, since a persistent infection with high-risk HPV is required for the development and progression of primary CIN lesions *(Chapter 2)*. We investigated the predictive value of HPV testing after treatment for CIN 2 or 3 and concluded that a high-risk HPV positive test 6 months after treatment was significantly more predictive for post-treatment CIN 2 or 3 than abnormal cytology (sensitivity 90% and 62%, respectively; *Chapter 5)*. The specificity of HPV testing and cervical cytology was similar during follow-up. In case of a high-risk HPV negative test and normal cervical cytology the risk of post-treatment CIN was very low (negative predictive value after 6 months of 99% and after 24 months of 100%). In conclusion, high-risk HPV testing should be used, in adjunction to cervical cytology, in monitoring initial treatment for CIN. In case of abnormal cervical cytology or a positive high-risk HPV test colposcopy is indicated. Testing at 6 months only appears to be sufficient. However, re-testing after 24 months as a safety-net should be considered to avoid missing severe lesions because of

detection problems or newly acquired infections with high-risk HPV *(Chapter 5, table 3)*. Moreover, higher acquisition rates of HPV are found in women with a history of CIN *(Chapter 2)*. Further research is necessary to investigate whether this strategy is cost-effective. A prospective study including women treated for high grade CIN and monitored by high-risk HPV and cervical cytology will be set up to test these proposed guidelines.

Proposed advice high-risk HPV testing in women treated for high grade CIN

- high-risk HPV testing and cervical cytology 6 months after treatment
- high-risk HPV testing and cervical cytology 24 months after treatment
- in case of abnormal cytology and/or high-risk HPV positive: colposcopy
- negative results at 6 and 24 months: population-based screening

2.4 Primary screening in underscreened women

The effect of primary cervical cancer screening programs is largely dependent on the performance of cervical cytology. However, the coverage is also of major importance. In the Netherlands it is known that about 50% of all cervical cancers develop in women who are not adequately screened. [1] Recently, studies have been carried out which compare the results of high-risk HPV testing on self-obtained vaginal samples with the cervical smear taken by a medical doctor. Sensitivities of 85% to 93% for high grade CIN in patient-obtained cervical swabs have been observed. [32-33] These studies took place at outpatient clinics, under optimal conditions. In our study, women were asked to test a cervico-vaginal lavage device for cervical cytology and high-risk HPV testing at home *(Chapter 6)*. Cervicovaginal lavage was no alternative for cytology because in most self-obtained samples no abnormal cells found in the smear could be identified. The sensitivity of a positive high-risk HPV test for high grade CIN (CIN 2/3) in the lavage was similar to that of the smear. The specificity was higher in the lavage. In most women the self-sampling device was acceptable and they would choose the device above the Pap smear as alternative screening tool in the next screening round. However, only on condition that both screening methods had similar performances in identifying underlying cervical lesions. So, self-sampling could be a tool to lower the threshold of screening and increase the participation. In future studies the performance and acceptability of the cervicovaginal lavage device will be compared to self-sampling by the vaginal swab.

Because of the high sensitivity for high grade CIN of high-risk HPV testing in self-sampling it can be advised in women who refuse to participate in screening programs. The classical

taken Pap smear (with additional HPV testing) remains the best option in women who do participate. In addition, the self-sampling method may open opportunities for screening in developed countries. In these countries it is difficult to initiate screening programs because of logistics, lack of educated personnel, and budgets. A simple, easy to perform screening tool for HPV testing could possibly reduce cervical cancer in developing countries.

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Summary

Several epidemiological studies have established a strong relation between infection with high-risk HPV types of Human Papillomavirus (HPV) and the development of cervical cancer and its precursors. Insight into the relation between HPV and the natural history of Cervical Intraepithelial Neoplasia (CIN) can lead to more efficient cervical cancer screening strategies by combining cervical smears with testing for HPV. Before implementation of the HPV test can be performed, some questions concerning this natural history have to be answered. A general introduction on CIN, HPV and cervical cancer screening is presented in **Chapter 1**. Five questions have been postulated and will be addressed in this thesis:

1. What is the natural course of high-risk HPV on the development of clinical progression and end histology CIN 3 in women with abnormal cervical cytology?
2. Is there a relationship between clearance of high-risk HPV and regression of cervical lesions?
3. Is there a difference in prevalence and clearance of high-risk HPV in pregnant and non-pregnant women?
4. Does addition of high-risk HPV testing contribute to a better risk assessment of post-treatment CIN?
5. Is cervicovaginal lavage performed by women useful as an alternative screening tool for cervical cytology and HPV detection and is it accepted?

In **Chapters 2, 3 and 4** of this thesis the results of a prospective, non-intervention study including 353 women referred for colposcopy because of abnormal cervical smears (mild to severe dyskaryosis) were described. Women were under tight clinical surveillance and closely monitored by cytological and colposcopic evaluation every 3-4 months. HPV genotyping was performed on cellular material from cervical smears by PCR using the primers GP5+/6+. During follow-up no cervical biopsies were performed to avoid interference with the natural course of the disease. Follow-up was ended, prior to the end of the study, when women reached clinical progression, which was defined as a colposcopic impression of CIN 3 covering three or more cervical quadrants or a cervical smear showing suspected microinvasive cancer.

Chapter 2 describes the influence of high-risk HPV status, i.e. persistent infection, acquisition and clearance or continuously negative for infection, on cervical lesions. We found that clinical progression of cervical lesions did not occur in the absence of high-risk HPV. Moreover, women with a persistent high-risk HPV infection were 327 times more at risk to develop CIN 3 than women who cleared the infection or were continuously negative for

high-risk HPV. We concluded that persistent infection with high-risk HPV is, therefore, required for development and maintenance of CIN 3. Most women cleared the infection during follow-up with a median time of 25 months. After 5 years of follow-up 67% was high-risk HPV negative. For women with mild to moderate dyskaryosis the diagnostic value for end histology CIN 3 of a second test for HPV and a second cervical smear was calculated at different time points. It appeared that a second high-risk HPV test at 6 months predicted end histology CIN 3 better than a second cervical smear. New guidelines on cervical cancer screening were suggested.

In **Chapter 3** cytological regression in relation to HPV clearance was studied. We found that cytological regression was seen more often in women without high-risk HPV than in women with an high-risk HPV infection. This was irrespective of the severity of the lesions; after 4 years of follow-up all high-risk HPV negative women with mild dyskaryosis and 85% of the women with moderate to severe dyskaryosis reached cytological regression. Moreover, regression time was longer in high-risk HPV positive women if lesions are more severe. These results indicated that regression of cervical lesions starts with HPV clearance. This relationship was confirmed since women in our study cleared their high-risk HPV infection on average 3 months earlier than regression of their smears occurred. Over-treatment in women with abnormal cervical smears may be prevented by implementing a wait-and-see period to allow for high-risk HPV clearance and subsequent regression of the lesion. However, the time-interval until re-testing should be traded off against the chance to regression and development of CIN 3. These data and the data described in **Chapter 2** gave us the opportunity to map both the risk to regression or progression in women with abnormal cervical smears. Since these risks were similar in women with mild and moderate dyskaryosis we concluded that these women should be treated as one group in contrast to current guidelines on cervical cancer screening. Thus, the current guidelines in women with mild dyskaryosis, i.e. re-testing after 6 months, should also be applicable to women with moderate dyskaryosis

In literature the influence of pregnancy on the natural course of infection with high-risk HPV types is not yet known. Some studies concluded that it alters the immune-response whereas others did not find any effect on HPV. **Chapter 4** describes HPV prevalence and clearance in pregnant and non-pregnant women. During follow-up 91 women were pregnant. During pregnancy high-risk HPV prevalence was higher than in the postpartum period. This effect was also demonstrated by the clearance rates; during the postpartum period women were

more at risk to clear high-risk HPV than non-pregnant women. However, at long term the course of HPV clearance did not differ between pregnant and non-pregnant women. These results suggest a lower immune-response against HPV during the first trimester of pregnancy with a catch-up postpartum.

Chapter 5

After treatment for high-grade CIN failure rates of 5-15% have been observed despite close cytological follow-up. We studied the rationale for the current follow-up policy of women treated for high grade CIN, and whether addition of high-risk HPV testing contributes to a better risk-assessment of post-treatment CIN (i.e. residual or recurrent disease). In an observational study of 184 women treated for CIN 2 or 3 the performance of both tests in predicting post-treatment CIN was calculated at different time-points after treatment. Our results showed that at 6 months after treatment a positive high-risk HPV test was more predictive for post-treatment CIN 2 or 3 than abnormal cervical cytology (sensitivity 90% and 62% respectively, with similar specificity). Moreover, the negative predictive value of a high-risk HPV negative cytomorphologically normal smear was very high (99% after 6 months and 100% after 24 months of follow-up). From these results we concluded that high-risk HPV testing is valuable in the early detection of post-treatment CIN 2/3 and we recommended the implementation of high-risk HPV testing in the follow-up after treatment.

In **Chapter 6** we studied self-sampling for cervical cytology and HPV DNA. Self-sampling is regarded as a possible tool to facilitate screening in women who refuse to participate in cervical cancer screening programs. A self-sampling method performed by the woman herself, without intervention by a doctor, could lower the threshold and increase the attendance to screening. Our main questions were whether self-testing for cervical cytology and HPV testing by women at home could be considered as an adjuvant screening tool and if it was acceptable in women. 71 women, 56 with abnormal cervical cytology and 15 with normal cytology, participated and were asked to obtain a self-collected cervicovaginal lavage at home and fill in a questionnaire on the use of the device. The acceptance for the self-sampling method was high; 77% of the women preferred it above the classical taken Pap smear for their next screening round on condition that both screening methods obtained equal result. We evaluated the performance of the lavage in comparison with cervical cytology and HPV testing by doctors. The agreement in cytology between the Pap smear and the lavage was low, less women with high grade CIN would be identified by cytology in the lavage than in the Pap smear. In contrast, high-risk HPV testing in self-obtained

cervicovaginal lavage was a feasible alternative method. The sensitivity for high grade CIN in women with a high-risk HPV positive test result in the lavage was lower, although not statistically significant (81% and 91%, respectively). However, the specificity of the HPV test in self-sampled material was higher (68% and 42% respectively). We concluded that, provided proper instructions are given, self-sampling for HPV DNA testing seems suitable as an alternative screening tool. Its use should be restricted to women who decline to participate in cervical cancer screening programs. In women who do participate the Pap smear (with additional high-risk HPV testing) remains the best option.

Chapter 7 provides a general discussion based on the five questions the research was focussed on and gives recommendations for new guidelines in cervical cancer screening and the follow-up after treatment for high grade CIN.

Samenvatting

Diverse epidemiologische studies beschreven reeds de relatie tussen een infectie met hoog-risico Humaan Papillomavirus (HPV) typen en het ontstaan van baarmoederhalskanker en voorlopers daarvan. Een beter inzicht in de relatie tussen HPV en het natuulijk beloop van deze voorlopers (Cervicale Intraepitheliale Neoplasia, CIN) kan bijdragen tot een efficiëntere opzet van het bevolkingsonderzoek op baarmoederhalskanker, waarbij het uitstrijkje van de baarmoedermond wordt gecombineerd met een test op HPV. Voordat implementatie van de HPV test echter kan worden uitgevoerd dienen belangrijke vragen aangaande dit natuurlijk beloop worden beantwoord. In **Hoofdstuk 1** van dit proefschrift worden CIN, HPV en screening op baarmoederhalskanker besproken. Vijf vragen ter nadere bestudering werden geformuleerd:

1. Wat is de rol van hoog-risico HPV in het ontstaan van klinische progressie en eind histologie CIN 3 bij vrouwen met afwijkende uitstrijkjes?
2. Bestaat er een relatie tussen klaring (genezing) van hoog-risico HPV en het in regressie gaan van afwijkingen aan de baarmoedermond?
3. Bestaan er verschillen tussen prevalentie en klaring van hoog-risico HPV tussen zwangere en niet-zwangere vrouwen?
4. Kan toevoeging van de hoog-risico HPV test na behandeling voor hoog-gradige CIN laesies (CIN 2 en 3) bijdragen tot een betere risicoschatting op residu en recidief afwijkingen?
5. Kan cervicovaginale lavage, uitgevoerd door de vrouw zelf, gebruikt worden als een alternatieve screeningsmethode voor baarmoederhalskanker en is deze methode acceptabel voor de vrouw?

De **Hoofdstukken 2, 3 en 4** beschrijven de resultaten van een prospectieve, non-interventie studie van 353 vrouwen verwezen voor colposcopisch onderzoek in verband met een afwijkend uitstrijkje (milde tot ernstige dyskaryosis). Vrouwen werden klinisch nauwlettend in de gaten gehouden. Iedere 3-4 maanden werden een uitstrijkje en colposcopisch onderzoek verricht. HPV genotypering werd verricht op celmateriaal van uitstrijkjes met een PCR waarbij gebruik werd gemaakt van de primers GP5+/6+. Gedurende de follow-up werden geen biopten van de baarmoedermond genomen omdat we niet wilden interfereren met het natuurlijk beloop van de ziekte. Vrouwen verlieten de studie indien de afwijking aan de baarmoedermond klinische progressie vertoonde. Dit was gedefinieerd als een colposcopische impressie van CIN 3 bevattende drie of meer kwadranten van de baarmoedermond of een uitstrijkje suspect voor microinvasief carcinoom.

Hoofdstuk 2 beschrijft de invloed van de hoog-risico HPV status, een persistente hoog-risico HPV infectie, acquisitie en klaring of continu negatief voor hoog-risico HPV, op baarmoedermond afwijkingen. Het bleek dat klinische progressie niet optrad indien hoog-risico HPV afwezig was. Tevens hadden vrouwen met een hoog-risico HPV positieve test 327 keer meer kans op het ontwikkelen van CIN 3 dan vrouwen die de hoog-risico HPV infectie klaarden of continu negatief waren. Daaruit concludeerden wij dat een persistente infectie met hoog-risico HPV noodzakelijk is voor de ontwikkeling en het in stand houden van CIN 3. De meeste vrouwen klaarden de infectie gedurende de studie met een mediane tijdsduur van 25 maanden. Na 5 jaar werden 67% van de vrouwen negatief voor hoog-risico HPV. De diagnostische waarde voor CIN 3 van een tweede hoog-risico HPV test en een tweede uitstrijkje op verschillende tijdstippen werd berekend voor vrouwen met milde en matige dyskaryosis. Het bleek dat een tweede test op hoog-risico HPV na 6 maanden CIN 3 aan het einde van de studie beter voorspelde dan een tweede uitstrijkje en voorstellen voor nieuwe richtlijnen voor het bevolkingsonderzoek op baarmoederhalskanker werden besproken.

In **Hoofdstuk 3** werd de relatie tussen cytologische regressie en HPV klaring bestudeerd. We zagen dat cytologische regressie vaker voorkwam bij vrouwen met een negatieve test uitslag voor hoog-risico HPV dan bij positieve vrouwen. Dit was niet afhankelijk van de ernst van de afwijking: na 4 jaar studie bereikten alle vrouwen met milde dyskaryosis cytologische regressie, tegen 85% van de vrouwen met matige tot ernstige dyskaryosis. Tevens bleek dat klaring van hoog-risico HPV afhankelijk was van de ernst van het uitstrijkje. Deze resultaten suggereerden dat regressie van baarmoedermond afwijkingen begint met klaring van HPV. Deze relatie kon worden bevestigd. Klaring van HPV trad gemiddeld 3 maanden eerder dan cytologische regressie. Overbehandeling van vrouwen met afwijkende uitstrijkjes kan worden tegengegaan door een herhalingsuitstrijkje in te voeren om eventuele HPV klaring en daarop volgende cytologische regressie een kans te geven. Bij de bepaling van het tijdsinterval voor deze tweede test moeten echter de kansen op enerzijds regressie en anderzijds het ontstaan van CIN 3 in deze periode worden afgewogen. De gegevens van deze studie, samen met de progressie gegevens beschreven in **Hoofdstuk 2** resulteerden in een risicoprofiel voor vrouwen met abnormale uitstrijkjes. Omdat de risico's gelijk waren bij vrouwen met milde en matige dyskaryosis concludeerden wij dat beide groepen in het bevolkingsonderzoek als één groep moeten worden beschouwd. Dit betekent dat, conform de huidige richtlijnen bij vrouwen met milde dyskaryosis, tevens een herhalingstest na 6 maanden voor vrouwen met matige dyskaryosis moet worden toegestaan.

De literatuur is niet eenduidig over de invloed van zwangerschap op het natuurlijk beloop van een infectie met hoog-risico HPV typen. Sommige studies beschrijven dat zwangerschap de immuun respons verandert, anderen vonden geen effect op HPV. **Hoofdstuk 4** beschrijft de klaring en prevalentie van HPV in zwangere en niet-zwangere vrouwen. Gedurende de studie waren 91 vrouwen zwanger. De prevalentie van HPV bleek tijdens de zwangerschap hoger te zijn dan postpartum. De HPV klaringssnelheden gaven dit ook weer: gedurende de postpartum periode hadden zwangere vrouwen meer kans om hun HPV infectie te klaren dan niet-zwangeren. Gedurende de gehele studietijd bleek er echter geen verschil in het beloop van HPV klaring te bestaan tussen deze vrouwen. Deze resultaten suggereren dat er een verminderde immuun respons bestaat gedurende de eerste trimesters van de zwangerschap met een inhaalslag postpartum.

Hoofdstuk 5

Na behandeling van CIN 2 of 3 blijkt dat in 5-15% van de gevallen de afwijking nog steeds aanwezig is (residu) of terugkeert (recidief). Ondanks goede cytologische follow-up wordt dit vaak pas laat ontdekt. Wij evalueerden het huidige vervolgbeleid na behandeling en bestudeerden of toevoeging van een hoog-risico HPV test in dit traject kan leiden tot een betere risicoberekening van residu en recidief laesies. In een observationele studie van 184 vrouwen behandeld voor CIN 2 of 3 werden de waarden van zowel het uitstrijkje als de hoog-risico HPV test in de voorspelling van residu of recidief laesies berekend op verschillende tijdstippen na behandeling. Wij vonden dat laesies beter werden voorspeld door een hoog-risico HPV positieve test 6 maanden na behandeling dan een afwijkend uitstrijkje (sensitiviteit 90% versus 62%, met gelijke specificiteit). Tevens bleek de negatief voorspellende waarde van een hoog-risico HPV negatief normaal uitstrijkje erg hoog (99% na 6 maanden en 100% na 24 maanden). Wij concludeerden hieruit dat een hoog-risico HPV test waardevol kan zijn in de vroege detectie van residu of recidief laesies en bevolen toevoeging van de HPV test aan in het follow-up traject na behandeling.

Hoofdstuk 6 beschrijft een studie naar een zelf-test voor cytologie en HPV. Een zelf-test kan worden gebruikt als een alternatieve screeningsmethode om deelname aan het bevolkingsonderzoek te stimuleren. Een zelf-test door de vrouw zelf, zonder tussenkomst van een doktor, kan de drempel om deel te nemen aan het bevolkingsonderzoek verlagen. De belangrijkste vragen in onze studie waren: kan een zelf-test, verricht door vrouwen thuis, gebruikt worden als een alternatieve screeningsmethode voor baarmoederhalskanker en is deze methode acceptabel voor de vrouw? 71 vrouwen: 56 met een abnormaal en 15 met een

normaal uitstrijkje, namen deel aan de studie. Ze werden gevraagd om thuis een cervicovaginale lavage te verrichten en een vragenlijst in te vullen over het gebruik van het apparaat. De acceptatie van de zelf-test was hoog: 77% prefereerden de zelf-test boven het klassieke uitstrijkje door een doktor onder voorwaarde dat beide testen dezelfde resultaten hadden. De prestatie van de lavage werd vergeleken met die van het uitstrijkje en de HPV test afgenomen door de dokter. De overeenkomst tussen de cytologische uitslagen van het uitstrijkje en de lavage was slecht; de lavage identificeerde significant minder vrouwen met ernstige premaligne afwijkingen aan de baarmoedermond. De lavage bleek echter wel geschikt om daarin HPV aan te tonen. De sensitiviteit voor CIN 2 en 3 bij vrouwen met een positieve hoog-risico HPV test in hun lavage was lager dan de test uitgevoerd door de dokter, echter niet significant (81% versus 91%). De specificiteit was echter duidelijk hoger (68% versus 41%). Wij concludeerden hieruit dat een zelf-test voor HPV geschikt lijkt als een alternatieve screeningsmethode, onder voorwaarde dat vrouwen adequaat worden geïnstrueerd over het gebruik hiervan. Het gebruik van de zelf-test moet echter beperkt worden voor vrouwen die weigeren deel te nemen aan het bevolkingsonderzoek. Voor vrouwen die wel deelnemen blijft het klassieke uitstrijkje (eventueel samen met een hoog-risico HPV test) de beste methode.

Hoofdstuk 7 geeft een algemene beschouwing gebaseerd op de vijf vragen die vooraf werden gesteld. Tevens worden er, naar aanleiding van de resultaten van dit proefschrift, aanbevelingen gedaan voor nieuwe richtlijnen van het bevolkingsonderzoek op baarmoederhalskanker en het traject na behandeling van CIN 2 en 3.

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Curriculum Vitae

De schrijfster van dit proefschrift werd op 4 juli 1968 geboren te Lichtenvoorde. In 1986 behaalde zij het Athenaeum diploma aan de R.K. Scholengemeenschap Marianum te Groenlo. In datzelfde jaar begon zij met de studie Geneeskunde aan de Rijksuniversiteit Leiden. Gedurende haar studie liep zij stage op the Department of Gynaecology/Oncology van het Groote Schuur Hospital in Kaapstad, Zuid Afrika (o.l.v. Prof. B. Bloch). In 1994 behaalde zij haar artsexamen. Daarna was zij achtereenvolgens werkzaam als AGNIO op de afdeling Verloskunde en Gynaecologie in het St. Lucas-Andreas ziekenhuis te Amsterdam (o.l.v. dr. J.Th.M. van der Schoot) en op de afdeling Verloskunde en Vrouwenziekten van het Academisch Ziekenhuis Rotterdam (o.l.v. Prof.dr. Th.J.M. Helmerhorst). In maart 1997 begon zij met promotieonderzoek op de afdeling Pathologie aan de Vrije Universiteit te Amsterdam o.l.v. Prof.dr. C.J.L.M Meijer, en op de afdeling Verloskunde en Vrouwenziekten van het Academisch Ziekenhuis Rotterdam o.l.v. Prof.dr. Th.J.M. Helmerhorst. Na beëindiging van het onderzoek begon zij in maart 2001 met haar opleiding tot gynaecoloog in het Reinier de Graaf Ziekenhuis te Delft (opleider: Dr. J.C. Kuijpers).