Risk reduction by HPV-testing in women with premalignant cervical lesions

Studies of clinical management
and observations on the immunological background

Aagje Bais

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Risico vermindering met behulp van een HPV-test bij vrouwen met premaligne afwijkingen aan de baarmoedermond

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en waarnemingen van immunologische veranderingen

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Contents of this thesis

Chapter 1

Introduction

1.1 GENERAL INTRODUCTION

1.1.1 Anatomy of the uterine cervix
1.1.2 Cytological and histological classification of precursor lesions
1.1.3 Diagnosis of (pre)malignant lesions
1.1.4 Treatment of CIN lesions

1.2 ETIOLOGY OF CERVICAL CANCER

1.2.1 Human Papillomavirus (HPV)
1.2.2 Role of HPV-testing in diagnosis and treatment

1.3 IMMUNOLOGY

1.3.1 Changes in the cytokine network

1.4 OUTLINE OF THIS THESIS

1.1 GENERAL INTRODUCTION

Cervical cancer is, after breast cancer, the second most common malignancy among women worldwide [1].The estimated total number of women diagnosed with cervical cancer in 2002 was 493,000, and 274,000 women died from the disease. The majority of these cases (83%) occur in developing countries, where cervical cancer accounts for 15% of newly diagnosed cancers in women. In developed countries with good screening options invasive cervical carcinoma is a relatively rare condition and accounts for only 3.6% of new cancers.

The incidence of cervical cancer in The Netherlands has decreased yearly by 2% since 1990. This has been attributed to cervical cancer screening [2].

In The Netherlands, 584 new cases of cervical cancer were diagnosed in 2003 with an age-standardized incidence rate of 7.4 new cases per 100,000 women [3]. A total of 214 women died in 2003 as a consequence of cervical cancer, with an age-standardized mortality rate of 7.1 women per 100,000.

1.1.1 Anatomy of the uterine cervix

The cervix is the lower third part of the uterus. The outer surface of the cervix (ectocervix) is covered with non-keratinizing squamous epithelium, whereas the inner part of the cervix (endocervix) contains glandular columnar epithelium. In late fetal life the uterus, developing from the Müllerian duct, is lined by the original columnar epithelium, which extends downwards into the cervical canal. There it comes into contact with the original squamous epithelium from the vagina [4-6]. The boundary between the original columnar and squamous epithelium is called the original squamocolumnar junction (SCJ). Under the influence of estrogen stimulation during late fetal life and in adolescence, the distal part of the original columnar epithelium is replaced with squamous epithelium by the physiological process of squamous metaplasia. The metaplastic squamous area between the squamous and columnar epithelium is referred to as the transformation zone.

Due to the high turnover of cells within this layer, the transformation zone is thought to be more susceptible to oncogenic influences. Cervical cancer and its precursor lesions develop particularly in this zone. Cervical lesions in the transformation zone can be identified by colposcopy and are diagnosed by histological examination.

1.1.2 Cytological and histological classification of precursor lesions

Cervical cancer develops from well-defined dysplastic precursor lesions. Dysplasia is characterized by a disturbed epithelial architecture and cellular atypia of the epithelial cells. In 1932 Broders recognized carcinoma in situ as a precursor

Table 1. Conversion of different terminology used for cytological and histological reporting[18]

CYTOLOGY

PAP	PAP 1	PAP 2	PAP3A1	PAP 3A2	PAP 3B	PAP 4	PAP 5
Bethesda 2001	negative	asc-us/agc	LSIL		HSIL		SqCa/ AdCa
Europe		borderline & mild dysplasia		moderate dysplasia	severe dysplasia	carc. in situ	invasive carcinoma

HISTOLOGY

Descript.	Normal	atypia	mild dysplasia	moderate dysplasia	severe dysplasia	carc. in situ	invasive carcinoma
CIN		CIN 0	CIN 1	CIN 2	CIN 3		invasive carcinoma
SIL			LSIL	HSIL			

asc-us: atypical squamous cells of undetermined significance, agc: atypical glandular cells, LSIL: low-grade squamous intra-epithelial lesions, HSIL: high-grade squamous intra-epithelial lesions. SqCa: squamous carcinoma, AdCa: adenocarcinoma, CIN: cervical intraepithelial neoplasia, SIL: squamous intra-epithelial lesion.

of invasive cervical cancer[5]. Papanicolaou and Traut demonstrated in 1941 that dysplastic changes could be diagnosed by exfoliation cytology and introduced the Pap-classification[6]. The earliest terminology used to classify abnormalities of cervical cytology was as follows: Pap 1: absence of atypical or abnormal cells, Pap 2: atypical cytology but no evidence of malignancy, Pap 3: cytology suggestive of, but not conclusive for, malignancy, Pap 4: cytology strongly suggestive of malignancy and Pap 5: cytology conclusive for malignancy. However, this classification was not used consistently by different laboratories and failed to correlate with histological findings.

An attempt was therefore made to introduce a terminology predictive of the histology. Dysplastic precursor lesions were originally divided into four categories: mild, moderate and severe dysplasia and carcinoma in situ.

The concept of cervical intraepithelial neoplasia (CIN) was introduced by Richart in 1966[9]. This classification emphasized that cervical cancer develops from these non-invasive pre-malignant stages. CIN lesions were classified into three grades according to the thickness of epithelial layer involved by neoplastic changes. CIN 1 lesions (mild dysplasia) show dysplasia in less than one third of the epithelial layer. In CIN 2 lesions (moderate dysplasia) one to two thirds of the epithelial layer consists of dysplastic cells, while in CIN 3 lesions (severe dysplasia or carcinoma in situ) two thirds to full thickness of the epithelial layer contains dysplastic cells[10]. The Bethesda classification was developed in 1988 to replace the various Papanicolaou designations and to standardize cytologic terminology to correlate with histology

reports[11,12]. A distinction was made between lesions likely to progress (high-grade squamous intra-epithelial lesions or HSIL) and lesions less likely to progress (low grade squamous intra-epithelial lesions or LSIL) to cervical cancer[12,13]. The Bethesda system was revised in 2001 aiming at a more efficient management of women with equivocal results of cervical cytology[14].

In The Netherlands a modified Papanicolaou system is used for classification of cervical cytology[15]: Pap 1 indicates normal cytology, Pap 2: borderline dyskaryosis, Pap 3a1: mild dyskaryosis, Pap 3a2: moderate dyskaryosis, Pap 3b: severe dyskaryosis, Pap 4: suspected of at least carcinoma in situ and Pap 5: suspected of at least microinvasive cancer. A new CISOE-A (in Dutch KOPAC-B) classification was introduced in 1996 to increase the efficacy of the screening program[15,16]. In this classification information on cell composition, inflammatory characteristics and adequacy of the smear are incorporated. Five items are scored: C (composition), I (inflammation), S (squamous epithelium), O (other abnormalities and endometrium) and E (endocervical columnar epithelium). A stands for adequacy of the smear. The CISOE code is in fact a descriptive extension of the Pap classification[18]. An overview of different cytological and histological terminology is shown in table1.

The preceding description of cytology refers primarily to precursors of squamous cell carcinoma, which is the most common type of cervical cancer. Other types are adenosquamous carcinoma and adenocarcinoma. Rare types include small cell carcinoma. Figure 1. demonstrates the relationship between the different cytological and histological classifications, during development of cervical cancer.

1.1.3 Diagnosis of (pre)malignant lesions

Women with abnormal cervical smears are referred to the gynecologist for colposcopic examination. Colposcopy was first described in 1925 by Hinselmann[19]. He evaluated his colposcopic findings by histopathology. Hinselmann's complicated nomenclature and controversial opinions on development of cervical cancer[20] led to reluctance amongst other gynecologists to accept colposcopy. It was not until the late 1960s that colposcopy became established for the detection of premalignant lesions, in combination with cytology and histology.

Modern binocular colposcopes with stereoscopic magnification between 6 and 40 times, allow visualization of microscopic changes. Most commonly used are 10 and 16 fold magnifications. The cervix and vaginal walls are inspected for abnormalities, and the transformation zone is visualized. The colposcopic examination is denoted 'satisfactory' when the entire transformation zone (including upper-endocervical limit) can be visualized.

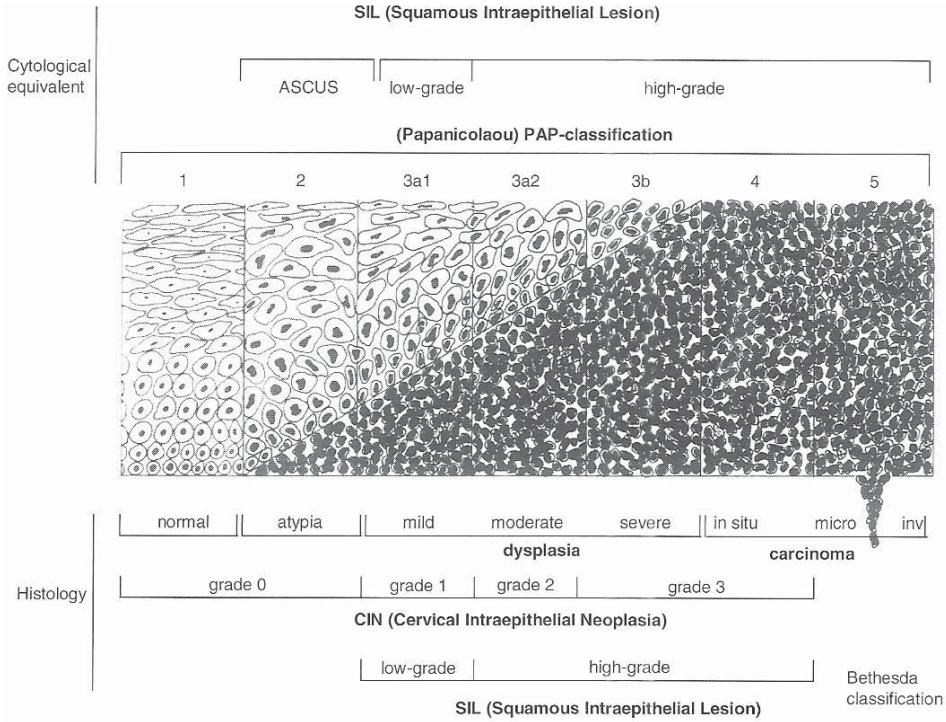

Figure 1. Illustrated cytological and histological classifications during development of cervical cancer.

Application of 3% acetic acid causes reversible coagulation or precipitation of the nuclear proteins and cytokeratins in cells, resulting in a white discoloration and swelling of columnar and abnormal epithelium. Dysplastic epithelium has many nuclei due to disturbed epithelial maturation, leading to visible discoloration of this abnormal epithelium. The Schiller test displays iodine staining in matured squamous epithelial cells that contain glycogen, thus leaving dysplastic, metaplastic and columnar cells unchanged. Other colposcopic features can be identified: vascular patterns, intercapillary distance, color tone, surface contour and epithelial borders.

It is the role of colposcopy to identify the most atypical site for biopsy in order to establish a histo-pathological diagnosis. Histology is the gold standard to define the severity of the cervical lesion. In addition, visualization of the transformation zone allows the method of treatment to be determined. If colposcopy is unsatisfactory an endocervical curettage is recommended in order to avoid unnecessary conization and to rule out endocervical (adeno)carcinoma.

1.1.4 Treatment of CIN lesions

It is generally accepted that treatment is indicated for high-grade CIN lesions (CIN 2/3), while for low-grade lesions (CIN 1) a conservative approach is recommended, since the majority will regress. In The Netherlands, women with CIN 1 are followed by regular cytological surveillance until there have been at least two consecutive normal smears. Women with CIN 2 and CIN 3 lesions are treated. Treatment options are: large loop excision of the transformation zone (LLETZ), laser evaporisation, cryocoagulation, cone biopsy or hysterectomy, depending on the severity of the lesion and the expertise of the gynecologist. In general, LLETZ is preferred, since this can be performed in an out-patient setting and allows histological examination of the removed lesion, while fertility is preserved. More than 90% of patients are cured after this procedure[21].

In cases where colposcopy was unsatisfactory (i.e. transformation zone not completely visible) cone biopsy is recommended in order to ensure removal of the entire transformation zone and to confirm histologically complete elimination of the dysplastic lesion.

According to the Dutch protocol, follow-up after treatment consists of cervical cytological smears 6, 12 and 24 months after treatment. Once there have been repeated normal smears, women return to the regular national screening program.

1.2 ETIOLOGY OF CERVICAL CANCER

The natural history of cervical cancer is characterized by development through premalignant lesions. These lesions are classified as CIN and are detectable by cervical cytology. It is estimated that the progression from CIN to cervical cancer generally takes 10-15 years[22,23]. Not all pre-invasive lesions will progress into cervical cancer. The mildest form, CIN 1 (mild cervical dysplasia) regresses in most cases (57%, with a persistence of 32% and progression of 11%), while 20-45% of the CIN 2/3 lesions will progress to cervical cancer if left untreated[24,25].

1.2.1 Human Papilloma Virus (HPV)

HPV is a sexual transmitted virus, with a life-time risk of 80%[26,27]. A persistent infection with high risk Human Papilloma Virus (hrHPV) is necessary for the development, maintenance and progression of primary CIN lesions[28-30]. Over 100 HPV types have been identified, 18 of them are associated with cervical carcinogenesis[31].

HPV is divided into low-risk (non-oncogenic) types and high-risk types (oncogenic). Low-risk HPV is mainly seen in genital warts and non-progressing low-grade CIN lesions, whereas high-risk types are associated with cervical carcinoma and high-grade CIN lesions. 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%)[31].

In women with normal cervical cytology the prevalence of hrHPV is age-related, decreasing from 20% in women between 18 and 25 years to 4% in women older than 35 years[30,32-34]. This pattern indicates that HPV infections are very common among young women and frequently resolve spontaneously. A higher HPV prevalence in the younger generation might be due to different sexual behavior. More likely, the influence of age reflects acquired immunity to various HPV types, leading to clearance of most primary HPV infections.

Most HPV-infected women will clear the infection within one to two years without developing cervical lesions[26,35-37]. After clearance of HPV regression of the lesion occurs. About 20% of women infected with hrHPV develop cervical lesions, which are transient in the majority of cases. Only 1-2% of these women ultimately develop cervical cancer, in about 12-15 years, if not properly treated[22,24,38]. The competence of the immune system and environmental factors may ultimately determine clearance of hrHPV or development of cervical cancer[39-41]. In fact, cervical cancer may be considered as a rare complication of a HPV infection[42].

1.2.2 Role of HPV-testing in diagnosis and treatment

HPV testing, as an adjunction to cytology, has potential value in different settings of screening. In the Dutch population-based cervical cancer-screening program women between 30 and 60 years of age undergo a cytological smear every fifth year. The efficiency of the screening may increase when HPV testing is used in conjunction with cytology[43]. Such an extension to screening is under investigation in a population based randomised trial in Amsterdam, The Netherlands (POBASCAM)[44].

HPV testing may also be of value in triage of borderline and mild dyskaryotic smears (BMD). Since only 10% of women with a BMD smear have a high-grade CIN lesion, referral is not necessary for the majority of these women. Triage with HPV testing can help to identify women at risk of development of high-grade CIN lesions and consequently to improve management of women with BMD smears[45-49].

In addition, HPV testing can be useful in follow-up after treatment for high-grade CIN. Follow-up after treatment for high-grade CIN consists of cervical cytological monitoring at 6, 12 and 24 months, to identify residual/recurrent CIN lesions (CIN 2/3). One of the drawbacks of follow-up by cervical cytology is the high number of false-positive findings. Approximately 20% of the women have abnormal cervical cytology at some point during the follow up. However in more than half of these women no underlying residual/recurrent CIN will be found, resulting in unnecessary diagnostic procedures. The use of combined testing by cytology and HPV can lead to a better selection of women at risk for residual/recurrent CIN. Combined

testing has been suggested in several retrospective and observational studies[21,50-56]. Improved selection leads to diagnostic procedures being performed only in those patients who have the necessary risk factor present for the development of recurrent/residual CIN lesions, and avoids unnecessary diagnostic procedures in patients without this risk factor. Consequently, this policy can lead to an important reduction in health costs and lessens anxiety for the women involved.

One further use for HPV testing as a contribution to screening is in self-sampling. Patients who do not respond to an invitation for screening (non-responders) are at increased risk of cervical cancer. Offering vaginal self-sampling for HPV testing for these women, may lower the threshold for women who do not participate in the regular cervical screening program[57-63]. Recent studies have shown that self-sampled material is usually not suitable for cervical cytology, but the self-sampled vaginal specimen contains enough cervical cells to detect a HPV infection[59-61,64,65]. Self-sampling for HPV might increase the participation rate for population based cervical screening programs.

1.3 IMMUNOLOGY

The human defense against viral infection is mediated by the early reactions of innate and the later responses of adaptive immunity. The effector cells of the innate response include neutrophils, granulocytes, monocytes, macrophages and natural killer cells. They recognize, internalize and/or phagocytose the invading virus or viral associated molecular patterns, and release soluble effector molecules, cytokines, which regulate and coordinate many of their activities.

There are two types of adaptive immune response, humoral and cell-mediated. The effector cells for humoral immune responses, B-lymphocytes, produce antibodies that specifically recognize and bind to the extracellular virus thus targeting it for elimination by various mechanisms. The cellular immune response targets the intracellular virus or viral antigens presented by antigen-presenting cells (APCs), especially dendritic cells. It is regulated by T lymphocytes [T-helper (Th) cells, cytotoxic T cells (CTLs) and regulatory T-cells (Treg cells)] in cooperation with APCs, and mediated through the release of cytokines which can influence each other's synthesis and actions in the setting of a complex immuno-regulating cytokine network.

Cytokines in immune responses to infection are often functionally classified as either immuno-stimulating (tumor-suppressing) Th1–type cytokines such as IFN-γ, TNF-α, IL-2 and IL-12, which mainly induce cell-mediated immunity, - or immunoinhibitory Th2-type cytokines (IL-4, IL-5, IL-6, IL-8, Il-10), which predominantly induce humoral immunity[66,67].

The increased incidence of HPV infection in individuals with cell-mediated immune response deficiencies (such as AIDS patients and transplant recipients) clearly demonstrates the crucial role that cell-mediated immune response plays in the control of HPV infections[68]. Although antibody responses are important for protection from HPV infection, cell-mediated immune responses are critical for lysing HPV-infected cells and have been implicated in regression of HPV lesions[69-71]. The importance of Th1-type cytokines in direct and indirect protection from both HPV persistence and HPV-associated disease progression has been highlighted in several studies[72,73]. Pro-inflammatory cytokines like TNF-α, IFN-γ and IL-12 down-regulate HPV gene expression and affect the growth of HPV-harbouring cells. Anti-inflammatory cytokines (IL-10, IL-4, IL-5, IL-6 and IL-13) down-regulate cell-mediated immune response, and their activity may suppress eradication of virus-infected and tumour cells[69].

1.3.1 Changes in the cytokine network

Qualitative and quantitative alterations in Th1-type and Th-2 type cytokine profiles have been used to analyze the immune response in HPV-associated CIN[74-76]. The general view favours a shift from Th1-type to Th2-type cytokines during the development of CIN and cervical carcinoma. Studies describing circulating cytokines in the plasma of patients with cervical dysplasia or cancer are scarce, deal with one or only a few cytokines, or are contradictory and only incidentally related to hrHPV. Possible changes in the capacity of the cytokine network during carcinogenesis of cervical cancer are not yet fully explored.

1.4 OUTLINE OF THIS THESIS

Detection of women at risk for development of cervical cancer is currently based on cytology. Sensitivity and specificity of cervical cytology for prediction of high-grade CIN varies: 55% to 86% for sensitivity, 62% to 98% for specificity. Although sensitivity is of major importance in the screening setting, current protocols demonstrate the adverse effects of low specificity. Unnecessary referral, treatment and costs can be reduced by improvement of detection methods.

Since the development of a reliable HPV test, risk reduction in women with CIN lesions can be investigated in the hope of establishing better clinical protocols.

In **chapter 2.1** triage with HPV testing in women with persistent borderline or mild dyskaryotic (BMD) smears is evaluated. In this prospective study, a wait-and-see policy was incorporated into the existing protocol to allow time for potential clear-

ance of hrHPV infection and regression of the cervical lesion. The use of triage with HPV testing to prevent unnecessary diagnostic procedures was evaluated.

In **chapter 2.2** the costs and the side effects of three strategies for managing women with persistent (defined as two consecutive) BMD smears using HPV testing as triage tool are described. In this study, both the side effects and the costs were compared to those associated with the conventional management (i.e., direct colposcopy of all women without first assessing their HPV status).

HPV testing could be used to improve monitoring of women for recurrent or residual disease after treatment of CIN 3. Several observational and retrospective studies for the implementation of HPV testing in monitoring of women treated for CIN 3 have been published.

In **Chapter 3.1** twenty studies, published between 1996 and 2003 are reviewed. These compare prediction of residual/recurrent disease by hrHPV testing, resection margins or cervical cytology. Eleven of these studies were used in a meta-analysis to establish an overview of recent data. This overview focusses on the utility of hrHPV testing in monitoring women after treatment for CIN 3. The overall conclusion of the post treatment review emphasized the necessity of a randomised controlled trial. In **chapter 3.2** addition of HPV-testing to cytological follow-up after treatment for high-grade CIN was investigated in a randomised clinical trail to select women at risk for residual/recurrent CIN.

The specificity and sensitivity for residual/recurrent CIN after treatment were both established for combined testing (cytology and HPV) and compared to conventional testing by cytology alone. Secondary, health-care costs and possible influence of hrHPV types were assessed.

HPV testing can also be used to reduce risk in women who refuse to participate in the national screening program. These so-called non-responders are at high particularly risk, since more than 50% of women with cervical cancer do not have a screening history.

In **chapter 4** the use of self-sampling for HPV in a regular screening programme was tested in non-responder women. In addition, we compared the screening history of women who submitted a sample with the history of age-matched participants in the regular screening programme. The yield of high-grade CIN as detected by hrHPV testing in self-sampling non-responders was compared to the yield by conventional cytology in screening responders.

In addition, costs per detected high-grade CIN lesion or cervical cancer in both groups of women were evaluated.

Observations on changes within the immune response to HPV infection during the development of cervical cancer are reported in **chapter 5**. Changes in the peripheral cytokine-network were investigated within different stages of CIN.

In **chapter 5.1** more insight is obtained into the deregulation of circulating cytokines. Changes in the capacity of cytokine release by immunocompetent cells are described in **chapter 5.2**.

The results of the previous chapters are summarized in **chapter 6**. Clinical consequences based on risk reduction by HPV testing and immunological observations are discussed.

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Chapter 2.1

Triage using HPV-testing in persistent borderline and mildly dyskaryotic smears: Proposal for new guidelines

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ABSTRACT

In The Netherlands 2% of cervical smears in the cervical cancer screening program are read as borderline or mildly dyskaryotic cytology (BMD smear). Only in about 10% of these women a high-grade CIN lesion (CIN II-III) is present, therefore referral is for the majority unnecessary. In this study triage with high risk HPV (hrHPV) testing was used to identify women at risk for development of high-grade CIN lesions after a repeat BMD smear.

A 'wait-and-see' period was incorporated allowing clearance of HPV and regression of the lesion. Women with a low-grade lesion, irrespective of their HPV-status were monitored at 12 months; women with a high-grade lesion at 6 and 12 months.

Fifty-one of the 105 women (49%) were hrHPV negative at baseline, none of them showed progression of the lesion within the first year of follow-up (NPV 100%). High-grade CIN was present in one patient who was HPV negative at baseline (2%), she demonstrated regression after 12 months. Nineteen of the hrHPV positive women (35%) demonstrated a high-grade CIN lesion at baseline, three cleared hrHPV after 6 months, with a subsequent regression of CIN. Ten women remained hrHPV positive with persistence of high-grade CIN and were eventually treated. At baseline 35 hrHPV positive women demonstrated a low-grade lesion, 19 remained hrHPV positive after 12 months, 5 developed high-grade CIN. Sixteen out of the 35 cleared the hrHPV infection without progression of the lesion.

In conclusion, triage using hrHPV testing for women with persistent BMD cytology can select women who are not at risk for development of high-grade CIN. We recommend return to the screening program without referral for colposcopic examination if hrHPV is absent. For hrHPV positive women a repeat hrHPV test after another six months is suggested. Referral is only required if persistence of hrHPV is established.

INTRODUCTION

In the Dutch population-based cervical cancer-screening program women between 30 and 60 years of age undergo a cytological smear every fifth year. Recent surveys showed that about 2% of these smears contained borderline or mildly dyskaryotic (BMD) changes[1,2]. According to current screening guidelines, in these cases cytology is repeated after 6 months and (if negative) after 12 months. When cytology is persistently abnormal, women are referred to the gynaecologist where colposcopic examination is performed and a biopsy is taken for histological examination. When histology shows no or low-grade dysplasia (≤ CIN I) women will be kept under cytological surveillance without immediate treatment. High-grade dysplasia (CIN II-III) or a worse lesion will be treated. Only ten percent of women with a single BMD smear show a high-grade CIN lesion[3-6]. Consequently, there is a need for an improved risk assessment of women with BMD.

Infection with high-risk HPV (hrHPV) has been established as an important etiological factor in the carcinogenesis of cervical cancer[7,8]. Persistence of hrHPV infection is required for development of cervical cancer[9-12]. Several studies have shown that HPV testing has a high sensitivity (approximately 95%) for identifying high-grade lesions and cervical carcinoma. Moreover, the negative predictive value of HPV testing for detection of a high-grade CIN lesion or even (micro)invasive carcinoma is nearly 100%[13,14]. Consequently, HPV-testing may play an important role in clinical practice, i.e. in triage of women with BMD cytology, as an adjunct to cytology in primary screening and in post-treatment protocols[15-17].

The prevalence of hrHPV in women with BMD smears is about 35%[18,19] and it is likely that hrHPV negative BMD women are not at risk for development of high-grade CIN lesions or carcinoma. Hence, triage with hrHPV testing may improve the selection of women at risk for development of cervical cancer and consequently the management of women with BMD smears[15,19-23]. This would not only have the advantage of improving the efficiency of the screening program through fewer referrals, but also reduce unnecessary anxiety among the majority of women with BMD who are not at risk for high-grade CIN[3,5,24].

In this prospective study, triage with hrHPV testing was performed in the follow-up of women referred with a repeated BMD established in the screening program. Unlike most published studies a wait-and-see period was incorporated in the present protocol to allow potential clearance of hrHPV infection and consequently regression of the cervical lesion. We evaluated the use of triage with hrHPV testing to prevent unnecessary diagnostic procedures and treatment.

MATERIAL AND METHODS

All women with a repeated BMD smear referred through the national screening program to gynaecological outpatient clinics were asked to participate in this study. Participating hospitals were the Erasmus University Medical Centre Rotterdam (December 1999 - May 2003), the Hospital Walcheren in Vlissingen (January 2000 - March 2002) and the VU University Medical Center Amsterdam (June 2000 - January 2003). Exclusion criteria were pregnancy at time of enrolment or during follow-up (n=3), non-Hodgkin lymphoma (n=1), age younger than 30 years or older than 60 years at time of abnormal smear (n=5), or insufficient Dutch or English language skills (n=1).

Figure 1 shows the trial design. At baseline women were asked to complete a questionnaire on education, ethnic background, smoking, number of sexual partners, sexarche, contraceptive use and history of sexually transmitted diseases. A cervical scrape was taken for HPV detection followed by colposcopy. Standard colposcopical assessment with acetic acid and iodine solution was performed by expert gynaecologists. Biopsies were taken from all colposcopic abnormalities. At this first visit no treatment was carried out.

Women who were hrHPV negative were reviewed after 12 months. HrHPV positive women were reviewed after 12 months if the colposcopically-directed biopsy was ≤ CIN I, and after 6 and 12 months if histology showed moderate to severe dysplasia (CIN II-III). During follow-up visits hrHPV-testing and colposcopy were performed. Women with a persistent hrHPV-positive high-grade CIN lesion during follow-up were treated by loop excision of the transformation zone (LETZ). The study endpoint was reached after 12 months, or after treatment, whichever came first. The study protocol was approved by a multicenter research ethics committee and by local committee's at all three hospitals. All women voluntarily gave signed informed consent before enrolment.

Histology

Lesions were histologically defined as mild dysplasia (CIN I), moderate dysplasia (CIN II), severe dysplasia (CIN III) and (micro) invasive cancer. Lesions ≤ CIN I are hereafter referred to as a low-grade lesion and CIN II-III as a high-grade lesion. Regression or progression was defined as histological change from a high-grade lesion to low-grade lesion or vice versa, detected in the biopsy material at two consecutive time points as scheduled by the trial design. All histological samples were read by expert pathologists.

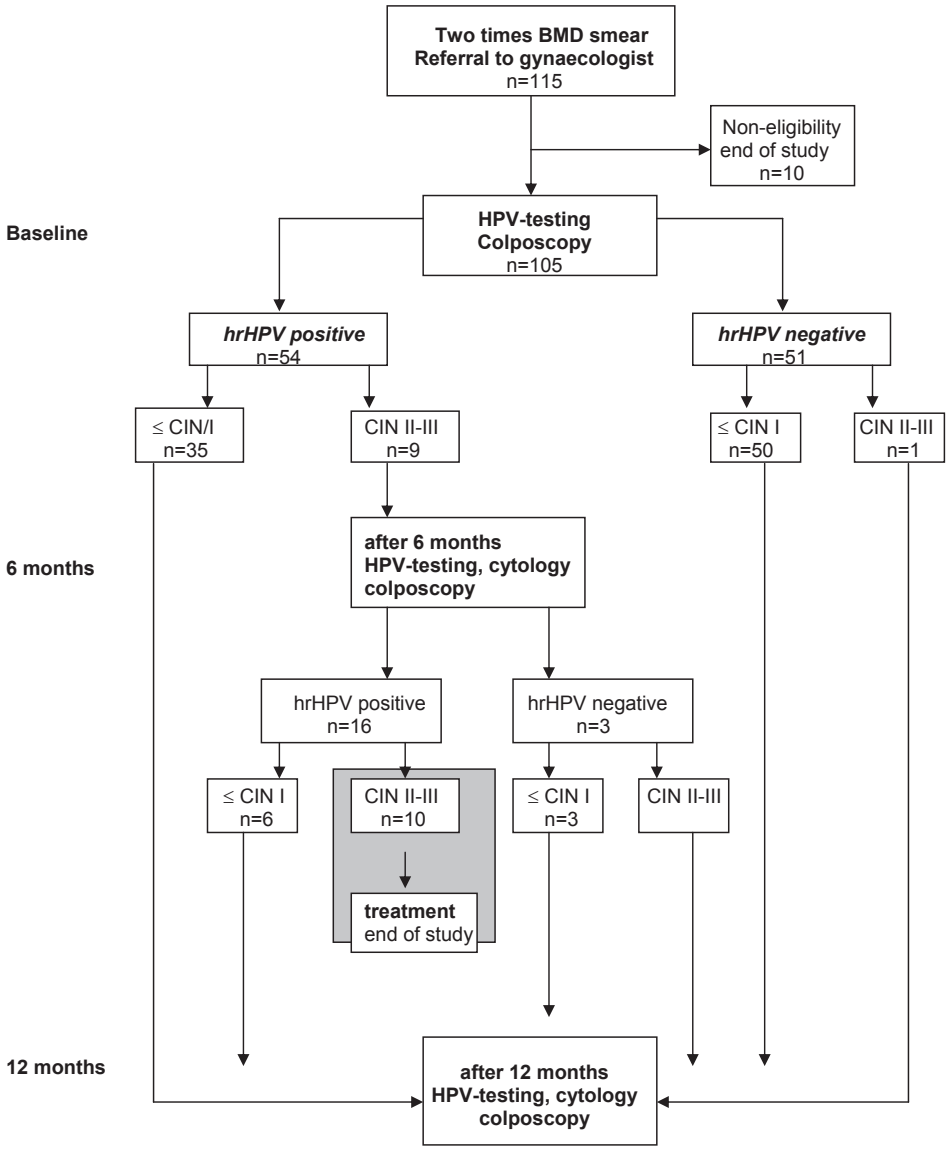

Figure 1. Trial design

Human papillomavirus testing

All HPV samples were taken by a cervical biosampler (Accellon Combi® Medscand Medical, Sweden). Testing for HPV was conducted by using a general/consensus primer based GP5+/ GP6+ polymerase chain reaction (PCR) enzyme immunoassay (EIA) for 14 high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68)[25]. This test has been clinically validated[11,26,27]. Additionally, reverse line blot

(RLB) analysis was used to identify individual HPV types in case the PCR-EIA was positive[25]. We used β-globin PCR to identify sampling errors and to monitor for PCR inhibitors.

Statistical analysis

The risk of development of high-grade CIN associated with HPV presence was assessed with the Fisher exact test and Chi-square. Confidence intervals of 95 percent and 2-sided P-values were used.

RESULTS

A total of 105 women met the inclusion criteria. The mean age was 39 years (range 30-60 years). The median time-lag between the two BMD smears on the basis of which women were referred was 7 months, with a range of 2-20 months. The median time-lag between the second BMD smear and colposcopic examination was 2 months (range 0-8 months).

At baseline 54 women (51%) were hrHPV positive, leaving 51 hrHPV negative subjects (figure 1). HPV 16 and 31 were the most frequently detected hrHPV types (40% and 20% of all infections, respectively). Other types were less common: HPV 18 (7%), HPV 33 (10%), HPV 35 (7%), HPV 42 (7%), HPV 45 (2%), HPV 51 (7%), HPV 52 (10%), HPV 56 (10%), HPV 58 (7%), HPV 59 (2%) and HPV 66 (2%). Of the observed HPV positive scrapings at baseline, 68% contained single infections and in the remaining 32% a multiple infection was detected. With one exception, all hrHPV negative women demonstrated no or a low-grade lesion at baseline biopsy: 27 women without dysplasia (53%) and 23 women with CIN I (45%). One hrHPV negative woman had a CIN II lesion at baseline.

Among hrHPV positive women at baseline, 35 cases (65%) had ≤ CIN I and 19 cases (35%) CIN II-III. The first group consisted of 10 women (19%) without dysplasia and 25 women (46%) with a CIN I lesion, whereas the high-grade CIN group comprised 14 women (26%) with a CIN II lesion and five women (9%) with a CIN III lesion.

Table 1 shows the characteristics of the study population stratified according to hrHPV presence at baseline. When age is stratified in three categories 30-40, 40-50 and 50-60 years respectively, we see a significant difference for the youngest group (30-40 years) where HPV is more frequently present ($p<0.01$). The presence of CIN II-III was significantly higher in women who were hrHPV positive at baseline, compared to hrHPV negative women ($p<0.01$ with an odds ratio of 27 (95% CI 3-211)). Women who were hrHPV negative were less likely to have a history of sexually transmitted disease ($p<0.01$). Other risk factors such as education, ethnic

Table 1. Baseline characteristics

	Total	hrHPV negative at intake	hrHPV positive at intake	Significance
	no. (%)	*no. (%)*	*no. (%)*	
	n=105	*n=51*	*n=54*	
Age at intake (years) *				***p<0.01***
30-40	58 (55%)	17 (33%)	41 (76%)	
40-50	26 (25%)	16 (31%)	10 (19%)	
50-60	21 (20%)	18 (35%)	3 (6%)	
Histology at intake *				***p<0.01***
low-grade	85 (81%)	50 (98%)	35 (65%)	
high-grade	20 (19%)	1 (2%)	19 (35%)	
Ethnic background				
Caucasian	82 (78%)	43 (84%)	39 (72%)	
Asian	4 (4%)	2 (4%)	2 (4%)	
Negroid	10 (10%)	4 (8%)	6 (11%)	
Mediterranean	4 (4%)	0	4 (7%)	
Other	5 (5%)	2 (4%)	3 (6%)	
Education				
Primary or less	9 (9%)	4 (8%)	5 (9%)	
Secondary, incomplete	55 (52%)	32 (63%)	23 (43%)	
Secondary or more	41 (39%)	15 (29%)	26 (48%)	
Age at first intercourse (years)				
≤15	23 (22%)	8 (16%)	15 (28%)	
16-18	51 (49%)	26 (51%)	25 (46%)	
≥19	31 (30%)	17 (33%)	14 (26%)	
No. of sexual partners last year				
0-1	89 (85%)	45 (88%)	44 (81%)	
2-4	16 (15%)	6 (12%)	10 (19%)	
Smoking				
no	56 (53%)	32 (63%)	24 (44%)	
yes	49 (47%)	19 (37%)	30 (56%)	
Oral contraceptive use				
no	62 (59%)	34 (67%)	28 (52%)	
yes	43 (41%)	17 (33%)	26 (48%)	
History of Sexual Transmitted disease *				***p<0.01***
no	84 (80%)	48 (94%)	36 (67%)	
Chlamydia trachomatis	9 (9%)	3 (6%)	6 (11%)	
Condylomata accuminata	5 (5%)		5 (9%)	
other	7 (7%)		7 (13%)	

* Fisher's Exact p< 0.01

background, sexarche, number of sexual partners in the preceding year, smoking and oral contraceptive showed no difference of statistical significance between groups with and without HPV infection.

Follow-up of hrHPV negative women

One woman acquired hrHPV infection after 12 months, without progression to high-grade CIN. All other women (50/51) remained hrHPV negative and did not develop a high-grade CIN lesion. The woman with a high-grade CIN lesion at baseline showed histological regression to no dysplasia after 12 months (Table 2).

Follow-up of hrHPV positive women

Nineteen of the 35 hrHPV positive women (54%) with CIN ≤ I at baseline demonstrated persistence of hrHPV infection after 12 months. Five out of these 19 (26%) showed progression to high-grade CIN. No (micro)invasion was detected. Fourteen women without HPV clearance continued to have CIN ≤ I. Clearance of hrHPV infection occurred in 16/35 women (46%), none of whom showed progression detected in histological biopsies, i.e. 14 women without dysplasia and 2 women who still had CIN I after 12 months.

Among the 19 women with a high-grade lesion three (16%) cleared the hrHPV infection after six months, with histological regression to a low-grade lesion, and remained hrHPV negative after 12 months. Amongst the 16 women who were still hrHPV positive after 6 months (84%) 10 (63%) revealed a (persistent) high-grade lesion. No (micro)invasion was detected. These 10 women were treated. The remaining six women (37%) demonstrated regression to a low-grade lesion. One of them was treated at her own request and therefore left the study group prematurely. After 12 months two out of five women remained persistently hrHPV positive, one of whom had a high-grade lesion (CIN II). The other three women showed clearance of hrHPV and a low-grade lesion on histology.

Table 2. Histology outcome related to HPV status at baseline

	baseline			*t=6 months**			*t=12 months*		
	≤ CIN I	CIN II-III	Total n (%)	≤ CIN I	CIN II-III	Total n (%)	≤ CIN I	CIN II-III	Total n (%)
HPV pos (n)	35	19	54 (51%)	9	10	19 (100%)	37	6	43 (41%)
HPV neg (n)	50	1	51 (49%)	-	-	-	51	0	51 (49%)
Total (n)	85	20	105	9	10	19	88	6	94**

pos=positive, neg=negative

*only HPV positive CIN II-III had t=6 months follow-up; **11 women treated (10%).

DISCUSSION

In this study progression to a high-grade CIN lesion was not seen in hrHPV negative women with a persistent BMD smear detected in the national screening program. All women with a high-grade lesion were hrHPV positive at baseline, except for one woman who had a CIN II lesion at baseline and was hrHPV negative. Histology from this patient showed regression to a low-grade lesion after 12 months, suggesting that for this woman clearance of hrHPV was already evident at baseline, with subsequent regression of the lesion becoming apparent after 12 months. This corresponds with the findings of other studies [17,20,28].

In our study, the negative predictive value (NPV) of hrHPV testing for having high-grade CIN was 98% at baseline for women with a repeated BMD smear, and 100% after 6 and 12 months follow-up. This suggests that hrHPV negative women, despite a persistent BMD cytology, are not at risk for high-grade CIN.

Various studies have shown that better results are obtained using HPV detection when compared with conventional cytology (i.e. NPV cytology 93-98%) [7,14,19]. Triage with HPV-testing as an adjunct to cytology has been proposed for women with minimal cytological changes [15,17,23]. Several studies have used different classifications of mildly abnormal cytology [17,19-20,23,30-32]. Cuzick et al. included women with borderline cytology and negative cytology but hrHPV positive (HART study) [23]. The ALTS study describes triage in women with ASCUS and LSIL cytology (ALTS Group) [29]. An overview of triage studies with corresponding data is shown in Table 3. Selection was based on HPV detection methods that were clinically validated in longitudinal studies (i.e. HCII and GP5+/6+ PCR), since the clinical rather than the analytical performance of HPV detection methods should be considered for triage policies [33]. The main benefit that can be concluded from all listed studies is a reduction in referral of hrHPV negative women with minimal abnormal cytology for colposcopy. For more severe cytological abnormalities the use of triage with HPV-testing is not indicated.

Our study was based on women referred to the gynaecologist for two times BMD smear according to the national guidelines. Based on our results we suggest that women with a persistent BMD smear and a concomitant negative hrHPV test result are not at risk for development of cervical cancer and should stay in the screening program, without colposcopic evaluation. In The Netherlands the next scheduled screening would then take place after 4.5 years. The risk that a woman will develop high-grade CIN within that period of time seems acceptable, since it will be exceptional to take place within 5 years given that a persistent hrHPV infection is required [4,34]. Precursor lesions are detectable within an average of 10-15 years before progression to cancer [35]. Thus, even though our study only covers 12 months of

Table 3. Triage in women with borderline or low grade cytological abnormalities (selected studies based on validated HPV detection methods)

Study	*Population*	*n*	*Mean age years*	*Follow-up*	*HPV +*	*HPV clearance*	*CIN 2/3*	*Sens %*	*Spec %*	*PPV %*	*NPV %*	*Reduction of referral*
(HART)				RCT								
Cuzick et al. 2003	borderline cytology negative cytology HPV+	825	42 y	6-12 months	27%	35-45%	3%	97	93	13		HPV- borderline cytology return to screening HPV+ borderline cytology retest HPV after 12 months HPV+ mild dyskaryosis refer
Ho et al. 2003	repeated mild dyskaryosis or less	149	28-32 y	observational study	59%	44%	24%	100				42% reduction based on HPV+
				every 6 months								HPV- mild dyskaryosis retest 6-12 months
				mean 3 years								retest HPV: total of 67% less referral
(ALTS)				RCT								
Solomon et al. 2001	ASCUS	2198	29 y	6-12-18 months	54%		12%	96		20	99	44% reduction based on ASCUS HPV +
Sherman et al. 2002	ASCUS-LSIL	3046	29-25 y		89%			96-97				46% reduction based on ASCUS HPV+, 15% reduction if LSIL HPV +
Guido et al. 2003	ASCUS	881	25 y	CIN I or less follow-up			18%	92				45% reduction based on HPV retest after 12 months
Cox et al. 2003	ASCUS HPV +	1132			51%		18%					49% reduction based on ASCUS HPV+
	LSIL	852			81%		18%					
Zielinski et al. 2001	single and repeated borderline or mild dyskaryosis	278	41 y	observational study	42%	45%	10%	96	63	22	98	58% reduction based on HPV +
				every 6 months median 1.4 years								
Current study	repeated borderline or mild dyskaryosis	105	39 y	intervention study: treatment postponement	51%	41%	19%	95	59	35	98	49% reduction based on HPV+
				6-12 months								

follow-up, it seems reasonable to return women with a hrHPV negative persistent BMD smear into the screening program without additional surveillance or treatment. However, an estimate of excess risk among hrHPV negative women with BMD cytology is expected within the next few years when e.g. the ongoing Dutch POBASCAM screening study and Swedish screening study are expected to yield final results [1,36].

In our study 54/105 women (51%) were hrHPV positive at baseline of whom 19 (35%) had a histologically confirmed high-grade CIN lesion. The overall frequency of high-grade CIN was 19%, which is comparable with the findings of Ho et al. 2003 [20]. In contrast, a previous study by our group demonstrated a prevalence of 10% [19]. In that study however, about half of the women were referred after one BMD smear in contrast to our current study where women were referred after two BMD smears in 6 months. Consequently, this group with BMD contains more women with mildly dyskaryosis than the previous group (38% vs. 26%) (data not shown). Therefore, our results can be explained by the fact that the prevalence of high-grade CIN increases with severity of abnormal cytology [12,19].

In this trial women with a high-grade lesion were not treated immediately. A 'wait-and-see' period of six months showed persistence of high-grade CIN only in women with persistent hrHPV infection. No patient developed (micro)invasive carcinoma. These findings suggest that a wait-and-see period for (at least) six months involves no additional health risks. In addition, 16% of these women showed clearance of hrHPV with a subsequent regression of the lesion. They were no longer at risk of development of high-grade CIN, and consequently, an expectative policy could result in a reduction of the need for treatment.

The majority of hrHPV positive women with a repeat BMD smear have low-grade CIN lesions (in our study 35 out of 54 (65%) at baseline). This is in agreement with the findings of previous studies [6,17,19,20,23,30-32]. Approximately half of these women cleared hrHPV in 12 months and none of them developed a high-grade CIN during this time. Fourteen percent developed high-grade CIN after 12 months (all were persistently hrHPV positive), without an observed development of (micro)invasive carcinoma. It should, however, be considered that the biopsies taken to establish the severity of premalignant lesions may have interfered with the natural course of disease. On the other hand, several natural history studies have demonstrated that HPV clearance and histological regression occur after 6-12 months [4,28,37]. Our follow-up time of 12 months should therefore cover the major part of regression. Consequently, referral can therefore be restricted to women with a repeat BMD smear who remain persistently hrHPV positive for at least another six months.

Although our proposal requires more hrHPV testing (100%), according to the present study it will lead to a 49% reduction in referrals for colposcopy. A 'wait-

and-see' period in the HPV positive group for at least six months will result in a supplement of 51% HPV-testing and a further 18% reduction in referrals and 9 percentage points treatment reduction. In summary, a total reduction of 67% referrals for colposcopic evaluation and 38% less treatment can be obtained by addition of 151% HPV-testing. The 6 months wait-and-see period in hrHPV positive

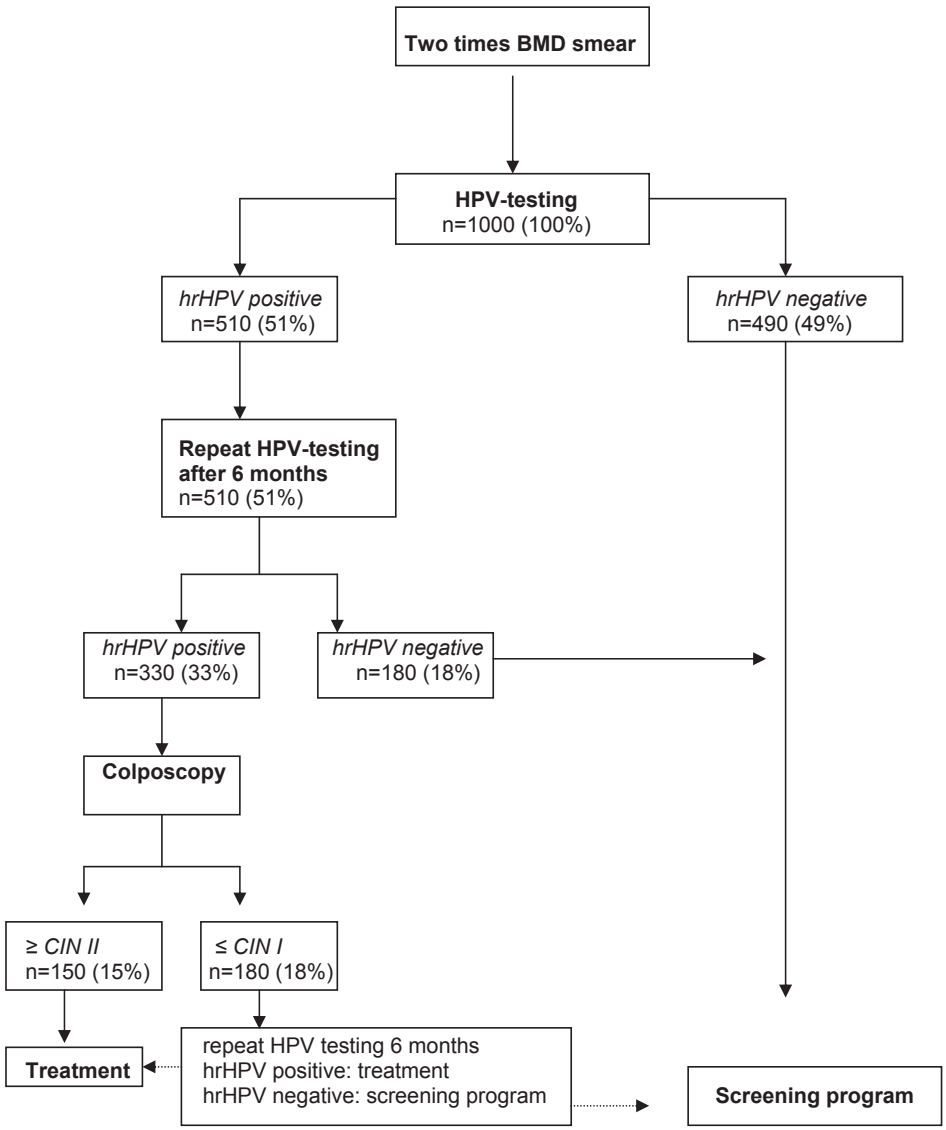

Figure 2. Policy proposal, illustrated by a hypothetical number of 1000 patients.
Reduction of 67% referral for colposcopic evaluation (49 + 18), 9 percentage points or 9/24*100=38% less treatment (current directions 25/105 (24%) treated vs. proposed proposal 16/105 (15%) treated) accomplished if 151% HPV-testing is added.

women may also have negative influences on the women involved. Some women might prefer a see-and-treat policy instead of a more conservative approach. Proper information and explanation of the natural development of premalignant lesions in relation to hrHPV infection by the physician is therefore required, after which patients preference may influence further choices especially in this group[38]. The potential gain of less treatment however, remains to be weighted against potentially changes in quality-of-life and effectiveness. Our recommendations are summarized in Figure 2.

In conclusion, triage using hrHPV testing for women with a persistent borderline or mildly dyskaryotic cytology is recommended. Women can stay in the screening program without referral to the gynecologist if no hrHPV infection can be determined. We suggest a repeat HPV test after another six months for hrHPV positive women. Referral for colposcopy is only required if persistence of hrHPV is demonstrated. Women who cleared hrHPV infection are no longer at risk of development of premalignant lesion. Further surveillance can take place within the screening program at the protocollized interval of five years.

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Chapter 2.2

Human papillomavirus triage of women with persistent borderline or mildly dyskaryotic smears: Comparison of costs and side effects of three alternative strategies

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ABSTRACT

The conventional direct referral to colposcopy of persistent borderline or mildly dyskaryotic (BMD) smears in cervical cancer screening leads to considerable unnecessary referrals and associated anxiety and costs. This may be improved by including testing for oncogenic human papillomavirus (HPV) in the triage. We assessed costs and side effects (referrals, treatments, time in follow-up) for three possible HPV triage strategies (immediate HPV testing, a 6-month delay in HPV testing, a two-stage combination of both) and compared them with the conventional strategy.

The assessments are based on recent Dutch data from various national databases and trials. We estimated that the referral rate could be reduced by 49%, 58% and 58% with immediate, delayed and two-stage HPV testing, respectively. As a consequence, the average length of follow-up, as well as average costs, also decrease.

Therefore we advocate including HPV testing before referring to colposcopy. Among the three HPV strategies, analysis of additional aspects favours implementation of immediate HPV testing.

BACKGROUND

Cytology-based cervical cancer screening prevents deaths by treating pre-invasive (cervical intraepithelial neoplasia - CIN) and early invasive disease. Low-grade abnormalities described as borderline and mild dyskaryosis (BMD) are the most common type of cytologic abnormalities, ranging in frequency from below 2% in The Netherlands to above 6% in Finland.[1] In several countries, it is currently recommended to follow up these women with a Pap smear in 6 months[2-4]. Women are then referred for a colposcopy if the BMD abnormality does not normalise[3]. In The Netherlands, about one third of the women with BMD primary screening smears is eventually referred[1], most often because of BMD persistence and less often because of cytological progression[5].

This diagnostic policy of following-up BMD smears induces a considerable amount of side effects in terms of a high number of referrals and a long follow-up period with associated costs and psychologic consequences. Sixty to ninety percent of women with BMD persistence have no high-grade lesion that needs to be treated[6-13]. The burden on women and the health care could be reduced if the subgroup at high risk for a significant lesion were identified.

It has been consistently shown in the literature that detection of an infection with one of the high-risk human papillomavirus types (HPV) can be used for a risk stratification of women with low-grade abnormal smears[14,15]. An infection with HPV is a necessary factor in the development of invasive cervical cancer[16]. Because no histological progression is seen in women who spontaneously clear the HPV infection[17], women without a detectable HPV infection do not need further follow-up. That could be the case in 40-60% of women with persistent BMD smears[7-13]. The discussion about the optimal strategy and time points of incorporating HPV testing into triage of women with BMD smears is still on-going[18-20].

To inform this discussion, we investigated the costs and the side effects of three strategies of managing women with persistent (defined as two consecutive) BMD smears using HPV testing as a triage tool. Both the side effects and the costs are compared to those associated with the conventional management (i.e., direct colposcopy of all women without first assessing their HPV status).

METHODS

Triage strategies

We analysed three possible HPV triage strategies of women with two consecutive BMD smears (Figure 1; corresponding to ASC-US+ASC-H+LSIL in the Bethesda 2001 classification[21]).

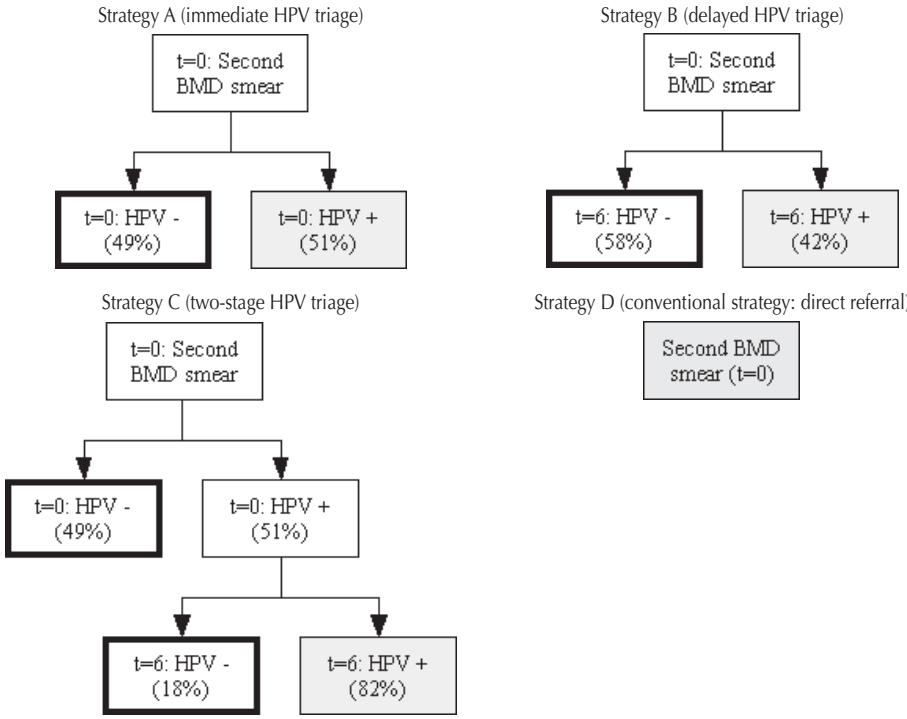

Figure 1. Strategies A, B, C, and D (conventional strategy) for triage of persistent BMD smears.
Note: Shaded rectangles represent women who need to be referred, whereas the rectangles with a bold border represent women who can return to the regular screening programme.

The difference among these strategies is in the timing of HPV testing, and the consequent referral to colposcopy:

- A – immediate HPV triage: the co-collected HPV sample is analysed immediately when BMD persistence is established (t=0), and all HPV-positive (HPV+) women are referred to colposcopy;
- B – delayed HPV triage: an HPV sample is collected six months after the second BMD smear (t=6), and all HPV+ women (i.e., those who have not cleared the virus) are referred to colposcopy;
- C – two-stage HPV triage: the co-collected HPV sample is analysed immediately when BMD persistence is established (t=0), HPV+ women are re-tested for HPV visit at t=6, and all women who remain HPV+ (i.e., those who have not cleared the virus) are referred to colposcopy.

We assumed that in strategies A and C the HPV samples are co-collected when the woman presents at the general practitioner (GP) to have a follow-up Pap smear after a BMD primary screening smear (both conventional cytology). These HPV samples are investigated if the follow-up smear is read as BMD. Women with one negative

HPV test return to a normal screening schedule. The three HPV strategies were compared for side effects and costs to:

- D – conventional strategy: direct colposcopy, i.e. a referral at t=0 of all women with two consecutive BMD smears without a prior assessment of their HPV status.

Quantification of side effects and costs

We assessed the side effects and costs per woman with two consecutive BMD smears for the period after the second BMD smear. This period includes all HPV sampling for triage and the complete post-referral management.

In the Dutch cervical cancer screening programme, roughly 1 in 4 women with a primary BMD smear has a follow-up (=second) BMD smear; the remaining 3 in 4 women have either a negative follow-up smear, or a highly abnormal (>BMD) smear[5]. Because of co-collection at t=0 in strategies A and C, when the outcomes of cytologic testing are not yet known, HPV samples would need to be taken from all women with a primary BMD smear. This represents extra costs compared to the conventional strategy, and needs to be taken into account in the analysis. Therefore, in these two strategies we assumed that three extra HPV samples need to be collected for each woman with two consecutive BMD smears. We further assumed that only those collected samples that are relevant for our analysis, i.e. 1 in 4, are read in the laboratory.

The proportion of women referred, the proportion treated and the time needed to complete the recommended follow-up are quantified from epidemiological data from a recent Dutch trial performed at the Erasmus Medical Center (Erasmus MC) reported earlier.[7] This trial aimed to evaluate the potential to prevent unnecessary diagnostic procedures and treatments by doing the HPV triage of women with two consecutive BMD smears corresponding to strategy C. At enrolment, the gynaecologist took an HPV sample and biopsies from all colposcopic abnormalities but treatment was deferred. Women with an HPV+ high-grade (CIN 2/3) lesion at enrolment were followed-up 6 months later with an HPV test and a colposcopically guided biopsy. If the HPV+ CIN 2/3 lesion persisted, the woman was treated. If instead within 6 months the woman cleared the virus or no CIN 2/3 lesion could be established anymore, she was seen at the exit visit 12 months after enrolment together with those women who tested HPV-negative at enrolment or had (at most) a low-grade lesion (CIN 0/1). At this exit visit, women were tested for HPV and underwent colposcopy.

Table 1. HPV prevalence and CIN lesions.

	Base case	Range (Literature)	Outcomes affected
(1) HPV prevalence at t=0	51%[7]	40-60%[8-13]	Equals the referral rate in strategy A.
(2) Persistence of HPV infection from t=0 to t=6	82%†	60-85%[9, 13]	Multiplied with *(1)*, it equals the referral rate in strategies B and C.
(3) CIN 2+ among women regardless of HPV status at t=0	19%[9,11-13]	10-40%[6,8-13]	Equals the proportion of women treated immediately upon referral in strategy D.
(4) CIN 2+ among women HPV+ at t=0	35%[7]	20-50%[9,11-13]	Multiplied with *(1)*, it equals the proportion of women treated immediately upon referral under strategy A.
(5) CIN 2+ among women with a persistent HPV infection at t=6	44%†	30-60%	Multiplied with *(2)*, it equals the proportion of women treated immediately upon referral under strategies B and C.
(6) CIN 0 : CIN 1 ratio, regardless of HPV status	44%:56%[7]		Multiplied with (1 - *(3)*) and the respective progression rates (Table 2), it equals the proportion of women treated at follow-up in strategy D. Multiplied with the cost per CIN 0 or CIN 1 case (Table 4), determines the total cost of referral of CIN 0/1 in strategy D.
(7) CIN 0 : CIN 1 ratio, HPV+ women	29%:71%[7]		Multiplied with (*(1)* × (1 - *(4)*)) for strategy A or (*(1)* × *(2)* × (1 - *(5)*)) for strategies B and C, and the respective progression rates, it equals the proportion of women treated at follow-up in strategies B&C. Multiplied with the cost per CIN 0 or CIN 1 case, determines the total cost of referral of CIN 0/1 in strategies A, B and C.
(8) CIN 2 : CIN 3 ratio	75%:25%‡		Multiplied with *(3)*, *(4)*, or *(5)* (depending on the strategy), the recurrence rates after treatment, and the cost per CIN 2 or CIN 3 case, determines the total cost of referral of CIN 2/3 in all strategies.

t=0: at time of the second BMD smear. t=6: six months after the second BMD smear.

† Estimated by the multi-state Markov model from the Erasmus MC trial (see Material and Methods).

‡ Not varied by HPV status, as observed in the Erasmus MC trial.

At enrolment, 51% of women had detectable HPV (Table 1). Nineteen percent of all enrolled women were found to have a CIN 2/3 lesion at t=0, i.e. 35% of all HPV+ and 2% of HPV- women. The observed 12-month progression and persistence proportions of (untreated) CIN 0/1 lesions (CIN 0 to >CIN 0, and CIN 1 to ≥CIN 1; both transitions may prompt treatment during post-colposcopic follow-up) were dependent on the HPV status at enrolment (Table 2). No cancer was found during follow-up.

In the Erasmus MC trial, the HPV status and the distribution of CIN lesions at t=6 were not directly observed for all women. We applied a multi-state Markov model on the longitudinal data of all HPV+ women at enrolment (n=54), using the HPV

Table 2. Proportion of women with persistence or progression of the initial lesion, as observed during 12 months in the Erasmus MC trial.

Initial CIN 0 lesion to >CIN 0[1]	14%
HPV- at enrolment	7%
HPV+ at enrolment[2]	30%
Initial CIN 1 lesion to ≥CIN 1[1]	38%
HPV- at enrolment	26%
HPV+ at enrolment[2]	48%
Post-treatment recurrent or residual lesions in CIN 2/3[3]	10%[26]

[1] Determined by pooling the trial outcomes for HPV+ and HPV- women. Used for evaluation of strategy D. [2] Used for evaluation of strategies A, B and C. [3] Used for evaluation of all four strategies.

and CIN prevalence observed at t=0 (for all 54 women), at t=6 (for the subgroup with HPV+ CIN 2/3 at enrolment), and at t=12 (for women with HPV+ CIN 0/1 at enrolment, and women without persistent HPV+ CIN 2/3 at t=6). The result of this model was an estimate of HPV and CIN prevalence for all 54 women at t=6. We used the msm package for the statistical program R version 0.6.3 (Christopher Jackson, Dept. of Epidemiology and Public Health, Imperial College, London). We allowed for the following transitions: HPV clearance, CIN progression and CIN regression, and assumed that women treated at t=6 would without treatment have remained in the same state until t=12. This lead to an estimate that 18% of women HPV+ at t=0 cleared this infection by t=6 (Table 1). With the same model we also estimated that 44% of women who do not clear the HPV by t=6 have CIN 2/3.

For the HPV prevalence and persistence rates and the CIN 2/3 prevalence, we extracted the plausible ranges from the literature[6,8-13] and used them as the basis for univariate sensitivity analyses. In all cases where independent observations were available, these formed an interval around the point estimates observed in the Erasmus MC trial (Table 1).

We defined the average duration of follow-up as the period following the second BMD smear in which women are further triaged through the HPV test (strategies A, B and C), referred and followed-up. We assumed compliance with the recommended follow-up (surveillance) after colposcopy. In The Netherlands, women with CIN 0/1 at initial referral are not treated but it is recommended to follow them up with 2 smears within 12 months; women with CIN 2/3 at initial referral are offered treatment and are then followed-up with 3 surveillance smears within 24 months[22]. If in either case at least one surveillance smear is abnormal, the woman is referred for colposcopy again and eventually (re-)treated.

We accounted for the extra follow-up time due to surveillance based on the progression/persistence rates of CIN 0/1 observed in the Erasmus MC trial, and on

post-treatment residual/recurrence rates for CIN 2/3 extracted from the literature (Table 2). In this way, we neglected the extra follow-up time because of negative colposcopies in women with (false-)positive surveillance smears. We assumed that the abnormal surveillance smear is found at the mid-point of the recommended follow-up interval. The assumed time needed between the successive management steps is then as follows: 2 months from the positive triage test to colposcopy, 1 month from colposcopy to treatment, 6 months for post-colposcopy surveillance of CIN 0/1, and 12 months for post-treatment surveillance of CIN 2/3.

We estimated that from the moment the referral advice is given it takes on average 10, 14, and 17 months to complete follow-up for CIN 0, CIN 1, and CIN 2/3, respectively. These estimates, used for the evaluation of the conventional strategy D, are based on the combination of the observed persistence/progression proportions (Table 2), and the recommended length of post-referral follow-up, both per CIN stage and regardless of the HPV status. In strategies A, B and C all referred women are HPV+. Untreated HPV+ CIN 0/1 lesions are more likely to be referred once again due to higher persistence/progression proportions of the lesion than the HPV- CIN 0/1 lesions (Table 2).

In these strategies, therefore, the estimated average follow-up time per CIN stage increases to 13 and 15 months for CIN 0 and CIN 1 lesions, respectively.

Direct medical costs, and the time and travel cost incurred by women were included in the analysis. The costs per procedure, and the average number of diagnostic and treatment procedures during post-referral management (diagnostics, treatment and follow-up) per CIN grade regardless of the HPV status, represent the recent Dutch situation[23,24] (Tables 3&4).

The average number of procedures per CIN stage[23] are based on the assumptions that (a) the given CIN grade is the maximum CIN grade, and that therefore women with CIN 0 are never treated, (b) 44% of women whose CIN 1 lesion is expected to persist or progress[25] are subsequently treated, (c) all women with CIN 2/3 are treated immediately, and (d) 10% of the women treated will need retreatment[26]. These assumptions and the estimated treatment modalities published earlier[24,27] were validated against the most recent available individualised national data on diagnoses, diagnostic procedures and treatments[5,28].

For the purpose of the present cost analysis, we also accounted for progression of CIN 0, and at least persistence of CIN 1 lesions by HPV status (see Table 2). We assumed that all women showing progression (or persistence of CIN 1) are treated.

Table 3. Average number of diagnostic and treatment procedures per CIN grade regardless of HPV status, based on national data for The Netherlands [23].

	Histology			
	CIN 0	CIN 1	CIN 2	CIN 3
Diagnostic procedures:				
Colposcopy	2.11	4.45	4.77	5.05
Smear by a gynaecologist	1.61	2.59	3.10	3.13
Biopsy	0.37	0.63	0.65	0.62
Endocervical curretage	0.13	0.37	0.35	0.38
Smear by a GP	0.40	0.51	0.44	0.32
Treatment procedures:				
LETZ[1]	0.13	0.42	0.93	0.75
Conization	0.01	0.04	0.15	0.30
Hysterectomy	0.00	0.02	0.02	0.04

[1] Loop excision of the transformation zone.

Table 4. Unit costs (€2005) of medical procedures, visits and hospital stays, based on the recent Dutch data [23].

Procedure	
Pap smear, taken by a GP[1]	45
Pap smear, taken by a gynaecologist[2]	25
Co-collected HPV test	1
HPV test at an extra GP appointment[3]	59
First colposcopy	86
Second or later colposcopy	63
Biopsy	50
Endocervical curretage	81
LETZ[4]	490
Conization[4]	1195
Hysterectomy[4]	4176
Average total cost (referral + post-referral management):	
- CIN 0	336 (420 if HPV+)
- CIN 1	828 (884 if HPV+)
- CIN 2	1239
- CIN 3	1432

[1] Includes the visit at the GP and the collection of sample material (€21), laboratory cost (€17) and costs of the woman (€6). [2] The laboratory cost. Collection of sample material and costs of the woman are included in the cost of colposcopy. [3] Includes visit at the GP and collection of sample material (€21), laboratory costs (€33), and costs of the woman (€6). [4] Treatment costs include the charge per type of treatment (LETZ €294, conization €477, hysterectomy €1062), cost of outpatient visit (if the procedure is performed in an outpatient setting; €64), cost of hospital days (day care €229, hospital day €359), preoperative diagnostics (for conization or hysterectomy €98) and costs for the woman (€9 for an outpatient visit, and €42 per treatment day).

RESULTS

While all women undergo a colposcopy under the conventional strategy (strategy D), only 51%, 42% and 42% would have been triaged to it by HPV under strategies A, B, and C, respectively (Table 5). The expected total detection rate of women with high-grade CIN lesions (CIN 2/3) would be 18%, 18%, 18% and 19% of the eligible women under strategies A, B, C, and D, respectively. The avoided referrals would therefore predominantly concern women with at most low-grade CIN lesions (CIN 0/1). While 81% of all women with two consecutive BMD smears turned out to have CIN 0/1 at referral in the trial (strategy D), this would only be 33%, 23% and 23% under strategies A, B, and C, respectively. As a consequence, the total treatment proportion would amount to 32%, 28%, 28% and 41% under strategies A, B, C, and D, respectively. This proportion includes immediate treatment of women with CIN 2/3 lesions at referral, and later treatment of initially untreated women (CIN 0/1 at referral) who show abnormalities in follow-up.

We estimated that the total follow-up after the second BMD smear takes 13 months on average under the conventional strategy D (Table 5). This period predominantly reflects the time needed to complete the recommended management after referral. Therefore, the average length of follow-up per strategy is strongly affected by the lower referral rates in the HPV strategies. These outweigh the prolongation of the pre-referral period due to the extra HPV triage, so that in the end the completion of follow-up after the second BMD smear would on average take 8, 12, and 10 months under strategies A, B, and C, respectively.

The expected average difference between strategies A and C is small though it should be noted that the subgroup that is referred based on delayed HPV testing (strategy C) is at a disadvantage. This is because they spend extra time in triage while the fact that they are referred and their post-referral management do not change.

We estimated that the total cost to manage a woman with two consecutive BMD smears under the conventional strategy (D) is €740 (Table 5). HPV testing itself would add to the triage costs, but these extra costs would be lower than the savings due to fewer referrals. The resulting difference is most favourable for strategy B (a decrease of 36% compared to strategy D), followed by strategies C (35%) and A (30%).

In the sensitivity analysis, we varied the epidemiologic assumptions with ranges from the literature reported in Table 1. In Table 6, we present the effects on the referral rate, the average length of follow-up and total costs. The ranking of strategies does not change. All three HPV strategies would remain more favourable than the conventional strategy. Under all investigated possibilities except when a lower HPV

Table 5. Results: Number of procedures, side effects, and costs (€2005) due to HPV triage, diagnostic assessment and treatment per strategy, and per woman with two consecutive BMD smears. Base case assumptions.

	TRIAGE STRATEGY			
	A (immediate triage)	B (delayed triage)	C (two-stage triage)	D (Direct colposcopy)
NUMBER OF PROCEDURES, SIDE EFFECTS				
HPV tests – co-collected samples[1]	4.00	0.00	4.00	n.a.
HPV tests – samples collected at extra GP visits[2]	0.00	1.00	0.51	n.a.
Proportion of women referred[3]	51%	42%	42%	100%
Detection of CIN 0[4]	10%	7%	7%	36%
Detection of CIN 1[4]	24%	17%	17%	45%
Detection of CIN 2[4]	13%	14%	14%	14%
Detection of CIN 3[4]	4%	5%	5%	5%
Proportion of women treated[5]	32%	28%	28%	41%
Average time in follow-up (months)[6]	7.7	12.4	9.5	12.9
COSTS				
HPV tests	37	59	67	n.a.
Referrals to colposcopy	478	412	412	740
Total	515	472	479	740
HPV tests (extra GP visits)				
- per avoided referral	0.0	1.7	0.9	n.a.
- per avoided treatment	0.0	7.8	4.0	n.a.

t=0: immediately after the second BMD smear. t=6: six months after the second BMD smear.
n.a. = not applicable

[1] E.g. for strategy C: It is observed in The Netherlands that 25% of all BMD primary smears have a repeat BMD smear.[5] This means that for every woman with the second BMD smear, 4 co-collected HPV samples need to be taken in total. [2] E.g. for strategy C: 51% of women with two BMD smears are HPV+ at t=0, and are retested for HPV at t=6. [3] E.g. for strategy C: 51% × 82% HPV persistence rate (Table 1). [4] Directly observed in the Erasmus MC trial for strategies A and D (see Table 1), and estimated for strategies B and C. [5] E.g. for strategy C: women with HPV+ CIN 2/3 (18%, see Table 5) + persistence or progression in women with initial CIN 0/1 lesions (7% × 30% + 17% × 48%, see Tables 2&5). [6] E.g. for strategy C: 51% HPV+ at t=0 × 6 months in triage + post-triage follow-up (7% × 13 months for CIN 0 + 17% × 15 months for CIN 1 + (14% + 5%) × 17 months for CIN 2/3).

Table 6. Results: Triage outcomes (referral rate, length of follow-up) after changes in assumptions on HPV prevalence and persistence, and CIN 2/3 prevalence. Sensitivity analysis.

	Strategy A			Strategy B			Strategy C			Strategy D		
	% Referred	Follow-up (months)	Total cost	% Referred	Follow-up (months)	Total cost	% Referred	Follow-up (months)	Total cost	% Referred	Follow-up (months)	Total cost
Baseline assumptions	51%	7.7	515	42%	12.4	472	42%	9.5	479	100%	12.9	740
Lower HPV prevalence[1]	40%	6.1	412	33%	11.0	383	33%	7.4	384	100%	12.9	740
Higher HPV prevalence[1]	60%	9.1	599	49%	13.5	544	49%	11.1	557	100%	12.9	740
Lower HPV persistence[2]	51%	7.7	515	31%	10.7	361	31%	7.8	369	100%	12.9	740
Higher HPV persistence[2]	51%	7.7	515	43%	12.6	487	43%	9.7	494	100%	12.9	740
Lower CIN 2/3 prevalence[3]	51%	7.6	474	42%	12.3	440	42%	9.4	448	100%	12.6	679
Higher CIN 2/3 prevalence[3]	51%	7.9	556	42%	12.6	508	42%	9.6	515	100%	13.9	882

[1] Baseline assumption: 51%. Range: 40%-60%. [2] Baseline assumption: 82%. Range: 60%-85%.
[3] Baseline assumption: 19% (all at t=0), 35% (HPV+ at t=0), and 44% (HPV+ at t=6). Range: 10%, 20%, 30% (low), 40%, 50%, 60% (high).

persistence rate is assumed (60% instead of 82%), strategy A would remain the most favourable in terms of the time needed to complete the total recommended follow-up. When the lower HPV persistence rate is assumed, strategy C could decrease the average time in follow-up to the same level as strategy A.

The proportion of women referred and treated, as well as the total cost, would remain the lowest under strategies B and C: the referral rate in the range of 31-49%, and the cost decrease compared to strategy D in the range of 33-51%. Our results are most affected by the changes in the assumptions on HPV prevalence and its persistence within 6 months (direct observations for the latter are not available). This is not surprising since these determine how many women will be ultimately referred for colposcopy.

DISCUSSION

Our analyses showed that compared to direct referral of women with two consecutive BMD smears to colposcopy based on HPV testing can prevent at least one out of two colposcopies and treatment in one of three women with at most low-grade lesions. It can decrease the average follow-up time by half a year, and reduce the associated average total costs by a third. In the population of 3,4 million women at risk (i.e., with a cervix) aged 30-60 in The Netherlands, around 8,700 annually have a BMD primary screening programme smear in the screening programme, of whom around 2,200 are referred due to BMD persistence[5]. Even if strategy A with the lowest expected cost decrease would be adopted instead of the currently recommended direct colposcopy (strategy D), total annual savings of close to €0.5 million could be attainable in this group of women in The Netherlands. BMD primary

smears outside of the screening programme account for roughly half of the BMD primary smears in The Netherlands [5], so savings could double if the recommendation for HPV triage would extend from smears within the screening programme to all smears. Savings per screened woman could be higher in areas where the proportion of BMD primary smears is higher (e.g. >5% in Finland and England compared to <2% in The Netherlands [1]).

None of the three analysed HPV strategies is optimal for both side effects and costs. Delaying the HPV testing by 6 months (strategy B) may have the lowest total costs (=-36% compared to direct colposcopy in strategy D) but for the combination strategy C the costs are only slightly higher. The risk selection is equally good for strategies B and C (both strategies establish HPV persistence before a referral), but the period of time in which women are kept in triage is on average 3 months shorter for strategy C. Strategy A eliminates the need for an extra triage period and extra GP visits, but it is less powerful in selecting women at higher risk for progression to cancer than strategies B and C.

The rationale for screening programmes is early detection and treatment of disease. In our analyses, we assumed equal effectiveness of each triage strategy, i.e. that there are not more cervical cancer deaths in the HPV strategies than in the direct colposcopy.

This assumption can be challenged for two reasons. First, in the HPV strategies only women with detectable HPV infections, i.e. women at risk for cancer, are referred for colposcopy. A recent meta-analysis estimated that 95.5% of all CIN 2+ lesions can be identified if women with primary ASCUS and LSIL smears (which approximately correspond to BMD smears) are tested for HPV [29]. Because at the time of histologic testing some women may have already cleared the HPV without yet having their lesion regress, or might still clear the HPV later, only (an unknown) part of the remaining 4.5% may represent potential loss of sensitivity in detecting CIN 2+ compared to direct colposcopy (strategy D).

On the other hand, recent data from the ALTS trial suggests that HPV testing may perform no worse than, or may even outperform colposcopy in identifying high-grade CIN lesions [30]. Follow-up data from the currently on-going randomised trials, e.g. that from the POBASCAM study expected shortly [31], will shed more light on the loss of sensitivity of HPV triage due to less frequent referral. Second, in The Netherlands the conventional guidelines of following up the BMD primary smear by a follow-up smear in 6 months implicitly accept the risk of postponing treatment to occult underlying cancers in 0.4 per 1000 women with BMD primary screening smears [5]. When HPV testing is delayed for another 6 months (i.e., to 12 months after

the BMD primary smear), the risk of diagnosing an invasive cancer is a further 0.9 case per 1000 women with a BMD primary screening smear [5]. Should strategies B or C be adopted, postponing referral would miss another six months of lead-time for treatment in these 0.9/1000 women. Given that these are screen-detected cancers, one may assume that despite this delay it is likely that they are still found at an early enough stage of invasion to retain the good 5-year prognosis of 90% survival [32]. To sum up, we can reasonably assume that the effectiveness of the three analysed HPV triage strategies and of the conventional strategy are comparable.

An alternative to the analysed triage strategies could be to drop the requirement of first establishing the persistence of the BMD smear and to instead triage solely through HPV, that is directly after one primary BMD smear. Per 1000 women with primary BMD smears, such an approach would expectedly increase the referral rate by 100, the CIN 2/3 detection rate by 10-15, and prevent 1-3.5 cancers (see Appendix). Given the very long (>10years) average duration of preinvasive lesions [33], some of these 1-3.5 per 1000 women will still have the chance to be managed early at the next screening round in less than 5 years. This balance is not straightforward, and remains uncertain. Again, the expected long-term follow-up data from randomised trials such as the POBASCAM study should in the near future help improve these estimates. Also, adding further markers to HPV-testing (e.g. typing for HPV 16 and 18, testing for mRNA) is currently under investigation as feasible ways to improve specificity, which would more likely favour HPV-testing above cytological triage.

This study has some limitations. First, some women who in the Erasmus MC trial had a persistent HPV+ CIN 2/3 lesion from t=0 to t=6 (this prompted treatment and censoring from further follow-up) could have cleared the HPV by t=12. Therefore the assumption we used to fit our interpolation model with – that women treated at t=6 would have remained in the same state until t=12 had they not been treated – may give an overestimate of HPV and CIN 2/3 persistence, and an overestimate of the referral rate and costs for strategies B and C. On the other hand, the quantification of strategies B and C is based on the data from a trial in which biopsies were taken at enrolment from all colposcopic abnormalities. Biopsies may have interfered with the development of the disease, e.g. by removing HPV-infected lesions which would not have cleared and/or regressed if left unbiopsied in the observed period. Consequently, the estimated 6-month HPV persistence rate may be too low, the CIN 2/3 prevalence rate at t=6 too low, and the expected decrease in the referral and total costs too high.

Though especially strategy C seems interesting for implementation, we are less certain exactly how well it would in reality perform relative to strategy A. Given

our assumptions, strategy C could avoid a referral to colposcopy in at most 9% more women than strategy A. The cost for this advantage is an extra GP visit and a 6-month longer triage period for 51% of women in strategy C. In the base-case calculation the extra GP visits in strategy C save 0.18 referrals and 0.07 treatments compared to strategy A. Strategy C would be preferred over strategy A only when it could be shown that the perceived burden of an extra GP visit and 6-month waiting time during triage will be less than 18% of that associated with a referral for colposcopy, and also less than 7% of that of treatment. The relative burden is thus far unknown but it could be studied in an implementation trial in which the 6-month natural history of HPV could be monitored for this group of women without early biopsy interference.

Second, because we wanted to study the optimal strategy to offer to women, we assumed compliance with follow-up in all strategies during the triage period. Lack of follow-up will decrease screening effectiveness. It has been shown in the literature that up to one third of the women do not comply with follow-up[34], and that the compliance decreases with longer time lags in recommended follow-up[35]. Since women in our study have already had to wait for 6 months for the follow-up smear, these findings are especially challenging for those strategies that involve extra prolonged periods of triage in which HPV is allowed to clear (strategies B and C). In The Netherlands, cervical cancer screening is performed at the primary health care level by general practitioners (GP). A large audit of GP practices in The Netherlands has shown that complex decision trees are important barriers to compliance of the GP with diagnostic guidelines[36]. All three HPV strategies increase the diagnostic complexity for the GP as they add to the currently routine practice a test of a different type, strategies B and C also at extra time points.

Third, women's preferences should play a role in optimising the triage strategies. In principle, triage through HPV could decrease anxiety in women with abnormal smears by decreasing the number of false-positive (i.e., HPV-negative) referrals. Still, follow-up of a large UK screening cohort showed that a negative HPV result does not significantly reassure women with a BMD smear[37]. Moreover, women who received a positive HPV report showed even higher anxiety levels after an abnormal smear[38].

Several surveys have shown that a significant proportion of women with cytologic abnormalities may prefer an early referral to wait-and-see approaches[39,40] and that a higher level of psychological distress in a woman is an important factor contributing to such a choice[41]. It seems then that it is especially the postponement of action in women who know that they tested HPV-positive in strategy C that could negatively affect their well-being, making strategy A more appealing to implement.

In conclusion, our analysis provides further evidence that HPV can improve the specificity of referral for colposcopy of women with persistent BMD smears, and decrease the burden on both the women and the health-care system. Given the established high sensitivity of HPV testing for progressive cervical neoplasia, we therefore advocate including HPV testing before referring women. For these women, who are already in follow-up for 6 months, analysis of additional aspects favours implementation of immediate HPV testing without waiting another 6 months for clearance.

APPENDIX: EXPECTED REFERRAL AND DETECTION RATES FOR HPV TRIAGE, WITH AND WITHOUT CYTOLOGY IN TRIAGE

Assume strategies A1, B1 and C1 such that relative to the primary BMD smear (found at t=-6) the timing of HPV testing remains equal as in strategies A, B and C but the cytologic triage (at t=0) is not done. As a consequence, HPV testing is done on all women with a BMD primary smear in strategies A1, B1 and C1, whereas it is only done on women with two consecutive BMD smears in strategies A, B and C.

The expected referral rates, expressed as the proportion of all women with a BMD primary smear, are:

- Strategy A: 18% (25% with BMD in follow-up[5] × 51% HPV-positive[7] + 5% with >BMD in follow-up[5]; Figure A1);
- Strategies B and C: 15% (25%[5] × 51%[7] × 77% (Table 1) + 5%[5]);
- Strategy A1: 28% (35% of the women with a BMD primary smear are HPV positive[31] × 81% HPV persistence rate in 6 months[42]; Figure A1); and

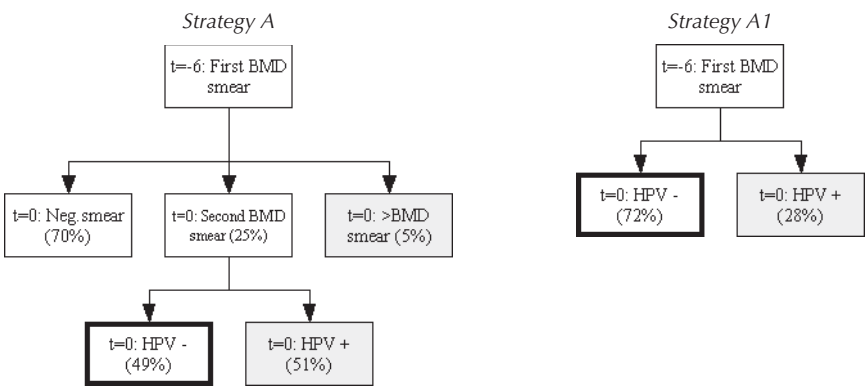

Figure A1. Calculation of expected referral rate in strategies A and A1, after a single BMD primary smear.

Note: Shaded rectangles represent women who need to be referred, whereas the rectangles with a bold border represent women for whom we assumed that they return to the regular screening programme.

- Strategies B1 and C1: 24-28% (35%[31] × [68% (18 months)[42] to 81% (6 months) HPV persistence rate[42]]).

The expected increase in the detection rate of CIN 2/3 lesions in strategies A1, B1 and C1 over those of strategies A, B and C is 13.5 per 1 000 women with a BMD primary smear ((95% - 80%) difference in sensitivity for CIN 2+ of HPV vs. cytology triage[29] × 9% prevalence of CIN 2/3 lesions in BMD primary smears[13] × 1000).

It has been estimated that 10-24% CIN 2/3 lesions eventually progress to cancer[25,43]. Therefore, substituting the combination of a follow-up smear and a HPV test for a stand-alone HPV test in women with BMD primary smears could prevent at most 1.4 to 3.2 ([10% to 24%] × 13.5) invasive cancers.

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Chapter 3.1

HPV testing and monitoring of women after treatment of CIN 3: Review of the literature and meta-analysis

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ABSTRACT

According to the current guidelines in most western countries, women treated for cervical intraepithelial neoplasia grade 3 (CIN 3) are followed for at least 2 years after treatment by cytology.

High-risk human papillomavirus (hrHPV) infections are necessary for the development and maintenance of CIN 3. HrHPV testing could be used to improve monitoring of women treated for CIN 3. This has prompted numerous studies for the implementation of hrHPV testing in monitoring of women treated for CIN 3.

Included in this review are twenty studies, published between 1996 and 2003 comparing hrHPV testing with either resection margins or cervical cytology to predict recurrent/residual disease, and 11 of them could be used in a meta-analysis.

In the meta-analysis of the 11 studies, the negative predictive value (NPV) for recurrent/residual disease of hrHPV testing was 98% (95% CI 97-99%), that of resection margins 91% (95% CI 87-94%) and that of cervical cytology 93% (95% CI 90-95%). When hrHPV testing was performed in conjunction with cytology, the sensitivity was 96% (95% CI 89-99%), specificity was 81% (95% CI 77-84%), the associated positive predictive value (PPV) was 46% (95% CI 38-54%) and the NPV was 99% (95% CI 98-100%). Combined hrHPV and cytology testing yielded the best test characteristics.

We propose to include hrHPV testing in conjunction with cytology for monitoring women treated for CIN 3. Some follow-up visits for women testing negative for both hrHPV and cytology can be skipped.

In western countries, this could mean that for women double negative at 6 months, retesting at 12 months should be skipped while keeping the 24-month follow-up visit.

INTRODUCTION

For over 10 years, it has been recognized that persistent high-risk human papillomavirus (hrHPV) infections are necessary for the development and maintenance of cervical intraepithelial neoplasia grade 3 (CIN 3), the most severe precursor lesion of cervical cancer, and for progression of CIN 3 to cervical cancer[1,2]. Awareness of the role of hrHPV has raised the possibility of using HPV testing in a number of clinical settings. The most important are the use of hrHPV testing in the triage of women with borderline or mild dysplasia (BMD) for colposcopically-directed biopsy and improvement of monitoring women for recurrent or residual disease after treatment of CIN 3[3-26].

This review will focus on addressing the utility of hrHPV testing in monitoring women after treatment of CIN 3.

Monitoring after treatment for CIN 3

Women with histologically-confirmed CIN 3 are treated to prevent the development of cervical cancer[27,28]. The treatment objective is to remove or destroy the abnormal epithelium of the transformation zone, the area of the cervix where most CIN lesions are located. Treatment modalities include Large Loop Excision of the Transformation Zone (LLETZ) or Loop Electrosurgical Excision Procedure (LEEP), cryocoagulation, laser evaporation, laser excision or cone biopsy.

In The Netherlands, women are monitored by cervical cytology at 6, 12 and 24 months, and, if necessary, yearly thereafter until three consecutive smears are read as normal[29]. In case of abnormal follow up cytology, re-treatment follows according to standard procedures. Reported treatment failure rates of high-grade CIN lesions vary between 5% and 25%[30-33].

In the United States, the American Society for Colposcopy and Cervical Pathology (ASCCP) published guidelines in 2001 which acknowledged there were no prospective randomized trials which have evaluate follow-up protocols[34]. They suggested that commonly used surveillance protocols after treatment for CIN 2/3 involved repeat cytology at 4-6 month intervals for up to 2 years and yearly thereafter. Prolonged follow up was recommended since late reoccurrences have been reported. It was noted that simultaneous colposcopy combined with cytology adds very minimally to the detection rate of persistent or recurrent CIN. The authors also point out that HPV testing has shown promise for surveillance and "unless a patient has risk factors for recurrent/persistent CIN such as a large lesion or endocervical extension, it would seem reasonable to perform HPV testing at 12 months after treatment."

HPV detection methods most frequently used for clinical use

HPV detection techniques depend on nucleic acid hybridization since HPV cannot be cultured *in vitro*. The tests most often used are the commercially available Hybrid Capture II (HC II) and polymerase chain reaction (PCR) methods using consensus primers. The HC II test is based on direct HPV DNA detection by hybridization of HPV target DNA with a cocktail of full-length HPV type specific RNAs, followed by capturing the hybrids to a solid phase. The following signal amplification is achieved by binding of multiple conjugated antibodies to the hybrids that specifically recognize DNA/RNA hybrids.

The HPV types detected by HC II include the low-risk types 6, 11, 42, 43 and 44 (5 types) and the high-risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (13 types)[35]. The HC II test includes more HPV types than the preceding HC I test, which improved the sensitivity of the HC testing method[35]. The PCR methods are based on amplification of HPV target DNA. Basically, two different methods of PCR amplification of HPV DNA can be used. First, type specific PCRs can be used. In this case, multiple PCRs are necessary to detect multiple HPV types using sets of type specific DNA primers. Second, consensus primers can be used. In this case, multiple HPV types are detected by a single or a limited set of DNA primers. This last approach has been explored extensively, as the L1 open reading frames of genital HPV genotypes is sufficiently conserved to detect the relevant HPV types using a single pair of primers. The most frequently used systems are the GP5+/6+ PCR (a single pair of primers), the MY09/11 PCR (5 primers) and the SPF1/2 system (6 primers)[36-38].

In a recent review by Snijders et al., it was shown that the analytical sensitivity of the SPF1/2 system is higher than that of the GP5+/6+ PCR and HC II, which actually results in a much lower clinical specificity for detecting CIN 3 because the SPF1/2 system detects more high-risk HPV in cervical smears than the other tests[39]. The negative predictive values of the HC II test, GP5+/6+ and SPF1/2 tests for CIN 3 were equally high. Therefore the GP5+/6+, MY09/11 and HC II test will have fewer positive test results than the SPF1/2 test while not missing CIN 3 lesions, making these tests more clinically useful for monitoring women treated for CIN 3. Many read out systems have been described for these assays, and enzyme immunoassays (e.g., EIA or DEIA) using type-specific oligoprobes, either individually or in a cocktail, or reverse line blot assay (e.g., LiPA or RLB) are commonly used[38,40-43]. The methods mentioned above have the potency to detect all relevant hrHPV types by adapting the composition of oligoprobes, and these methods have been applied with different numbers of probes (varying from 14 to 37).

MATERIAL AND METHODS

For the purpose of this review, and in line with current views, abnormal cytology is considered to be present if a cervical smear is classified as borderline dysplasia or worse. For the histological classification of premalignant cervical lesions or cervical intraepithelial neoplasia (CIN), the terms mild dysplasia (CIN 1), moderate dysplasia (CIN 2), severe dysplasia and carcinoma in situ (CIN 3) are used. In the studies reviewed, cervical carcinoma originating from the surface epithelium is limited to histopathologically-diagnosed squamous cell carcinoma, adenocarcinoma, and adenosquamous carcinoma of the cervix.

The English language medical literature between 1996-2003 was searched in MEDLINE, using the following Medical Subject Headings: *'HPV testing and CIN lesions', 'HPV testing and post-treatment', 'HPV testing and cervical dysplasia', 'HPV testing and squamous intraepithelial neoplasia and treatment'* and *'HPV infection and '* and *'HPV testing and CIN treatment'*. Additional potentially relevant studies were identified from the references of identified studies. No attempt was made to identify unpublished studies or studies only presented in meeting abstracts.

By the MEDLINE search, 1110 papers were identified. Only the reports that compared hrHPV testing to either resection margins or cervical cytology to predict recurrent/residual disease were included, and an overview of these 20 papers is presented in Table 1 [7-26]. In this table, we also included reason(s) why some of the studies were considered not to be suitable for further analysis in this review. Reasons to exclude studies from further analysis and the meta-analysis include: HPV testing not performed on a cervical smear, no histological confirmation of recurrent/residual disease, but also missing data about cytology results, initial resection margins or recurrent/residual disease after treatment and studies in which recurrent/residual disease does not occur during follow-up.

For the comparison of the performance of the different tests to predict recurrent/residual disease, 12 articles were left (Table 2) [7,8,13,14,16-18,20,21,24-26]. The data from these 12 articles concerning initial resection margins status and cervical cytology and HPV to predict recurrent/residual disease are presented in Table 2. Also an estimate of test properties such as sensitivity, specificity, positive and negative predictive value, positive and negative likelihood ratios is given in this table. Among these 12 articles, there were 2 case-control studies, and since the NPV and PPV are dependent of the prevalence, the NPV and PPV had to be corrected. Because of supposed overlap between the studies of Jain, et al. and Lin, et al., these studies were not included in the same analysis [14,16]. Therefore, 9 remaining prospective/cohort studies were used to estimate the presence (Pcase) and absence (Pcontrol) of recurrent/residual disease in the population for the correction of NPV and PPV. In

Table 1. Prospective clinical studies comparing hrHPV testing post treatment of CIN with initial resection margins and/or cervical cytology.

Reference, year of publication	Technique	HPV types	Study design
Elfgren[7] 1996	GP 5+/6+ PCR	6,11,16,18, 31,33	Prospective study with a follow up of at least 27 months, including 23 women (mean age 35.8 years, range 20-51) treated by conization for CIN 2-3. Recurrent/residual disease was predicted from cytology and HPV testing 16 months after treatment.
Chua[8] 1997	MY 09/11, GP 5+/6+ PCR on archival smears	16,18,X	Retrospective case-control study, including 26 case women (38.2 ± 10.7 years of age) with recurrent/residual CIN 2-3 and 22 consecutive control women (33.5 ± 9 years of age) without recurrent/residual disease for more than 46 months after treatment for CIN 3 by conization. Recurrent/residual disease was predicted from conus excision margins and from cytology and HPV testing 3 months after treatment.
Strand[9] 1997	GP 5+/6+ PCR	6,11,16,18, 31,33,35,45, 51,52,56,58	Prospective case-control study with a follow-up time of up to 12 months, including 82 cases treated for an HPV infection by local podophyllotoxin 0.5% and CO_2 for persistent lesions (mean age 24 years range 17-38 years) and a reference group of 30 women treated by laser for CIN 1-3 (mean age 33 years range 20-59 years). The first follow-up was 6-12 months after treatment. This study is not included in our analysis, because no information is available about post-treatment histologically confirmed recurrent/residual disease.
Bollen[10] 1999	CP I/II G PCR	6,16,18,31, 33,35,45,51, 54,64,58, 68,X	Prospective study with a mean follow up of 48 months (range 13-206 months), selecting 43 consecutive women (mean age of 31 years range 17-46 years) treated by different techniques for CIN 1-3, who had abnormal cytology after treatment. The first follow up by cytology and HPV testing was performed 6-11 months after treatment. Endpoint of the study was histologically confirmed CIN 1-3. This study is not included in our analysis, because only women with abnormal cytology after treatment had been selected.
Izumi[11] 2000	PCR	16,18,31,33, 35,52b,58	Prospective study, including 80 women (age not specified) treated by laser ablation for CIN 1-3 and cervical cancer. Cytology and HPV testing was performed at 2, 4 and 12 weeks and every 6 months after treatment (mean follow-up time is not specified). This study is not included in our analysis, because no recurrent/residual disease occurred during follow up.
Kjellberg[12] 2000	MY 09/11, GP 5+/6+ PCR	11,16,18, 33,X	Prospective study with a mean follow up of 35.4 months (range 22-46 months), including 112 women (mean age of 37.7 years range 19-58 years) treated by CO_2 laser excision or evaporation for CIN 1-3. The first follow up for cytology and HPV testing was 4 months after treatment if margins were positive and 1 year after treatment if margins were negative. This study is not included in our analysis, because recurrent/residual disease as detected in cervical smears was not histologically confirmed.
Nagai[13] 2000	PCR type specific	6,11,16,18, 31,33,35, 52,58	Prospective study with a mean follow up of 31.8 months (range 12-73 months), including 58 women (mean age 38.7 years, range 23-28) treated by conization for CIN 3 and immediately followed by endocervical curettage (ECC) if colposcopy was unsatisfactory. Recurrent/residual disease was predicted from cytology and HPV testing 6 months after treatment.

Table 1 Continued. Prospective clinical studies comparing hrHPV testing post treatment of CIN with initial resection margins and/or cervical cytology.

Reference, year of publication	Technique	HPV types	Study design
Jain[14] 2001	HC II	High-risk*	Prospective study, including 79 women (age not specified) treated for CIN 3 by LLETZ/conization resulting in a one-piece cone specimen and immediately followed by endocervical curettage (ECC). The women had completed their family and agreed with hysterectomy 2 months after conization as end-point of the study. Residual disease (CIN 1 or worse) was predicted from conus excision margins including positive ECC samples and from HPV testing 6 weeks after treatment.
Kucera[15] 2001	HC II	High-risk*	Prospective follow-up study with a follow up of 12 months, including 142 women (median age 33 years, range 17-68) treated by LLETZ for CIN 1-3. This study is not included in our analysis, because there is no information about resection margins or cytology after treatment.
Lin[16] 2001	HC II	High-risk*	Prospective study, including 75 women (mean age 54.1 years, range 31-82), who had been treated for CIN 3 by LLETZ or cold knife conization and endocervical curettage above the site of cone excision, and who have elected to undergo hysterectomy 2-7 weeks after conization as final treatment because of positive results on conization margins and/or endocervical curettage. In addition, women who had coexisting benign indications for a hysterectomy and those who were over 50 years of age were also included. Cytology and HPV testing was done immediately before hysterectomy. Recurrent/residual disease (CIN 1 or worse) was predicted from conus excision margins and HPV testing 3-6 weeks after initial treatment and before hysterectomy.
Nobbenhuis[17] 2001	GP 5+/6+ PCR	High-risk*,66	Prospective observational study with 2 years of follow up, including 184 women (mean age 34 years, range 21-70), who had been treated for CIN 2-3 by LLETZ or cold knife conization. Cytology and HPV testing was performed 3, 6, 12 and 24 months after treatment. Recurrent/residual disease (CIN 2 or worse) was predicted from cytology and HPV testing 6 months after treatment.
Paraskevaides[18] 2001	GP5+/6+ PCR on archival smears	16,18,31,33	Retrospective case-control study, including 41 case women (40.3 ± 5.6 years of age) with recurrent/residual CIN 1-3 or cancer and 82 randomly selected control women (31.5 ± 5.8 years of age) without recurrent/residual disease for at least 5 years of follow up after treatment for CIN 1-3 by LLETZ. Recurrent/ residual disease was predicted from conus excision margins and from cytology and HPV testing 4.2 months after treatment.
Acladious[19] 2002	GP 5+/6+ PCR	6,11,16,18, 31,33,52, 58,X	Prospective nested case-control study with a follow-up time of up to 2 years, including 77 cases with histologically confirmed treatment failure (median age 27 years, IQ range 9 years) and 154 controls (median age 27 years) with 2 consecutive normal smears after treatment, by different techniques for CIN 1-3. Post-treatment HPV results were available for 47 cases and 69 controls. Endpoint of the study was histologically confirmed CIN. This study is not included in our analysis, because information about post-treatment cervical cytology results or resection margins is either not available or incomplete.

Table 1 Continued. Prospective clinical studies comparing hrHPV testing post treatment of CIN with initial resection margins and/or cervical cytology.

Reference, year of publication	Technique	HPV types	Study design
Bekkers[20] 2002	SPF PCR type specific	High-risk*, 53,54	Prospective study with a median follow up of 32 months, including 90 women (age not specified) who had been treated for CIN 2-3 by conization. Cytology and HPV testing was performed 3 and 6 months after treatment. Endpoint of the study was histologically confirmed CIN 2-3. Recurrent/residual disease was predicted from cytology and HPV testing 6 months after treatment.
Bodner[21] 2002	HC I	6,11,42-44, 16, 18, 31, 33, 35, 45, 51, 52, 56	Prospective study with 2 years of follow up, including 37 women (median age 34 years, range 23-56) treated for CIN 2-3 by conization. Recurrent/residual disease (not specified) was predicted from re-conization or hysterectomy specimens and HPV testing 3 months after treatment.
Cruisshank[22] 2002	PCR type specific	16,18	Retrospective nested case-control study with a follow up of 6 months, including 107 case women with a biopsy proven recurrence (CIN 2-cervical cancer) and 98 control women (median age of all women 31 years, range 19-49 years) without recurrent/residual disease after treatment of CIN 3 by laser ablation or LLETZ. HPV testing was performed on archival smears taken 6 months after treatment. This study is not included in our analysis, because no information is available about post-treatment cervical cytology results or resection margins.
Elfgren[23] 2002	GP 5+/6+ PCR	High-risk*, 66	Prospective study with a mean follow-up time of 15.7 months, including 109 women (mean age 32.5 years, 20-71 years) treated by cryotherapy or conization for CIN 1-3 and microinvasive cancer. The first follow up was 3 months after treatment, followed by cytology and HPV testing at 6, 9, 12 and 24 months. Endpoint of the study was clearance of the HPV infection. This study is not included in our analysis, because no information is available about post-treatment histologically confirmed recurrent/residual disease.
Bar-Am[24] 2003	HC I	6,11,42-44, 16, 18, 31, 33, 35, 45, 51, 52, 56	Prospective study with a follow-up time of 5 years, including 67 women (median age 34 years, range 23-56 years) treated by LETZ for CIN 2-3. The first follow up was at 6 months intervals during the first 3 years and then annually for another 2 years after treatment. Endpoint of the study was recurrent/residual disease. This study is not included in our analysis, because no information is available about post-treatment histologically confirmed recurrent/residual disease in relation to cytology or HPV of all 67 women included in the study.
Houfflin[25] 2003	HC II	High-risk*	Prospective study with an average follow-up time of 14.9 months, including 205 women (age range 17-69 years) treated by LEEP for CIN 2-3. The first follow-up visit was 6 weeks after treatment, followed by cytology and HPV testing at 3 months and thereafter with 6 months intervals. Endpoint of the study was recurrent/residual disease. This study is included in our analysis.
Zielinski[26] 2003	HC II	High-risk*	Prospective study with a median follow-up time of 29 months (range 2-65 months), including 108 women (median age 35 years, range 23-56 years) treated by cone biopsy or LETZ for CIN 3. Follow-up visits were scheduled at 3, 6, 12 and 24 months after treatment, and yearly thereafter until 3 serial visits with normal cytology. Endpoint of the study was recurrent/residual disease. This study is included in our analysis.

* High-risk HPV types includes HPV types 16, 18 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68.

Table 2. Parameters (margins, cytology and hrHPV testing) predicting recurrent/residual disease

Reference	Parameter	Cases/ test positive	Cases/ test negative	Sens (%)	Spec (%)	PPV (%)	NPV (%)	LR+	LR-
Chua [8]	Margins	13/15	13/33	50	91	49*	91*	5.5	0.6
1997	Cytology	13/15	13/33	50	91	49*	91*	5.5	0.6
	HPV	24/24	2/24	92	100	100*	99*	∞	0.08
Jain [14]	Margins	31/78	1/33	97	41	40	97	1.6	0.08
2001	Cytology	26/32	5/15	84	63	81	67	2.2	0.3
	HPV	32/76	0/35	100	44	42	100	1.7	0
Lin [16]	Margins	23/63	4/12	85	17	37	67	1.0	0.9
2001	HPV	27/52	0/23	100	48	52	100	1.9	0
Paraskevaidis [18]	Margins	16/34	25/89	39	78	24*	88*	1.8	0.8
2001	Cytology	20/31	21/92	49	87	39*	91*	3.6	0.6
	HPV	38/51	3/72	93	84	51*	98*	5.8	0.09
Bodner [21]	Margins	3/6	0/31	100	91	50	100	11	0
2002	HPV	3/10	0/27	100	79	30	100	4.9	0
Houfflin [25]	Margins	22/74	9/131	71	70	30	93	2.4	0.4
2003	HPV	26/71	5/134	84	74	37	96	3.2	0.2
Elfgren [7]	Cytology	3/3	1/19	75	100	100	95	∞	0.3
1996	HPV	4/4	0/18	100	100	100	100	∞	0
Nagai [13]	Cytology	4/4	1/54	80	100	100	98	∞	0.2
2000	HPV	5/11	0/47	100	89	45	100	8.8	0
Nobbenhuis [17]	Cytology	18/32	11/152	62	91	56	93	6.9	0.4
2001	HPV	26/39	3/145	90	92	67	98	11	0.1
Bekkers [20]	Cytology(2x)	10/13	0/77	100	96	77	100	27	0
2002	HPV (1x)	7/34	3/56	70	66	21	95	2.1	0.5
Bar-Am [24]	Cytology	8/25	1/42	89	71	32	98	3.0	0.2
2003	HPV	9/27	0/40	100	69	33	100	3.2	0
Zielinski [26]	Cytology	5/18	1/90	83	87	28	99	6.5	0.2
2003	HPV	5/23	1/85	83	82	22	99	4.7	0.2

* The PPV and NPV for the case-control studies were corrected at a prevalence of recurrent/residual disease of 15%.

the 9 prospective/cohort studies, the prevalence of recurrent/residual disease was about 15% (124/846) [7,13,16,17,20,21,24-26]. Therefore, for the 2 case-control studies, the PPV and NPV were corrected, according to Baye's theorama with PPV = (sensitivity x Pcase)/(sensitivity x Pcase + (1 – specificity) x (1 – Pcase)) and NPV = (specificity x Pcontrol)/(specificity x Pcontrol + (1 – sensitivity) x (1 – Pcontrol)) with a Pcase of 15% and a Pcontrol of 85% (722/846) (44).

Sensitivity is defined as the proportion of cases with disease with a positive test result, specificity is the proportion of cases without disease with a negative test result, positive predictive value (PPV) is the proportion of cases with a positive test result who have disease and negative predictive value (NPV) is the proportion of cases with a negative test result who are free of disease[44]. The likelihood ratio for a positive result (LR+) indicates the odds of the disease increase when a test is positive. The likelihood ratio for a negative result (LR-) indicates the odds of the disease decrease when a test is negative.

Meta-analysis

Three meta-analysis were performed. In these meta-analyses, the study of Jain, et al. was excluded if the study of Lin, et al. was included, because the studies of Lin, et al. end Jain, et al. were performed in the same institution and their data might overlap[14,16]. Since the negative and positive predictive values cannot be calculated directly the case-control studies by Chua, et al. and Pareskevaides, et al., the first meta-analysis (Table 3A) consists of 9 prospective studies, whereas in the second meta-analysis the case-control studies of Chua, et al. and Pareskevaides, et al. were included.

The third meta-analysis concerns combinations of two tests to predict recurrent/residual disease. In the second and third meta-analysis, the NPV and PPV have been adapted according to Baye's theorama with the same Pcase of 15% and Pcontrol of 85% as used before (Table 3B and 3C)[44].

RESULTS AND DISCUSSION

Failure of treatment for CIN 3 has been reported to vary between 5 and 25%. For this reason, after treatment of CIN 3 all these women are kept under close gynaecological surveillance. This is rather inefficient because the majority of them will not have recurrent/residual disease[30-33]. If a test was available that could identify the majority of women not at risk of recurrent/residual disease, more efficient surveillance schemes could be designed.

Various studies have addressed the issue of how to select women not at risk for recurrent/residual disease from parameters such as the status of cervical resection margins at initial treatment and cervical cytology and hrHPV testing post-treatment[7-23,33]. In this review, we compared the accuracy to predict recurrent/residual disease from these parameters (see Material and Methods; Table 1). For the comparison of the performance of the different tests to predict recurrent/residual disease 12 articles were left (Table 2)[7,8,13,14,16-18,20,21,24-26]. The data from these 12 articles are pre-

Table 3A. Parameters (margins, cytology and hrHPV testing) predicting recurrent/residual disease, excluding the case-control studies of Chua et al. and Pareskevaides, et al.

Reference*	Parameter	Cases/test positive	Cases/test negative	Sens (%) (95% CI)	Spec (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	LR+ (95% CI)	LR- (95% CI)
Lin[16], Bodner[21], Houfflin[25]	Margins	48/143	13/174	79 (66-88)	63 (57-69)	34 (26-42)	93 (87-96)	2.1 (1.7-2.6)	0.3 (0.2-0.6)
Elfgren[7], Nobbenhuis[17], Bekkers[20], Nagai[13], Bar-Am[24], Zielinski[26]	Cytology	48/95	15/434	76 (64-86)	90 (87-92)	51 (40-61)	97 (94-98)	7.6 (5.6-10.2)	0.3 (0.17-0.41)
Nagai[13], Lin[16], Nobbenhuis[17], Bekkers[20], Elfgren[7], Bodner[21], Houfflin[25], Bar-Am[24], Zielinski[26]	HPV	112/271	12/575	90 (83-95)	78 (75-81)	41 (35-47)	98 (96-99)	4.1 (3.5-4.8)	0.1 (0.07-0.2)

* The study of Jain, et al.[14] was excluded from the meta-analysis if the study of Lin, et al. was included, because of supposed overlap between these two studies (14;16).

Table 3B. Parameters (margins, cytology and hrHPV testing) predicting recurrent/residual disease, including the case-control studies of Chua et al. and Pareskevaides et al. with corrections

Reference*	Parameter	Cases/test positive	Cases/test negative	Sens (%) (95% CI)	Spec (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	LR+ (95% CI)	LR- (95% CI)
Chua[8], Lin[16], Pareskevaides[18], Bodner (21), Houfflin (25)	Margins	77/192	51/296	60 (51-69)	68 (63-73)	25** (19-32)	91** (87-94)	1.9 (1.5-2.3)	0.6 (0.5-0.7)
Elfgren[7], Chua[8], Nagai[13], Nobbenhuis[17], Pareskevaides[18], Bekkers[20], Bar-Am[24], Zielinski[26]	Cytology	81/141	49/559	62 (53-71)	89 (87-92)	51** (42-60)	93** (90-95)	5.9 (4.5-7.8)	0.4 (0.3-0.5)
Elfgren[7], Chua[8], Nagai[13], Lin[16], Nobbenhuis[17], Pareskevaides[18], Bekkers[20], Bodner[21], Houfflin[25], Bar-Am[24], Zielinski[26]	HPV	174/346	17/671	91 (86-95)	79 (76-82)	44** (38-49)	98** (97-99)	4.4 (3.8-5.0)	0.1 (0.07-0.2)

* The study of Jain, et al.[14] was excluded from the meta-analysis if the study of Lin, et al. was included, because of supposed overlap with the study of Lin et al.[14,16]

** Since case-control studies (Chua, et al. and Paresevaides, et al.) are included, the PPV and NPV were corrected at a prevalence of recurrent/residual disease of 15%.

Table 3C. Meta-analysis for combinations of cytology and resection margins, hrHPV and resection margins and hrHPV and cytology predicting recurrent/residual disease, including the case-control study of Pareskevaides et al. with corrections

Reference	Parameter	Cases/test positive	Cases/test negative	Sens (%) (95% CI)	Spec (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	LR+ (95% CI)	LR- (95% CI)
Chua[8], Paraskevaides (18)	Cytology and resection margins	45/75	22/96	67 (54-78)	71 (61-79)	29* (18-42)	92* (85-96)	2.3 (1.6-3.3)	0.5 (0.3-0.7)
Jain[14], Paraskevaides[18], Bodner[21]	HPV testing and resection margins	74/163	2/108	97 (90-99)	54 (47-61)	27* (21-36)	99* (95-100)	2.1 (1.8-2.5)	0.05 (0.01-0.2)
Elfgren[7], Nagai[13], Nobbenhuis[17], Paraskevaides[18], Bar-Am[24], Zielinski[26]	HPV testing and cytology	90/181	4/381	96 (89-99)	81 (77-84)	46* (38-54)	99* (98-100)	4.9 (4.1-5.9)	0.05 (0.02-0.1)

* Since a case-control study is included, the PPV and NPV were corrected at a prevalence of recurrent/residual disease of 15%.

sented in Table 2. The test properties of the 2 case-control studies were computed on a prevalence (Pcase) of 15% and a Pcontrol of 85%[8,18].

Resection margins versus cervical cytology post-treatment

The negative predictive value of free resection margins and cervical cytology could be compared in three studies[8,14,18]. In the studies of Chua, et al. and Paraskevaidis, et al. the negative predictive values of resection margins (91% and 88%, respectively) were comparable to those of cervical cytology (91% and 91%, respectively). In the study of Jain, et al., the negative predictive value of resection margins was 97% and that of cervical cytology was 67%. In this study, the negative predictive value for resection margins was considerably higher (97%) than that of the other two studies mentioned earlier[8,14,18]. This discrepancy between the first 2 studies and the latter might be explained by the addition of endocervical curettage to the resection margins in the study by Jain, et al.[14].

Resection margins versus HPV testing pos-treatment

Six studies were found in which the negative predictive values of resection margins and hrHPV testing could be compared[8,14,16,18,21,25]. In the studies of Chua, et al., Lin, et al. and Pareskevaidis et al.,the negative predictive values of resection margins (91%, 67% and 88%, respectively) were considerably lower than those of hrHPV testing post-treatment (99%, 100% and 98%, respectively)[8,6,18]. In contrast, in the study of Bodner, et al., Jain, et al. and Houfflin, et al., the negative predictive value of resection margins (100%, 97% and 93%) and hrHPV testing (100%, 100% and 96%) did not differ (14, 21, 25). The small difference between the NPV of resection margins and HPV detection in the study by Bodner, et al. can be explained by the small number of women included in the study[3].

Cervical cytology versus HPV testing posttreatment

Nine studies were found in which the negative predictive values of cervical cytology and hrHPV testing could be compared[7,8,13,14,17,18,20,24,26]. In the studies of Nagai, et al., Bar-Am and Zielinski et al., the negative predictive values of cytology (98%, 98% and 99%, respectively) was somewhat lower than that of hrHPV testing (100%, 100% and 99% respectively)[13,24,26]. In the study of Bekkers, et al., the NPV of cytology (100%) was slightly higher than that of hrHPV testing which can be explained because they compared two serial cytology tests versus a single hrHPV test (95%)[20]. In the remaining studies, the negative predictive values of hrHPV testing to predict recurrent/residual disease was higher than that of cervical cytology[7,8,14,17,18]. Therefore, hrHPV testing could improve selection of women not at risk for recurrent/residual disease.

Meta-analysis

In the first meta-analysis with the results of the 9 prospective studies taken together, the prevalence of recurrent/residual disease was 15%. The negative predictive value for recurrent/residual disease post-treatment for hrHPV testing (98%, 95% CI 96-99%) was found to be higher than that of resection margins status (93%, 95% CI 87-96%) and cervical cytology (97%, 95% CI 94-98%) (Table 3A)[7,13,16,17,20,21,24-26]. The difference in test performance was even more evident when all 11 studies were included in the second meta-analysis, with a negative predictive value for recurrent/residual disease for hrHPV testing of 98% (95% CI 97-99%), compared to an NPV for initial free resection margins of 91% (95%, CI 87-94%) and that of cervical cytology of 93% (95%, CI 90-95%) (Table 3B)[7,8,13,16-18,20,21,24-26].

In the meta-analysis of combined testing for cytology and free resection margins post-treatment, the negative predictive value was low (92%, 95% CI 85-96%), when compared to that of combined testing for hrHPV and cytology (99%, 95% CI 98-100%) or hrHPV and resection margins (99%, 95% CI 95-100%). Even though the sensitivities of combined testing for hrHPV testing and resection margins (97%, 95% CI 90-99%) or cytology (96%, 95% CI 89-99%) were comparable, the specificity of combined hrHPV testing and cervical cytology post-treatment (81%, 95% CI 77-84%%) was much higher than that of hrHPV testing and resection margins (54%, 95% CI 47-61%). Therefore, combined testing of hrHPV and cytology is the best test combination to monitor women post-treatment.

Overall conclusions

This review confirms the advantages of combined cytology and HPV testing for follow up. The combined tests increase the sensitivity of detecting persistent or recurrent CIN and increase the negative predictive value which identified women at little or no risk for persistence or recurrence. Combined cytology and HPV testing proved more effective than either test alone or resection margin status.

Based on the analysis and our own studies[26], we recommend monitoring women treated for CIN 3 with cervical cytology and HPV testing at 6 months. Women testing positive by cytology and/or HPV testing should be referred for colposcopy and kept under close gynaecological surveillance since they are at high risk for residual/recurrent CIN. However, 70% of women treated for CIN 3 will have both negative cytology and a negative HPV tests at 6 months follow up. Because these women have no significant risk of persistent or recurrent CIN, they can omit the 12-month follow-up visit.

We recommend a 24-month examination with both HPV testing and cytology since these women are at high risk to re-acquire a high-risk HPV infection[45]. If both tests are negative at 24 months, they may be referred to the routine screening program.

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Chapter 3.2

Post treatment CIN: Randomised Clinical Trial using hrHPV-testing for prediction of residual/recurrent disease

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Submitted

ABSTRACT

Objectives To determine whether addition of hrHPV-testing (high-risk Human Papilloma Virus) to cytological follow-up after treatment for high-grade CIN (Cervical Intraepithelial Neoplasia 2/3) leads to a better selection of women at risk for residual/recurrent CIN.
Design Randomised Clinical Trial
Setting Outpatient clinics of three hospitals in The Netherlands
Patients 210 women with high-grade CIN undergoing treatment
Intervention Follow-up based on cytology alone and cytology combined with hrHPV detection
Main outcome measures Primary: improving specificity for residual/recurrent CIN after treatment. Secondary: health-care costs, impact of individual hrHPV type on the risk of residual/recurrent CIN.
Results Follow-up by abnormal cytology alone (6,12 and 24 months after treatment according to the Dutch protocol) showed a lower specificity for detection of residual/recurrent CIN than follow-up by abnormal cytology and presence of hrHPV (80% vs. 91%, relative risk 0.87 (95% CI 0.77 to 0.99)). Both methods showed no significant difference in sensitivity (86% vs. 100%) relative risk 0.86 (95% CI 0.63 to 1.16)). Comparing different post hoc modifications in the strategy of combined testing showed similar test characteristics when low-risk women (normal cytology and hrHPV negative at 6 months) omitted the 12 months visit (specificity 95%, p=1.00 z=0.00). Typing of hrHPV had no value for predicting development of residual/recurrent CIN. Total health-care costs using cytology and hrHPV testing during follow-up decrease when low-risk women omit the 12 months visit.
Conclusion Follow-up after treatment for high-grade CIN can be improved by combining cytology with hrHPV testing. We advise combined cytology and hrHPV testing at 6, 12 and 24 months after treatment. Low-risk women may omit the 12 months visit, resulting in cost reduction.
Trial registration The Dutch Cochrane Center ISRCTN31244687

INTRODUCTION

According to current Dutch standards follow-up after treatment for high-grade CIN (Cervical Intraepithelial Neoplasia 2/3) consists of cervical cytological monitoring at 6, 12 and 24 months, to identify residual/recurrent CIN lesions (CIN 2/3)[1,2]. Colposcopic examination is performed where there is abnormal cervical cytology. One of the drawbacks of cervical cytological follow-up after treatment is the high number of false-positive findings. Approximately 20% of the women have abnormal cervical cytology within the first two years of follow up. However in more than half of these women no underlying residual/recurrent CIN will be found, resulting in unnecessary diagnostic and therapeutic procedures[3-7]. The reported residual/recurrent high-grade CIN rates vary between 5% and 35%[3,4,8-10]. A persistent infection with high risk Human Papilloma Virus (hrHPV) is necessary for the development, maintenance and progression of primary CIN lesions[11-13]. It is assumed that effective treatment for CIN lesions results in the eradication of the hrHPV infection, while in residual/recurrent CIN disease hrHPV infection is still present[5,9,10,14].

Currently, only cytology is used in the follow-up after treatment. Previous studies have established the value of hrHPV testing for detection of high-grade CIN lesions[9,5,15-17]. The use of combined testing by cytology and hrHPV can lead to a better selection of women at risk for residual/recurrent CIN after initial treatment for high-grade CIN lesions. This has been confirmed in several retrospective and observational studies[3,9,14,18-23].

This selection results in diagnostic procedures being performed only in those patients who have the necessary risk factor present for the development of recurrent/residual CIN lesions, and avoids unnecessary diagnostic procedures in patients without this risk factor. Consequently, this policy can lead to an important reduction in health costs and anxiety. We investigated in a randomised clinical trial the role of hrHPV testing in addition to cytology during follow-up of post treatment CIN.

MATERIALS AND METHODS

Women who were to be treated for high-grade CIN lesions were asked to participate in the study. From July 2002 till September 2004 patients were enrolled at the outpatient clinic of the department of Gynaecology and Obstetrics of the Erasmus University Medical Center Rotterdam, the VU University Medical Centre Amsterdam and the Albert Schweitzer Hospital in Dordrecht. Exclusion criteria were previous treatment for high-grade CIN, immune compromising conditions, or previous or current cancer.

At baseline women were asked to complete a questionnaire on education, smoking, number of sexual partners and history of sexually transmitted diseases. Before treatment a cervical scrape was taken for hrHPV detection in all study participants. Treatment was performed according to standard methods such as: loop excision of the transformation zone (LETZ), cold-knife-exconisation or laser-exconisation. All treatment was performed by experienced gynaecologists. After treatment patients were randomised by computer in two groups: A, follow-up by cytology and B, follow-up by cytology and hrHPV testing at 6, 12 and 24 months after treatment.

Colposcopic examination was performed in group A if cervical cytology was abnormal (according to the standard Dutch protocol) and in group B if abnormal cytology was accompanied by a positive hrHPV-test.

At the end of the study all women, irrespective of the test results, underwent colposcopic examination for end-histology to exclude residual/recurrent CIN. The study endpoint was reached 24 months after treatment or in the case of re-treatment, whichever came first. The study protocol was approved by a multicentre research ethics committee and by local review boards at all hospitals. All women gave signed informed consent before enrolment.

Outcome measures

The primary outcome measure is an increase in specificity achieved by combined testing. This will secondarily result in a decrease in unnecessary examinations and treatment. Health-care costs and the possible impact of hrHPV type on the risk of residual/recurrent CIN were assessed.

Sample size calculation

Under the H_0 hypothesis it is assumed that the specificity of cytology and combined testing (cervical cytology and hrHPV-testing) is equal. In order to prove a higher specificity of combined testing in comparison to cytology alone, we used power of 80% and 10% level of significance. Assuming the specificity of combined testing being 10% points higher than the specificity of cytology alone, 148 women treated for CIN lesions without residual/recurrent CIN were needed for inclusion. Given the incidence of residual/recurrent CIN of 10-20%[3,4,8-10], 185 women treated for CIN lesions had to be included. Taking into account some patients lost to follow-up, 200 women were finally included.

Randomisation

We used block randomisation with random blocks of four and six. The patient's physician reported each study candidate to the independent study coordinator through a central telephone number. This coordinator assigned participants to group A or B.

The results of randomisation were not blinded since the different groups used distinct follow-up policies based on their results. The results of hrHPV testing in group A were blinded until the end of the study.

HPV testing

Cervical scrapes for HPV detection and typing were taken using a cervical biosampler (Accellon Combi® Medscand Medical, Sweden). HPV testing was performed with the consensus GP5+/GP6+ PCR enzyme immunoassay (EIA) using a cocktail probe covering all (probably) hrHPV types, as previously described[24]. This test is clinically validated[25]. We used β-globin PCR to identify sampling errors and to monitor for PCR inhibitors. Additionally, reverse line blot (RBL) analysis was performed on PCR-EIA positive cases to identify individual HPV types.

Cytology, Colposcopy and Histology

Cervical smears were read according to the Dutch CISOE-A (KOPAC-B) classification, the standard classification used in The Netherlands[26,27]. The relationship between CISOE-A and histological CIN and SIL classification (Bethesda 2001) has been previously described[26].

Smears were classified as normal (Pap 1) or abnormal, i.e., borderline dyskaryosis (Pap 2), mild dyskaryosis (Pap 3a1), moderate dyskaryosis (Pap 3a2), severe dyskaryosis (Pap 3b), suspected for carcinoma in situ (Pap 4), or suspect for invasive cancer (Pap 5). Experienced gynaecologists performed standard colposcopic assessment using acetic acid and iodine solution for the identification and delineation of the lesions. Biopsies were taken from all colposcopic abnormalities. Histology results were defined as mild dysplasia (CIN 1), moderate dysplasia (CIN 2), severe dysplasia (CIN 3) or (micro) invasive cancer. High-grade CIN and residual/recurrent CIN were defined as ≥ CIN 2. A gynaecological pathologist revised all cytological and histological samples.

Post hoc modifications in the use of hrHPV testing during follow-up

In earlier retrospective and observational studies various modifications in the use of hrHPV testing in the follow-up after treatment for high-grade CIN have been suggested (14,19,21,28). We assessed the data from group B also according to these different modifications.

The modifications were defined as B1: follow-up by combined testing whereby low-risk women (normal cytology and hrHPV negative at 6 months after treatment) omit the 12th months visit, B2: follow-up by hrHPV testing alone at 6, 12 and 24 months after treatment, and B3: follow-up by hrHPV testing at 6 months and combined testing at 24 months after treatment.

Health-care costs

For calculations of the health-care costs we looked at two major sources of cost difference. These were reduction in unnecessary examinations (colposcopy and treatment) and increase in hrHPV tests. Additionally in the case of modification B1 the omission of cytology at the 12th months visit was taken into account. The reduction in unnecessary examinations was calculated through the observed difference in specificity and adjusted for the proportion of residual/recurrent CIN. We assumed that all women in group B underwent hrHPV testing 3 times during 24 months of follow-up. We compared group A, group B and modification B1 within group B. The average total cost per referral per CIN grade (including all related follow-up) was established in an earlier study [29], and reflects the current Dutch situation. As costs were calculated from a socio-economic point of view, they included both direct medical (material, lab, consultations) and non-medical costs (time and travel costs of the women), which were valued according to Dutch guidelines [30,31]. In this study, average costs of (unnecessary) examinations were estimated at €582, or €652 if hrHPV was present. The costs of cytology and hrHPV testing were estimated at €89 and €34, respectively [32,33]. All costs are calculated at price levels for 2005.

Statistical analysis

The specificity and sensitivity of both follow-up policies for residual/recurrent CIN were determined on basis of the outcome of the colposcopic examinations. Women who were lost to follow-up were excluded.

In the analyses two groups were compared:

A. women with residual/recurrent CIN detected by abnormal cervical cytology.

B. women with residual/recurrent CIN detected by as well abnormal cervical cytology as a positive hrHPV-test. We used two-by-two tables to assess the diagnostic value of the different follow-up policies for post-treatment CIN 2/3. In these analyses women without a suspect cervical lesion on colposcopic examination, or with CIN 0 (no CIN) or CIN 1 (mild dysplasia) in the biopsy were considered as 'negative'. Relative risk and 95% confidence interval (CI) were calculated. We used McNemar's test for paired proportions to establish possible differences between variations in the use of hrHPV-testing during follow-up with respect to sensitivity and specificity.

RESULTS

Recruitment

A total of 210 women who attended the outpatient clinics for treatment of high-grade CIN were eligible for participation. Six of them were excluded: 4 women with previous treatment of CIN, 2 women diagnosed with cancer in the past.

The remaining 204 women were randomised (Figure 1). No important differences in baseline characteristics were found between the two groups or between women who were successfully treated and women who developed residual/recurrent CIN (Table 1). The residual/recurrent CIN rates were comparable in both groups (6.9% vs. 8.8%, relative risk 0.77 (95% CI 0.43 to 1.37)).

Primary outcome measures

Abnormal cytology was seen after 6 months in 14/96 women in group A (Figure 1, Table 2). Three of them had residual/recurrent CIN, leaving 11 women with a false-positive cytology result. After 12 months 5/90 women had abnormal cytology, none of them had residual/recurrent CIN. After 24 months 4/89 women had abnormal cytology, of which one had residual/recurrent CIN. In group B 7/96 women had abnormal cytology and hrHPV after 6 months, 4 of these had residual/recurrent CIN, leaving 3 women with false-positive test results. After 12 months 5/87 women had abnormal cytology and hrHPV, of whom two had residual/recurrent CIN, leaving 3 false-positive combined tests. After 24 months 4/85 women had abnormal cytology and hrHPV, 3 of these had residual/recurrent CIN, leaving 1 false-positive combined test. In a total follow-up of 24 months we found 17/23 false-positive cytology results in group A compared to 7/16 false-positive combined test results in group B. With the exception of one woman, all cases of residual/recurrent CIN that occurred during follow-up were picked up in both strategies. After 24 months, one woman in group A had residual/recurrent CIN with normal cytology.

Follow-up by conventional cytology (strategy for A) had a lower specificity for detection of residual/recurrent CIN compared to follow-up by cytology and hrHPV testing (strategy for B) (80% vs. 91%, relative risk 0.87 (95% CI 0.77 to 0.99)) (Table 3). There was no significant difference in sensitivity (86% vs. 100%, relative risk 0.86 (95% CI 0.63 to1.16)). PPV for strategy A was 26% compared to 56% for strategy for B (relative risk 0.46 (95% CI 0.21-1.05)). NPV was 99% en 100% (relative risk 0.99 (95% CI 0.96-1.01)), respectively.

Post hoc modifications in the use of hrHPV-testing during follow-up

Since the test characteristics were more favourable in the strategy for B, we compared combined testing with various modifications of hrHPV testing (Table 4). First,

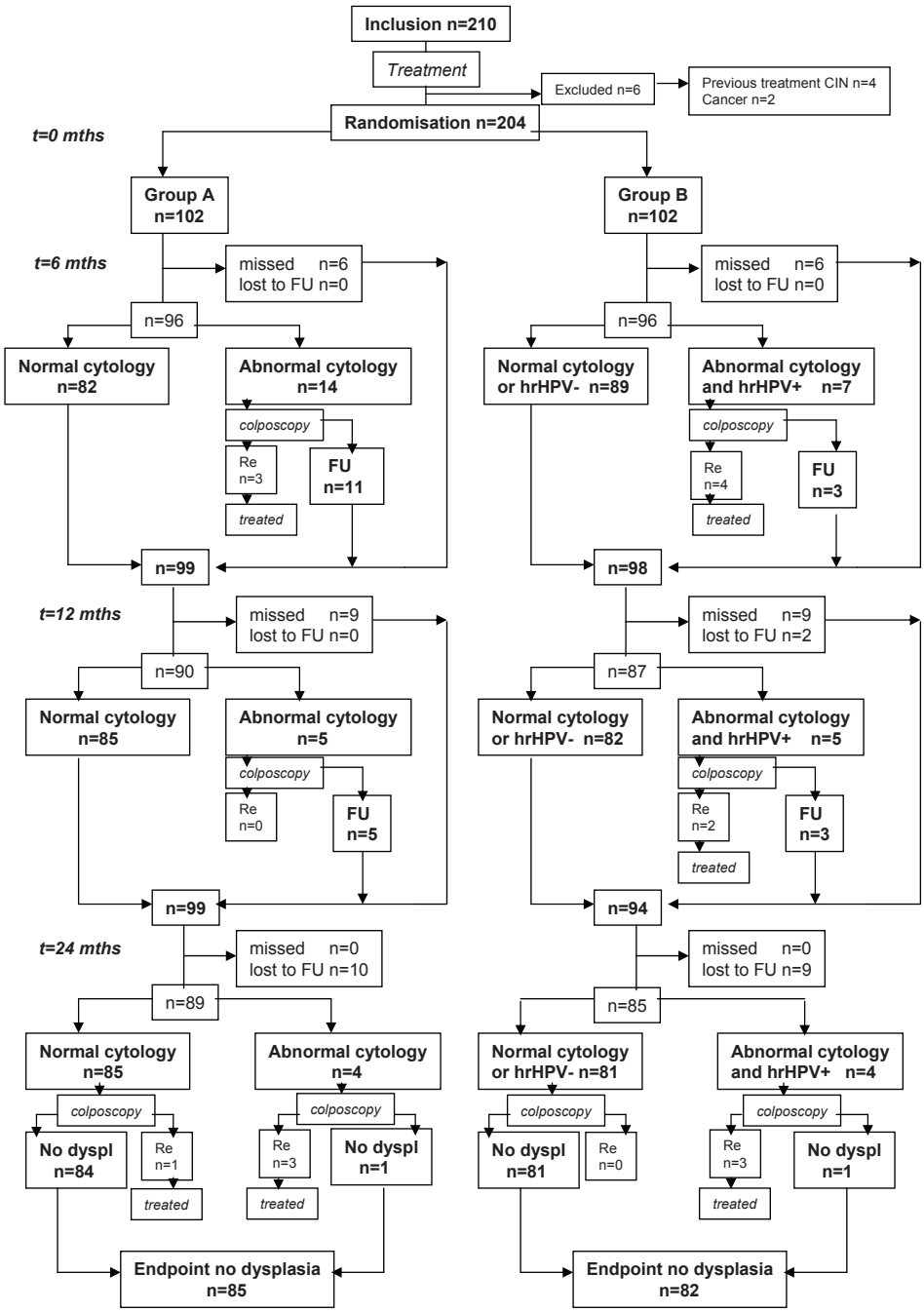

Figure 1. Trial design

mths=months, missed=patient absence, FU=follow-up, Re=residual/recurrent high-grade CIN, No dyspl= no cervical dysplasia.

Table 1

	Total numbers (%)	Successfully treated (no residual/recurrent CIN) (%)	Residual/recurrent CIN (%)
	n=183 (100)	n=167 (91)	n=16 (9)
Age at intake (years)			
<30	10 (6)	10 (100)	0
30-40	108 (59)	98 (91)	10 (9)
40-50	53 (29)	47 (89)	6 (11)
>50	12 (7)	12 (100)	0
No. of sexual partners last year			
0-1	157 (86)	144 (92)	13 (8)
2-4	26 (14)	23 (89)	3 (12)
Smoking			
No	95 (52)	90 (95)	5 (5)
Yes	88 (48)	77 (88)	11 (13)
Education			
Primary or less	29 (16)	29 (100)	0
Secondary	87 (48)	78 (90)	9 (10)
More than secondary	67 (37)	60 (90)	7 (10)
History of sexual transmitted disease			
No	136 (74)	125 (92)	11 (8)
Chlamydia Trachomatis	25 (14)	22 (88)	3 (12)
Condylomata acuminata	16 (9)	13 (81)	3 (19)
Other	11 (6)	11 (100)	0
Treatment			
LETZ	172 (94)	158 (92)	14 (8)
Exconisation	11(6)	9 (82)	2 (18)

CIN=Cervical Intraepithelial Neoplasia, LETZ=loop excision of the transformation zone.

Table 2. Test results in relation to residual/recurrent CIN

	t=6 months		*t=12 months*		*t=24 months*	
	test+/total	**res/rec**	**test+/total**	**res/rec**	**test+/total**	**res/rec**
	(n)	(n)	(n)	(n)	(n)	(n)
A	14/96	3/14	5/90	0/5	4/89	1/4
B	7/96	4/7	5/87	2/5	4/85	3/4

Group A: follow-up by cytology, group B: follow-up by cytology and hrHPV testing, test+=abnormal cytology for group A, abnormal cytology and hrHPV positive for group B, res/rec=residual/recurrent CIN.

Table 3. Test characteristics for detecting residual/recurrent CIN

	Randomisation groups						
	Group A			Group B			RR (95%CI)
	%	(n)	(95% CI)	%	(n)	(95% CI)	
Sensitivity	86%	(6/7)	(65-100%)	100%	(9/9)	(72-100%)	0.86 (0.63-1.16)
Specificity	80%	(68/85)	(70-88%)	91%	(75/82)	(83-97%)	0.87 (0.77-0.99)*
PPV	26%	(6/23)	(10-48%)	56%	(9/16)	(30-80%)	0.46 (0.21-1.05)
NPV	99%	(68/69)	(92-100%)	100%	(75/75)	(96-100%)	0.99 (0.96-1.01)

RR=relative risk, PPV=positive predictive value, NNP=negative predictive value, CI=confidence interval, n=number of women,* statistically significant
Group A: follow-up by cytology, group B: follow-up by cytology and hrHPV testing.

Table 4. Test characteristics of post hoc modifications in the use of hrHPV testing for detecting residual/recurrent CIN

	B1	p-value*	**B2**	p-value*	**B3**	p-value*
Sensitivity	90%	0.5	78%	1.0	86%	0.5
Specificity	95%	1.0	88%	0.0002	84%	0.003

Post hoc modifications within Group B
B1: follow-up by combined testing whereby low-risk women (normal cytology and hrHPV negative at 6 months) omit the 12th months visit, B2: follow-up by hrHPV testing alone at 6, 12 and 24 months after treatment, and B3: follow-up by hrHPV testing at 6 months and combined testing at 24 months after treatment. * McNemar's test for paired proportions.

HrHPV type 16 was present in the majority of women with residual/recurrent CIN before treatment (n=12/16) and at time of presence of residual/recurrent CIN (n=11/16).

DISCUSSION

Follow-up of women after treatment for high-grade CIN is more efficient and effective using both cytology and hrHPV testing. Compared to the conventional protocol using cytology alone at 6, 12 and 24 months after treatment (current national guidelines) a higher specificity can be obtained using cytology in combination with hrHPV (i.e., 80% vs. 91%). As a consequence, fewer false-positives lead to fewer unnecessary renewed examinations. This is in agreement with previous observational studies where hrHPV testing has been proposed as a screening tool for follow-up after treatment for high-grade CIN [14,19-21,23,28].

Since residual/recurrent CIN only develops when a hrHPV infection is present [5,22] our proposed policy includes colposcopic examination when both abnormal cytology and hrHPV are detected. Thus, cases with only hrHPV infection and no

cytological cervical changes, or those with abnormal cytology without a hrHPV infection do not need further investigation [20,23,34,35].

In group A (follow-up by cytology) we found one woman with residual/recurrent high-grade CIN (CIN 2) after 24 months but with normal cytology and presence of hrHPV. Since this was our study endpoint including colposcopic examination the residual/recurrent CIN was detected. In the current national follow-up protocol this lesion would have been left undetected and the patient would have been returned to the national screening program where she would have been screened 2-3 years later. According to the standard Dutch protocol this is considered an accepted level of risk, as the probability of developing invasive cervical cancer within these 2-3 years is extremely low [13,36,37].

Post hoc modifications in the use of hrHPV testing after treatment for high-grade CIN were also evaluated. Some studies suggest omitting the 12th months visit for low-risk women (normal cytology and hrHPV negative 6 months after treatment) [14,19,35,38]. According to our evaluation, the test characteristics (described as modification B1) were comparable to values obtained for combined testing at 6, 12 and 24 months after treatment. Other modifications such as hrHPV testing alone (B2) and hrHPV testing at 6 months with combined testing at 24 months (B3) showed less favourable test characteristics. As a result of the low specificity rates of hrHPV as a stand-alone test, unnecessary examinations could not be prevented in these groups. Although Coupé et al. previously recommended the latter strategy on basis of a simulation prediction model we could not confirm the advantage in our study [28]. An explanation might be the difference in follow-up time for residual/recurrence, which was 5 years in the prediction model in contrast with 2 years follow-up in this randomised clinical trial. Conversely, our proposed modification (B1) of combined testing at 6 and 24 months for low-risk women and combined testing at 6,12 and 24 months for high-risk women was not evaluated in the prediction model.

Taking the costs into account, follow-up after treatment for high-grade CIN using both cytology and hrHPV testing seems to be slightly more expensive. Although unnecessary examinations could be prevented, the total costs of the additional hrHPV testing were higher, even though this is only a fraction of the total costs of treatment for high-grade CIN (≤ 10% extra costs) [29]. Yet, in the future these costs may decrease (using liquid based cytology). In addition to costs, effects on quality-of-life must also be taken in account. When low-risk women omit the 12th months visit, reduction in health costs can be achieved.

HrHPV genotyping may potentially be a useful tool to determine the development of residual/recurrent CIN [39,40]. Gök et al. have suggested an increased risk for residual/recurrent CIN for HPV 16 [39]. In this study we could not confirm these results, although the majority of women who developed residual/recurrent CIN had

modification B1 showed a specificity of 95% and sensitivity of 90%, which was not statistically different from group B (specificity p=1.00, z=0.00, sensitivity p=0.5 z=0.71). Evaluating modifications B2 and B3, the test characteristics were even less favourable compared to combined testing (specificity 88% p=0,0002 z=3.75, sensitivity 78% p=1.0 z=0.00, and specificity 84% p=0.003 z=3.02, sensitivity 86% p=0.5 z=0.71, respectively).

Secondary outcome measures

Health costs

For group A, the total average cost of unnecessary referral [(1-specificity=) 20% x (1-residual/recurrent CIN) 93%= 18.6% x €582=] was €108 per woman treated for high-grade CIN. For group B this cost was €50 per woman and for modification B1 €23 per woman. The extra cost for hrHPV testing in group B is €102 (3 times follow-up x €34).

At 6 months, 62% of the treated women had normal cytology with hrHPV negative test results. Therefore, 2 times hrHPV testing and cytology are required for 62% in modification B1. The extra cost of hrHPV testing is [(62% x €34 x 2 + 38% x €34 x 3)=] €81, whereas the reduction in cost of cytology at the 12th months visit is 62% x €89= €55. In total, the cost of unnecessary referral for group A was €108, for group B €152 and for modification B1 €49. For group B the cost was on average €44 more than for group A. However for modification B1 the cost decreases by €59.

HrHPV typing

A total of 182 women were hrHPV positive at baseline, 10 women were hrHPV negative (Table 5). The most common types were HPV 16, 31 and 33, although a wide variety of other types was present. There was no significant difference in the distribution of hrHPV types (data not shown). These findings persisted during follow-up. Single infections were more common than multiple infections for all women (72% vs. 28%); this ratio remained stable during follow-up. No differences between groups A and B were found for individual hrHPV types and baseline characteristics, including residual/recurrent CIN (data not shown). In 21 of the 49 (43%) hrHPV positive women after treatment for high-grade CIN we found newly acquired hrHPV types. In 28 out of 49 (57%) the same hrHPV type was detected. For women who developed residual/recurrent CIN, 11 out of 16 (69%) revealed the same hrHPV type as seen before treatment, the remaining 5 (31%) showed newly acquired types.

Table 5. Prevalence of hrHPV types during follow-up

	t=0 (before treatment)	t= 6 months	t=12 months	t=24 months
Total hrHPV positive	n=182	n=37	n=24	n=16
hrHPV type (n)	**S/M**	**S/M**	**S/M**	**S/M**
16	79/16	16/2	10/1	5/0
18	2/6	1/2	0/0	0/0
31	11/6	2/0	1/0	0/1
33	11/5	1/0	1/0	1/0
35	3/1	0/0	0/0	0/0
58	6/3	2/0	2/0	0/0
other	19/14	6/5	6/3	6/3
Total	131/51	28/9	20/4	12/4
(%)	*72/28*	*76/24*	*83/17*	*75/25*

S/M= single/multiple infection

others=hr types 30,39,40,42,45,51,52,53,56,59,66,67,68,70,71,73,82

hrHPV type 16 present before treatment and at time of residual/recurrent CIN. The difference might be explained by the small number of residual/recurrent CIN. Also the number of follow-up visits was distinct; we evaluated patients after 6, 12 and 24 months and Gok et al. evaluated additionally after 3 and 18 months. Van Ham et al. have reported a high level of newly detected hrHPV types after treatment[40]. Different hrHPV types were detected in 75% of all hrHPV positive women 6 months after treatment, without description of presence or absence of residual/recurrent CIN. We found newly acquired hrHPV types in 21 out of 49 (43%) hrHPV positive women after treatment during follow-up of 24 months. Women with residual/recurrent CIN showed a newly acquired hrHPV type in only in 5 out of 16 (31%). It thus remains unconfirmed whether hrHPV genotyping has clinical value in follow-up policies after treatment for high-grade CIN.

Summarised, combined testing at 6, 12 and 24 months after treatment, with adjustment for low-risk women who can omit the 12th months visit, represents in our opinion the best follow-up strategy. Even the total health-care costs will decrease as a consequence of less unnecessary examinations. Aspects of quality-of-life (e.g. less anxiety) remain to be evaluated.

Conclusion

Based on our randomised clinical trial we recommend a follow-up policy for women treated for high-grade CIN consisting of both cytology and hrHPV testing at 6, 12 and 24 months after treatment. Low-risk women (with normal cytology and a negative hrHPV test at 6 months) may omit the 12th months visit during follow-up, resulting in cost reduction.

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Chapter 4

HPV testing on self-sampled cervico-vaginal brushes: An effective alternative to protect non-responders in cervical screening programmes

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ABSTRACT

Women not attending cervical screening programmes are at increased risk of cervical cancer. We investigated in these non-responders to what extent offering self-sampling devices for cervico-vaginal brushes for high-risk human papillomavirus (hrHPV) testing would induce participation and if so, what the yield of precursor (i.e. CIN2+) lesions following self-sampling would be. In addition, we assessed screening history of participants and costs per detected high-grade CIN (CIN2 or worse) lesion in comparison to the regular programme in The Netherlands.

Non-responders received a device for hrHPV testing (self-sampling group, n=2546) or an extra recall for conventional cytology (control group, n=284). The percentage of self-sampling responders was compared with those of cervical screening participants. HrHPV positive self-sampling responders were invited for cytology and colposcopy. CIN2+ yield and costs per detected CIN2+ were evaluated.

Active response was higher in the self-sampling than in the control group (34.2% vs. 17.6%; $p<0.001$). HrHPV positive self-sampling responders were less likely to have a prior screening history than screening participants ($p<0.001$), indicating that they are regular non-responders. HrHPV prevalence was similar (8.0% vs. 6.8%; $p=0.11$), but CIN2+ yield was higher in self-sampling responders compared to screening participants (1.67% versus 0.97%; OR=2.93, 95% CI 1.48-5.80; $p=0.0013$). Costs per CIN2+ lesion detected via self-sampling were in the same range as those calculated for conventional cytological screening (€8,836 versus €7,599).

Offering self-sampling for hrHPV testing in non-responders is an attractive adjunct to effectively increase population coverage of screening without the adverse effect of markedly increased costs per detected CIN2+ lesion.

INTRODUCTION

Screening programmes have contributed to a decline of incidence and mortality of cervical cancer[1-4]. However, non-response remains an important problem of current screening programmes[5-7]. In The Netherlands women between 30-60 years are invited to cervical screening at 5 year intervals. The active participation rate is 63%. Of the remaining women, 9% respond by declining the invitation for various reasons such as pregnancy, breastfeeding, history of hysterectomy or smear having been taken by any other occasion, leaving 28% women who do not respond at all (hereafter referred to as 'non-responders')[8].

Non-responders in the screening programme are at high risk for development of cervical cancer, since at least 50% of women diagnosed with cervical cancer in the United States, the UK and The Netherlands had no history of participation in cervical screening[1,2,9-11].

Women who do not respond to invitations for conventional smears may be inclined to respond to a self-sampling technique[12-18], but this has never been tested in the setting of a regular screening programme. Although the sensitivity for high-grade cervical intraepithelial neoplasia (CIN) of cytological specimens obtained from self sampled vaginal material is lower than the sensitivity of conventional cytology, studies have shown that self-sampled cervico-vaginal specimens (SSVS) are highly representative for the high-risk human papillomavirus (hrHPV) DNA status of the cervix[14-16,19-21]. Since hrHPV infection has been established as the primary cause of cervical cancer in nearly all cases[22-24], hrHPV detection on SSVS could be a valuable tool to identify non-responder women at risk of cervical cancer.

In the present study we investigated within the setting of a regular screening programme to what extent offering hrHPV testing on SSVS leads to participation of non-responder women. In addition, we compared the screening history of the hrHPV positive women that submitted a sample and compared this with that of age matched-, regular screening programme participants. Finally, the yield of high-grade CIN as detected by hrHPV testing on SSVS in non-responders against that found by conventional cytology in screening responders was evaluated as were the costs per detected high-grade CIN lesion or cervical cancer (CIN2+) in both groups of women.

MATERIALS AND METHODS

Study participants

The study was initiated as an intervention trial in addition to the regular population based cervical screening programme. The protocol was approved by the

multi-centre research ethics committees of the Erasmus University Medical Center, Rotterdam and VU Medical Center, Amsterdam. We selected 2,830 non-responder women between 30 and 50 years of age at invitation who, according to the Regional Health Council Database (in the area Amstelland/de Meerlanden and Kennemerland) had neither responded to the regular invitation nor to the first 6 months reminder between January 2003 and April 2004. Upper age limit of 50 was chosen because of low hrHPV positivity in women aged 50 or more. The time from the last 6 months reminder to the start of the study was at least 5 and at most 24 months. Women were assigned to either the self-sampling cohort or the control group at a 9 to 1 ratio, based on their randomly assigned invitational-procedure number in the database. The 9:1 ratio was chosen because the control group was used only for comparison of response rates whereas the study group size was based on our estimate of the expected yield of CIN2+ lesions. Since an initial small pilot study (n=100) revealed that 30% of non-responders submitted a SSVS (unpublished data), we calculated that at least 2,500 women should be included in the self-sampling group to detect CIN2+ with a standard error of 0.5%. For comparison of CIN2+ yield and viral positivity with screening participants, data were compared with those of age-matched women (n=6,208) who participated in a population based screening (i.e. POBASCAM) trial in the same region. The POBASCAM trial is a randomised prospective cohort trial that was conducted within the regular screening population in the same area (1998 to 2002)[25].

Women of the self-sampling group received a self-sample kit with instructions and an explanatory letter, whereas those of the control group received an extra recall for regular cytology with an explanatory letter. The kit for SSVS consisted of a Viba-brush® (Rovers Medical Devices, Oss, The Netherlands), a collection tube containing 5 ml Universal Collection Medium (UCM; kindly provided by Digene Corporation, Gaithersburg, USA), instructions (written and drawn) and a return envelop. UCM is a preservation medium with universal properties that allows not only liquid based cytology but also several DNA-based assays such as hybrid capture 2 and PCR. The latter has been tested in a recent study[26]. Women were asked to return the SSVS collection tube (with the brush) within a padded envelope to the laboratory for hrHPV testing. As outcome measure for the response rate we included all women that responded within 6 months after sending the kit, or, in case of the control group, that responded within 6 months after having received their second reminder.

Procedures

Upon arrival in the lab, SSVS samples were vortexed for 10 seconds and the brush removed, after which the sample was concentrated to 1 ml by spinning down for

10 min at 3,000g and removing 4 ml of the supernatant. For PCR purposes 150 µl of the concentrated sample was taken and centrifuged in a 1.5 ml reaction tube for 10 min at 3,000g. The supernatant was subsequently removed and the pellet resuspended in 1 ml 0.01 M Tris-HCL pH 7.4. This suspension was frozen for at least 1 hour at -80°C, thawed and than boiled at 100°C for 10 min. After centrifugation for 1 min at 3,000g followed by a short vortexing step, 10 microliters of this crude suspension was ultimately used in the PCR. Testing for hrHPV was conducted by the consensus primer GP5+/GP6+-PCR with an enzyme immunoassay read-out using a cocktail oligoprobe mix for 14 hrHPV types (HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), as described before[27]. A β-globin PCR was performed for quality assessment of the samples.

All women received a written test result and explanation. In case of an invalid sample (i.e. β-globin PCR negative material) women were asked to repeat the self-sampling test. All hrHPV positive women were invited for additional cytology, colposcopy and biopsy.

Cervical smears were classified according to the CISOE-A classification, the standard classification in The Netherlands for cervical cytology, which can be easily translated into Bethesda nomenclature[28]. Histology results were classified in a five tiered classification system consisting of the following categories: no dysplasia (CIN 0), mild dysplasia (CIN 1), moderate dysplasia (CIN 2), severe dysplasia or carcinoma *in situ* (CIN 3), and invasive carcinoma.

During the entire study period a telephone number was available for questions about hrHPV infection, cervical cancer and the self-sampling test. After receiving kits, some 20 telephone calls were received. This amount almost doubled after sending out results of the hrHPV test. Main questions were about acquisition of the virus and perceived gravity of the situation.

Statistical analysis

Response rates of the self-sampling and control group were compared with the Chi-square test. Differences in hrHPV prevalence's and in CIN2+ detection rates between the self-sampling group and the POBASCAM cohort of age-matched responders were assessed using Cochran-Mantel-Haenszel testing. Regarding CIN2+ detection, women in the POBASCAM cohort were directly referred for colposcopy in case of a cytological reading of moderate dyskaryosis or worse (which is equivalent to HSIL according to the Bethesda classification). Since for these women hrHPV status was irrelevant for direct referral, the blinded and unblinded groups were pooled.

HrHPV-positive women in the self-sampling group who had been invited for at least one prior screening round were tested for differences in historical screening

attendance. A difference in attendance at the previous screening round 5 years earlier was assessed by Cochran-Mantel-Haenszel testing. Women who had been invited for at least 2 screening rounds were also tested on a difference in attendance at any of the previous 2 screening rounds. Attendance at the previous screening round or at any of the 2 previous screening rounds (regular interval is 5 years) was assumed if a smear was taken within the last 7 or 12 years, respectively.

Cost-effectiveness calculations

We compared the costs and effects of conventional cervical cancer screening in the POBASCAM cohort to the additional costs and effects of offering self-sampling to non-responders. The effects were measured by the number of detected CIN2+ lesions found in the POBASCAM and self-sampling study group at baseline. Calculated costs per medical procedure included direct medical costs and indirect costs of travelling and production loss[29]. Cost calculations involved summing the costs of the screening procedures, colposcopies, biopsies, CIN treatments and follow-up after treatment. To assess the impact of self-sampling on the country level, total costs and detected CIN2+ were rescaled to the whole population in The Netherlands where 750,000 women are yearly invited and 63 percent attend screening[30].

RESULTS

Response rates

A total of 2,546 SSVS packages were sent to non-responder women belonging to the self-sampling group, whereas a second reminder (consisting of a regular invitation form plus a letter explaining the importance of attending the screening programme) was sent to 284 women in the control group. A flowchart of the study design is given in Figure 1.

Of the self-sampling group 194 (7.6%) women responded by returning a prepaid postcard indicating their reason for not participating, which included breastfeeding /pregnancy, previous hysterectomy, treatment by a gynaecologist or other reasons. Of the remaining 2,352 women of the SSVS group, 70 (3.0%) responded to the self-sampling package by visiting the general practitioner for conventional cytology (without using the SSVS kit), whereas 736 women (31.3%) returned SSVS samples to the lab. 1,546 (65.7%) women did not respond. From the 736 samples received at the laboratory, 17 had a β-globin PCR negative test result and the corresponding women received a second SSVS kit. Eight of them resubmitted a second SSVS (all of them being hrHPV negative), whereas 9 women did not respond the second time, yielding ultimately a total of 727 valid tests. Twelve (4.2%) women in the control group responded by returning a prepaid postcard. Of the remaining 272 control

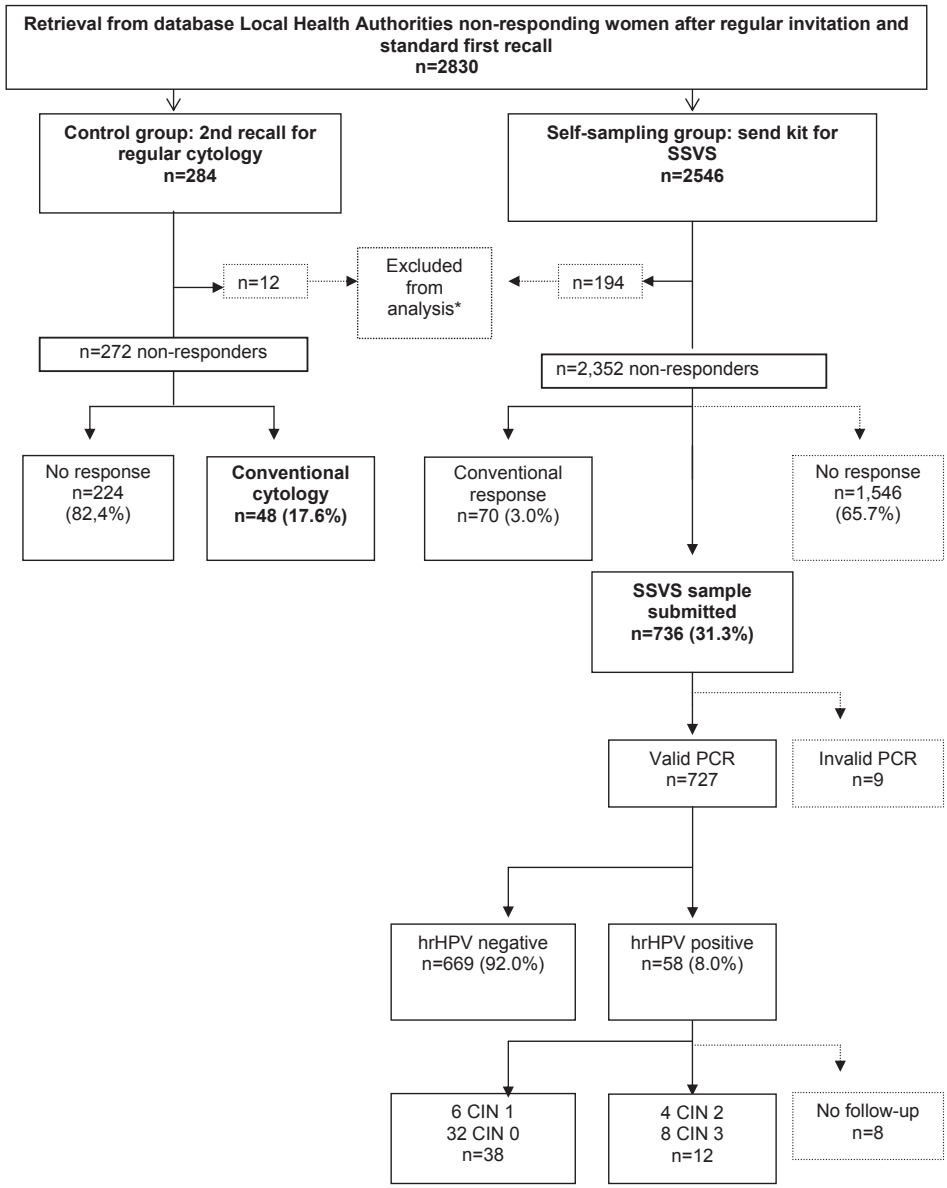

Figure 1. Flowchart of the intervention trial for non-responders in cervical screening programme. *Excluded from analysis were women who responded by returning a form, indicating the reason for not taking a self-sample test or responded to a second recall for cytology because of one the following reasons: pregnancy, breast feeding, prior hysterectomy, treatment by gynaecologist or 'other reason' (e.g. emigration, deceased, other illness, etc).

women, 48 responded to the second recall for regular cytology (17.6%). Active participation in the SSVS self-sampling group (including 3.0% conventional cytology) was statistically significantly higher than in the second recall group (34.2% vs. 17.6%, p<0.001).

HrHPV test results on self-sampled vaginal specimens

HrHPV DNA was detected in 58 (8.0%) of 727 women with a valid hrHPV SSVS test (Figure 1). The overall percentage of hrHPV DNA positivity in age matched women of the POBASCAM cohort was 6.8% (i.e. 422 of 6,208 women). The prevalence of hrHPV was slightly higher in the self-sampling group than in the POBASCAM responder cohort but the observed difference was not statistically significant (OR_{MH} 1.28; 0.93-1.76; 95% CI; p=0.11).

Comparison of screening history in self-sampling group and screening cohort

As the invitational database of the municipal health council functions since 2003 and is restricted to invitational smears only (i.e. containing no smears on other grounds), we obtained screening history concerning all smears for both test and control group from the National Pathology Registry (PALGA). However, owing to tight legislation in The Netherlands concerning privacy, records became fully anonimized for both test- and control group, thus impeding any proper group-allocation (i.e. control group, HPV-negative group and non participants in this study). We were unable, therefore, to obtain insight into screenings histories of women who had not responded by submitting a self sampled specimen.

Only for HPV positive women who had undergone further cytological- and histological testing, full screen history could be compared to the Pobascam control group.

In addition, since invitational screening starts in The Netherlands at 30, we could compare screening histories of hrHPV positive women who were at least 35 years of age (from either self-sampling group or age-matched responder group). These women were chosen because, in contrast to younger women, they had been invited for at least one prior screening round. Given the known attendance rate of women aged 30-34 in the population based screening programme in The Netherlands, it can be expected that about 80% of the women aged 35 or more have been screened at least once before. Indeed, the screening history of hrHPV positive women of the POBASCAM cohort was comparable to this figure: 279 of 356 (78%) of these women had at least one screening smear taken ≤7 years earlier (Table 1).

In contrast, only 10 out of 37 (27%) women in the self-sampling group had at least one smear taken within the last 7 years (ORMH: 0.09; 95% CI: 0.03-0.23;

Table 1: hrHPV positive women in self-sampling group have less screening history than hrHPV positive women in age matched cohort (historical control).

Screening history*	HrHPV positive women in self-sampling cohort	HrHPV positive women in POBASCAM cohort	Odds ratios
At least one screening smear ≤ 7 years ago**	13/37 (35%)	279/356 (78%)	OR 0.102 (95% CI: 0.042-0.247) p<0.001
At least one screening smear ≤ 12 years ago***	6/14 (43%)	20/26 (77%)	OR 0.118 (95% CI: 0.021-0.666) p=0.026

* For this analysis women belonging to age groups 31 and 35 were excluded since these women had not been invited for an earlier screening round. ** Only women that had been invited for at least one previous screening round. *** Only women that had been invited for at least two previous screening rounds.

p<0.001), indicating a significantly lower rate of prior screening in the self-sampling group.

This difference between both cohorts remained when screen history was surveyed over a longer period, i.e. over at least 12 years of women who were old enough for having a long screening history (i.e. 41 years and older): In the self-sampling group, only 6 out of 14 (43%) women had at least one screening smear ≤12 years earlier, which was significantly less than in the POBASCAM cohort where the attendance rate within the last 12 years was 20/26 (77%) (OR_{MH}: 0.12; 95% CI: 0.02-0.67; p:0.026; Table 1).

Cytology and histology results of women with hrHPV positive SSVS

Eight (14%) of the 58 women who had a hrHPV positive SSVS did not respond to the written (and reminded) alerts on their SSVS hrHPV positive test result. From the remaining 50 hrHPV positive women, all underwent cervical cytology. Cervical cytology was normal in 30 (60%). Sixteen women with normal cytology declined colposcopy-directed biopsy and thus had no histology. They opted for follow-up by cytology. The remaining 14 women with normal cytology underwent colposcopy-directed biopsy, which resulted in 1 CIN 3, 3 CIN 2, 2 CIN 1 and 8 CIN 0 cases. Cytological abnormalities were found in the remaining 20 (40%) women who had hrHPV positive SSVS. Fourteen of them had borderline or mild dyskaryosis (BMD) and 6 of them had moderate dyskaryosis or worse (>BMD). Of the 14 women with BMD 3 women refused colposcopy-directed biopsy and opted for cytological follow-up. Eleven remaining women with BMD underwent colposcopy-directed biopsy, yielding 1 CIN 3, 1 CIN 2, 4 CIN 1 and 5 CIN 0 cases. All 6 women with >BMD had CIN 3. In total 12 of 50 hrHPV positive women presented with an underlying high-grade CIN lesion.

Comparison of CIN2+ detection rates in self-sampling group and regular screening programme (POBASCAM cohort)

Together, 12 of 727 (1.6%) women with a valid SSVS test had underlying high-grade CIN. We compared this figure with the total yield of histologically confirmed CIN2+ diagnoses in 6,208 age matched women participating in the POBASCAM trial, who were immediately referred for colposcopy upon a cytological test result of >BMD. The overall detection rate of CIN2+ was higher in the self-sampling group than in the POBASCAM cohort (61/6208= 0.97%)(OR_{MH} 2.59, 95% CI 1.31-5.12; p=0.0047). The CIN2+ detection rate was still increased in the self-sampling group, although not statistically significantly, after including histology data of women with BMD in the POBASCAM cohort who were referred for colposcopy when having an abnormal smear upon repeat cytology at 6 and/or 18 months (OR_{MH} 1.68 (0.88-3.21; 95%CI; p=0.11; data not shown), as is conventionally done in the Dutch national programme. There was no association between odds ratio and age ($\chi 2$=0.45; p=0.93), i.e. there was no significant difference between any of the age strata.

Cost-effectiveness of offering SSVS

The detection rate of 0.97% CIN2+ lesions found by immediate colposcopy after >BMD in the POBASCAM cohort can be translated into an absolute figure of 4,567 (95%CI 3,295 – 5,872) CIN2+ lesions in The Netherlands (i.e. 4,567 CIN 2+ lesions = 750,000 invited women × 63% screening response × 0.97% detection rate). Based on our figures, when offering self-sampling to non-responders, the number of detected CIN 2+ lesions would increase by 1,085 (95%CI 519 – 1,650; 1,085 CIN 2+ lesions = 750,000 × 28% screening non-response × 31.3% SSVS response × 1.65% ≥ CIN 2 detection rate). The total direct and indirect costs of offering conventional cervical screening (including diagnosis and eventual treatment of CIN

Table 2: Costs per CIN2+ detected in conventional programme compared to SSVS as 'adjuncprogramme'.

	Conventional procedure	Self sample test per CIN2+
Cost per CIN2+ detected*	€7,599 (95% CI €5,910 – 10,532)	€8,836 (95% CI €5810 – 18472)

* specification per medical procedure in italics (index 2005; indirect costs of travelling and production loss included; see text and[29] for further details. Conventional response from[30]). Cost are assessed on organisation invitation, costs involved with testing and cost for diagnosis and treatment.

Screening organization (organisation-invitation) €15 / smear (similar for both conventional and ss test and dependent on response). Costs involved with testing: Smear taking at general practitioner and cytological evaluation €39 / smear. Self-sample material and High-risk HPV testing €2 / invitation + €33 / test. Adjunct smear taking at general practitioner and cytological evaluation €43 / smear. Cost involed with treating: diagnosis/treatment/and follow-up ≥CIN2 €1,889; Diagnosis/treatment/follow-up CIN1 €1,434 ; Diagnosis/follow-up CIN0 €335/ Colposcopy without biopsy €171.

after >BMD) in The Netherlands was calculated at €34,703,000 annually and the total costs of offering SSVS to non-responders was calculated at €9,587,000. The resulting costs per detected CIN2+ lesion were in the same range for cytological screening and self-sampling, i.e. €34,703,000 / 4,567 = €7,599 (95% CI €5,910 – 10,532) for conventional screening versus €9,587,000 / 1,085 = €8,836 (95% CI €5810 – 18472) for self-sampling (see table 2).

DISCUSSION

Half of the cases of cervical carcinoma is found in women who do not attend regular cervical screening. Our results show that offering self-sampling of vaginal specimens for hrHPV testing led to a higher response rate than a second recall in the group of non-responders of the nation wide cervical screening programme. The hrHPV positive women who responded by submitting a SSVS were also likely to have refrained from participation in previous screening rounds, and consequently can be considered regular non-responders. The relevance of these findings are emphasized by the observation that hrHPV testing on SSVS is highly effective in detecting CIN2+. We found that the detection rate of CIN2+ lesions was significantly higher in the hrHPV positive SSVS group than in age matched women participating in a regular screening programme that were referred immediately because of >BMD. Importantly, the costs per CIN2+ detected via hrHPV testing on SSVS in the non-responders are in the same range as those calculated for conventional cytological screening (€8,836 versus €7,599). Thus, offering self-sampling for hrHPV testing to recruit non-responders is likely to increase the effectiveness of the screening programme markedly.

The high response rate to offering SSVS in non-responders may be attributed to the fact that these women prefer this self-sampling procedure above visiting a general practitioner. Indeed, in a questionnaire filled in by the women referred to the colposcopy clinic (n=30), 29 women marked that the self-sampling procedure was easy and 25 of them indicated that they would prefer this test to conventional cytology. These data warrant further epidemiological investigation of non-responder women into reasons for declining the invitation of the regular screening programme.

Non-responders in screening programmes are considered to be a high-risk group for cervical cancer[1,2,9-14]. In line with this, an increased OR for prevalent CIN2+ in hrHPV positive non-responder women was evident, confirming that these women represent a group with a higher risk of CIN2+ than regular screening responders. Although there was a reasonable increase in hrHPV detection rate in non-responders compared with regular screening participants (7.8% versus 6.7%), this increase did

not reach statistical significance may be attributed to the fact that the sensitivity of detecting hrHPV is somewhat lower on self-collected samples compared to classical cervical scrape samples[21]. Furthermore, the ratio between clinically relevant versus clinically irrelevant hrHPV infections may be increased in non-responders of the screeningprogramme versus responders of the screeningprogramme, due to programme effect. It should be kept in mind that we compared the yield of CIN2+ in non-responders following hrHPV testing on SSVS with corresponding age matched POBASCAM responders who were directly referred for colposcopy because of a smear with >BMD cytology. However, POBASCAM responders with BMD (2.4% of the screened population) have two repeat smears (after 6 and 18 months) and are referred for colposcopy if any of the two repeat smears show cytological abnormalities. In 10% of these indirectly referred POBASCAM responders, CIN2+ is detected. When BMD women with CIN2+ diagnosed following cytologically abnormal repeat smears at 6 and 18 months were added to the CIN2+ cases detected after direct referral of women with >BMD, the overall detection of CIN2+ in the hrHPV positive self-sampling responders appeared as good as that in the conventional screening programme (p=0.11).

Thus, our study underscores the potential value of SSVS for hrHPV testing in cervical screening programmes as a method for enhancing the effectiveness of the screening programme[16-20]. If we extrapolate our results to the situation in The Netherlands where the annual non-response involves 210,000 women (about 300 of which contain cervical carcinomas), offering self-sampling for hrHPV testing could result in the early detection of 1,085 extra CIN2+ lesions, leading to approximately 100 cervical cancers being prevented or detected earlier. This is a substantial figure if we consider that annually about 700 cervical carcinomas are diagnosed in The Netherlands[31]. Therefore, hrHPV testing on SSVS clearly merits further attention, although efforts to improve the response remain certainly mandatory, since still 69% of the non-responder women did not participate at all.

Moreover, if SSVS for hrHPV testing of non-responders were to be considered as an adjunct in a cervical screening programme, a clear follow-up strategy must be adhered to in order to limit redundant colposcopic referral of hrHPV positive women with a negative test result in reflex cytology. Given our experience we presently advocate a protocol in which hrHPV positive women with abnormal cytology are directly referred for colposcopy directed biopsy. HrHPV positive women with normal cytology could be invited for repeat hrHPV and cytological testing after 6 months and referred for colposcopy when one or both tests is positive. The costs of detecting CIN2+ in self-sampling women could even be decreased when follow-up of those with hrHPV positive normal smears would be done solely by hrHPV testing.

In summary, offering hrHPV testing on SSVS is an attractive adjunct to offer protection to women that are not reached by the cervical screening programme. SSVS can increase the effectiveness of the regular cervical screening programme significantly at nearly the same costs per detected CIN2+ lesion.

Finally, in the era where prophylactic HPV vaccination is likely to be offered to young adolescent women in the near future, HPV testing on SSVS might also be highly attractive for these women since it allows them to control the effectiveness of vaccination and their risk of cervical lesions by themselves without intervention by the general practitioner.

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Chapter 5.1

A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions

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ABSTRACT

Aims: Based on the hypothesis of a shifted balance between Th1-type and Th2-type cytokines in cervical dysplasia we evaluated a possible deregulation of the cytokine network. We estimated expression of peripheral cytokine levels in different stages of cervical disease and in relation to presence or absence of high risk human papillomavirus (hrHPV).
Materials and Methods: 21 hrHPV positive women with high-grade cervical intraepithelial neoplasia (CIN II-III) and 12 patients with invasive cervical carcinoma formed the study groups. Two control groups consisted of 10 hrHPV positive and 11 hrHPV negative women without CIN. Differences in leukocyte subgroups were evaluated with a differential leukocyte count. Plasma concentrations of TNF-α, TNF-α receptors TNFRI and TNFRII, IFN-γ, IL-2, IL-12, IL-4 and IL-10 were determined by ELISAs.
Results: Leukocyte counts in patients with CIN III and carcinoma were significantly higher than in controls. Plasma IFN-γ concentrations in CIN III and carcinoma patients were significantly decreased when compared with values for women with CIN II or controls. Plasma levels of IL-12, IL-2, IL-4 and TNF-α did not differ significantly between groups, but a significant decrease in plasma levels of TNFRII was observed between CIN II and CIN III or carcinoma. IL-10 was detected with increased frequency in plasma of patients with CIN III and carcinoma.
Conclusions: Our data indicate that a shift to a Th2-type cytokine pattern during the carcinogensis of cervical cancer occurs in women with CIN III lesions.

INTRODUCTION

It is well established that high-risk (hr) HPV types are causative for the development of cervical cancer[1-3]. The majority of HPV infections are cleared without further consequences for the host, but a certain percentage of untreated infections with (hr) HPV types may give rise to high-grade cervical intraepithelial neoplasia (CIN II-III) and cervical cancer[4-6].

There is evidence that cell-mediated immune responses of the host, both systemic and local, are important determinants for the course of the infection[7]. Cell-mediated immune responses are regulated by T lymphocytes [T-helper (Th) lymphocytes and cytotoxic lymphocytes (CTLs)] in cooperation with antigen-presenting cells (APCs). The immune response is mediated through the release of different cytokines, which can influence each other's synthesis and actions in the setting of an immunoregulating cytokine network. Cytokines in immune responses to infection are often classified as immuno-stimulating (tumor-suppressing) Th1-type cytokines such as IFN-γ, TNF-α, IL-2 and IL-12, which mainly induce cell-mediated immunity, and Th2-type cytokines (IL-4, IL-5, IL-6, IL-8, Il-10), which are immuno-inhibitory for cell-mediated responses and predominantly induce humoral immunity[8,9].

Qualitative and quantitative analysis in Th1-type and Th2-type cytokine profiles have been used to determine the immune response in several human diseases, including HPV-associated CIN[10,11]. Studies describing circulating cytokines in plasma of patients with cervical dysplasia or cancer are either scarce, deal with one or only a few cytokines, or are contradictory and only incidentally related to hrHPV[12-20]. In the present study we analysed changes in the peripheral cytokine pattern in hrHPV positive women with different stages of CIN compared to hrHPV positive and negative controls.

MATERIALS AND METHODS

Patients and controls

Women with abnormal cervical cytology referred to the outpatient clinic of the Gynaecology Department of the Erasmus University Medical Center Rotterdam between July 2000 and August 2002 were selected as the study group. HPV sampling and a cervical biopsy were obtained from all participating women. Histology results were defined as mild dysplasia (CIN I), moderate dysplasia (CIN II), severe dysplasia (CIN III) or (micro) invasive cancer. An experienced pathologist revised all histological samples.

Our selection of study persons was based on the presence of hrHPV and the severity of cervical intraepithelial neoplasia. Women with CIN I lesions (mild dysplasia)

were excluded, since more than fifty percent of our patients with CIN I turned out to be hrHPV negative. Healthy women who entered the outpatient clinic for a regular sterilisation procedure were recruited as controls. Cervical cytology, histology and HPV sampling were obtained. Exclusion criteria for all participants were: postmenopausal state, pregnancy at time of sampling, chronic diseases (diabetes, allergy, auto-immune), signs of acute infection at time of sampling and an immune-compromised state. Two women with allergy, one woman with an auto-immune disease and one women with an acute infection at time of sampling were excluded. With the exception of oral anticonceptives patients did not use medication on a regular base. Pain-medication (NSAID's) was omitted for at least two weeks before sampling.

The study protocol was approved by the Hospital and University Ethics Committee and all women voluntarily gave signed informed consent.

HPV-sampling and determinations

Cervical scrapes for HPV detection and typing were taken by a cervical biosampler (Accellon Combi® Medscand Medical, Sweden). HPV testing was performed with the consensus GP5+/ GP6+ PCR enzyme immunoassay (EIA) for 37 (sub)types as previously described[21]. This test is clinically validated[22]. We used β-globin PCR to identify sampling errors and to monitor for PCR inhibitors. Additionally, reverse line blot (RLB) analysis was performed on PCR-EIA positive cases to identify individual HPV types.

Blood sampling and processing

Peripheral venous blood samples were collected between 8 and 12 a.m. in sterile endotoxin-free vacutainers (Becton Dickinson, Meylan) with ethylene-diaminetetra-acetic acid (EDTA) as anticoagulant and immediately centrifuged at 1500 g for 10 min at 4°C. Plasma samples were stored at –80°C until assayed. A differential leukocyte count was performed in all blood samples with a Sysmex XE-2100.

Cytokine determinations

All plasma samples were analyzed by commercially available enzyme-linked immunoassays (Biosource Europe, Nivelle, Belgium) for the cytokines TNF-α, soluble TNF-α receptors RI and RII, IFN-γ, IL-2, IL-4, IL-10 and IL-12.[23] The detecting antibody in the immunoassay for IL-12 recognized the bioactive heterodimeric (p40+p35) cytokine as well as the subunit p40 homodimer. According to the manufacturer the minimal detectable concentrations (MDCs) and intra- and inter - assay coefficients (CVs) of variation were as follows: TNF-α: MDC = 3 pg/ml, CVs <6 and <10%; sTNFRI: MDC = 50 pg/ml, CVs <7 and <10%; sTNFRII: MDC = 100 pg/ml,

CVs <7 and <10%; IFN-γ: MDC = 2 pg/ml, CVs <5 and <10%, IL-2: 7 pg/ml, VCs <6 and <10%; IL-4: MDC = 2 pg/ml, CVs <5 and < 7%, IL-10: MDC = 1 pg/ml, CVs <5 and <10%; IL-12+p40: MDC = 1.5 pg/ml, CVs <10 and <10%.

For calculations of cytokine ratios values below the detection limit were assigned concentrations equal to one-half of the lower detection limit of each assay.

Statistical analysis

Cytokine data are presented as medians with ranges unless stated otherwise. The non-parametric Kruskal-Wallis test and Mann-Whitney's U-test were used as appropriate to assess differences in cytokine levels between groups, Spearman's rank test to investigate correlations between cytokine variables. A two-tailed p-value of 0.05 was chosen to represent statistical significance. Differences in leukocytes and leukocyte subpopulations were evaluated by unpaired two-tailed T-tests.

RESULTS

The study groups

Thirty-three women were included into our study groups: 10 women with moderate dysplasia (CIN II), 11 women with severe dysplasia (CIN III) and 12 women with cervical carcinoma (10 squamous cell carcinoma, 2 adenocarcinoma). The control groups consisted of 11 women without cervical dysplasia without hrHPV and 10 women without cervical dysplasia but with a positive hrHPV test. The mean age was 32.4 ± 6.2 years for hrHPV-negative control women, 27.9 ± 7.4 years for the hrHPV-positive control group, 33.4 ± 5.7 years for CIN II patients, 32.3 ± 5.5 years for CIN III patients and 35.6 ± 7.2 years for women with invasive carcinoma.

Results of HPV determinations

HPV 16, 18 and 31 were the most frequently detected hrHPV types [n=18 (42%), n=7 (16%) and n=8 (19%) of all infections respectively]. Other high risk types were less common: HPV 33 n=2, HPV 35 n=1, HPV 39 n=2, HPV 45 n=1, HPV 51 n=1, HPV 52 n=2, HPV 56 n=6, HPV 58 n=1, HPV 59 n=2, and HPV 82 n=1. HPV 70 (lr type) was detected together with HPV 16 in one woman with CIN III and as single infecting agent in one of 12 carcinoma patients. There were 77% single and 23% multiple HPV infections. HPV-typing within study groups is summarized in table 1.

Table 1. HPV-typing in patients with cervical dysplasia (CIN II, CIN III, Carcinoma) and in hrHPV positive controls.

CONTROLS	CIN II	CIN III	CARCINOMA
n=10	n=10	n=11	n =12
hrHPV-type	hrHPV-type	hrHPV-type	hrHPV-type
31	**31**	**31,56**	**16**
31	**31**	**31,18**	**16**
31	**16**	**16**	**16**
31,51,56	**16**	**16**	**16**
56	**16**	**16**	**18**
59,58,56	**16**	**16**	**18**
59	**16,45,56**	**16**	**18**
35,56	**18,39**	**16**	**18**
52	**39**	**16**	**18**
16	**52**	**16,(70)**	**33**
		33	**(70)**
			82

The differential white blood cell count

The total leukocyte count was higher in patients with CIN III and carcinoma than in CIN II - and control groups; the difference reached statistical significance between CIN II and CIN III. The neutrophil count showed a similar course with a significant increase between CIN II and carcinoma patients. Absolute monocyte - and lymphocyte counts varied significantly between groups, with a maximum in women with CIN III. The results of differential white blood cell counts are presented in Figure 1.

The cytokine profile

The results of cytokine assays were calculated per 10^6 leukocytes, in order to stratify for possible different amounts of cytokine-producing leukocytes between study subjects.

Analysis of the peripheral cytokine profile in our study groups revealed different courses for each of the eight investigated cytokines. Differences between groups were observed for IFN-γ levels, with a significant decrease from CIN II to CIN III and carcinoma ($p \leq 0.01$) as summarized in Figure 2.

There were no statistically significant differences in plasma levels of TNF-α between control and study groups. Both TNF receptors were significantly correlated in all groups [r=0.973, p=0.000 for hrHPV negative controls, r=0.855, p=0.002 for hrHPV positive controls, r=0.979, p=0.001 for patients with CIN II, r=0.791, p=0.004 and r=0.748, p=0.005 for patients with CIN III and invasive carcinoma,

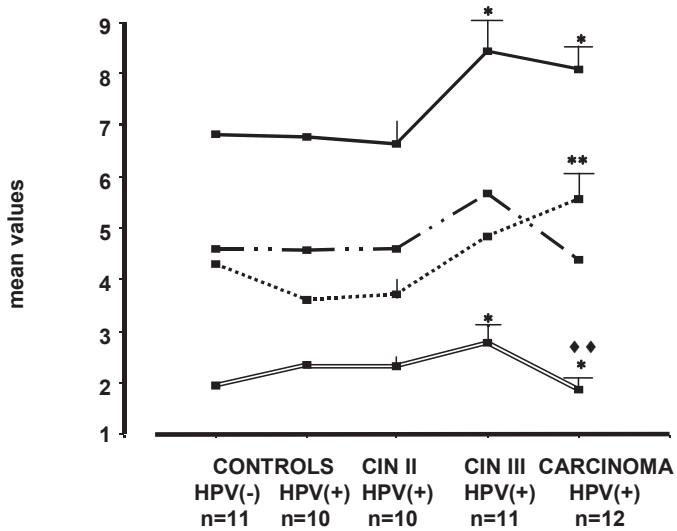

Figure 1. Differential white blood cell counts in hrHPV positive patients with cervical dysplasia and in hrHPV negative and hrHPV positive controls.
—— :leukocytes (x 10^6/ml); –··–··– : neutrophils (x 10^6/ml); ····· : monocytes (x10^5/ml); ═══ :lymphocytes (x10^6/ml). Data are presented as mean ± S.E.M
∗ $p \leq 0.05$ when compared with CIN II ∗∗ $p < 0.01$ when compared with CIN II
♦ $p \leq 0.05$ when compared with CIN III ♦♦ $p < 0.01$ when compared with CIN III

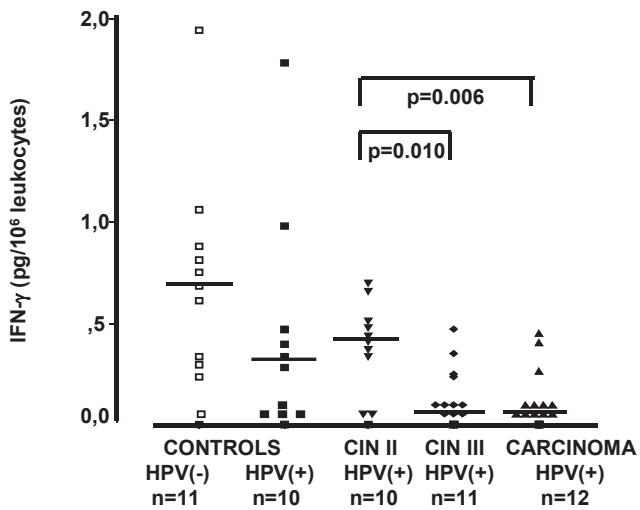

Figure 2. Plasma concentrations of IFN-γ in hrHPV positive patients with cervical dysplasia and in hrHPV negative and hrHPV positive controls. Median values are indicated by a horizontal line.

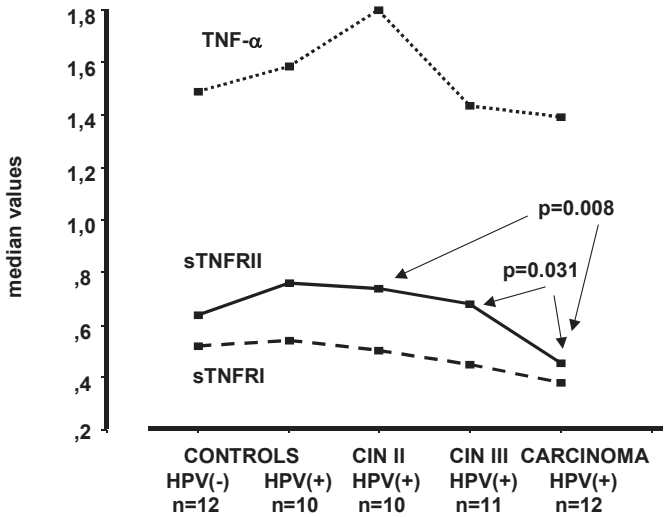

Figure 3. Plasma concentrations of TNF-α and its soluble receptors sTNFRI and sTNFRII in hrHPV positive patients with cervical dysplasia and in hrHPV negative and hrHPV positive controls. Median concentrations of TNF-α as 10^6 pg/ml; of sTNFRI as 10^6 ng/ml; of sTNFRII as 10^6 ng/ml.

Table 2. Plasma concentrations of IL-4 and IL-10 in hrHPV positive patients with cervical dysplasia and in hrHPV negative and positive controls

Group	n	IL-4 > det.limit* in x/n plasmas	IL-10 > det.limit* in x/n plasmas
Controls hrHPV(-)	11	2 / 11	2 / 11
Controls hrHPV(+)	10	1 / 10	0 / 10
CIN II hrHPV(+)	10	2 / 10	0 / 10
CIN III hrHPV(+)	11	1 / 11	4 / 11
Carcinoma hrHPV(+)	12	3 / 12	7 / 12

*Detection limit for IL-4: 2 pg/ml *Detection limit for IL-10: 1 pg/ml

respectively]. TNF-receptor RII levels showed a maximum in hrHPV positive controls. A significant decrease was seen in patients with cervical carcinoma compared to cases with CIN II. The same course as for receptor RII was observed for plasma concentrations of receptor RI but differences did not reach statistical significance. Median plasma concentrations of TNF-α and its two soluble receptors sTNFRI and sTNFRII in all study groups are presented in Figure 3.

There was a significant increase (p=0.037) in plasma concentrations of IL-2 between hrHPV negative and hrHPV positive controls but no other significant changes in IL-2 levels. IL-12 showed no significant differences between groups. IL-4 concentrations were below the detection limit in most of the investigated plasmas. IL-10

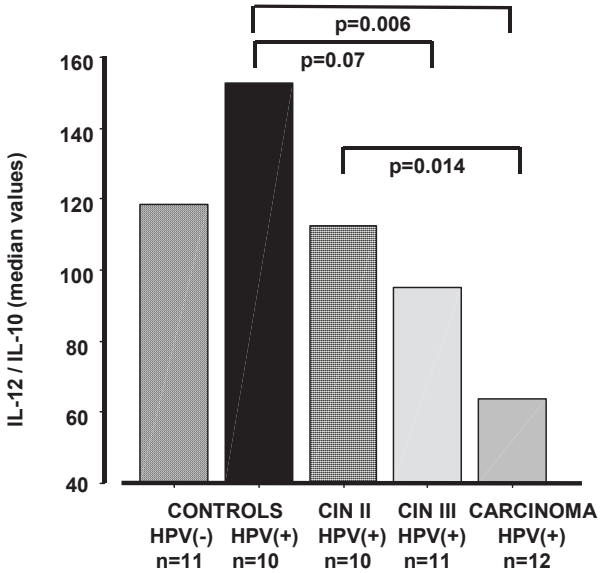

Figure 4. IL-12/IL-10 ratio in plasma of hrHPV positive patients with cervical dysplasia and in hrHPV negative and hrHPV positive controls.

values were below the detection limit in most control women and women with moderate dysplasia, but were detectable in 4/11 patients with CIN III (36%) and 7/12 invasive carcinomas (58%). Data for IL-4 and IL-10 are presented in Table 2.

A possible shift to an increased expression of Th2-type cytokines in patients with carcinoma was evaluated by calculating the ratio of Th1-type cytokines IL-12, IFN-γ, TNF-α and IL-2 to Th2-type cytokine IL-10 for all study groups. These ratios decreased significantly ($p<0.01$ for IFN-γ / IL-10, $p=0.02$ and $p<0.01$ for TNF-α / IL-10, $p=0.03$ and $p=0.06$ for IL-2 / IL-10) when patients with carcinoma were compared to hrHPV positive controls or patients with CIN II. Figure 4 shows as an example the ratio of IL-12/IL-10.

DISCUSSION

Our data for the differential white blood cell count in our patients are in agreement with data described by Onsrud et al. who reported increased leukocyte and neutrophil counts in cervical carcinoma, probably induced by inflammation and necrosis, and a decrease in absolute lymphocyte numbers in comparison with healthy controls[24]. Studies by Gemignani and Balaram confirm these results[25,26]. Stratification of cytokine data per 10^6 leucocytes seems justified.

Our study showed a significant decrease in circulating IFN-γ levels in women with severe dysplasia and invasive carcinoma. IFN-γ, secreted by Th1 cells, CTLs and stimulated NK-cells, is a major contributor to an effective Th1-type cellular immune response against HPV infections[7]. Defective IFN-γ production may be associated with persistent HPV infection and development of HPV-related neoplasia[27]. Our data for circulating IFN-γ agree with these reports.

TNF-α has been shown to act synergistically with IFN-γ on cervical cancer cells by inducing apoptosis or necrosis[28]. Biological effects of TNF-α are mediated by its two receptors TNFRI and TNFRII. Both receptors are present on many cell types and tissues; receptor RI is especially expressed on cells that are sensitive for cytotoxic actions of TNF-α, whereas receptor RII is strongly expressed in stimulated T- and B cells[29]. Several agents including TNF-α itself down-regulate cell surface expression of both receptors by proteolytic cleavage of the extra cellular domains that are shed as soluble proteins sTNFRI and sTNFRII into the circulation. Measurement of both soluble receptors, especially of sTNFRII, is often used to assess Th1-type activity in cell-mediated immune response[29-31]. Our study did not show significant differences in TNF-α values between study groups. However, in the plasma of carcinoma patients we observed a decrease in both soluble receptor levels that reached statistical significance for sTNFRII. This result reflects a decrease in circulating TNF-α and is in accordance with a disturbed Th1-type like immune response after progression to CIN III and invasive carcinoma.

There were no significant differences in peripheral concentrations of the Th1-type cytokine IL-2 between our study groups, except a slight increase (p=0.037) between hrHPV negative and hrHPV positive controls that could indicate an inflammatory response to the viral infection.

A possible shift to Th2-type cytokines in course of development of cervical neoplasia may be reflected by increased plasma concentrations of Th2-type cytokines IL-4 and IL-10. Although, we did not observe any significant differences in IL-4 concentrations between our study groups. IL-10 concentrations increased significantly with the severity of dysplasia. IL-10 is a potent immunosuppressive cytokine, which inhibits T cell activation and Th1 cell differentiation. This shift to Th2-type activity was characterized by Jacobs et al. as a significant decrease in the IL-12 / IL-10 ratio in patients with high-grade SIL[20]. IL-12, a key cytokine in the induction of cell-mediated immunity promotes T-cell differentiation into IFN-γ producing Th1 cells and induces IFN-γ production in NK cells. The significant decrease in IL-12, IFN-γ, TNF-α and IL-2 relative to IL-10 in CIN III and carcinoma patients as observed in our study supports and extends the finding of Jacobs et al. of a disturbed cellular immune response in the development of severe cervical dysplasia and cervical carcinoma. The presence of diseases that could influence circulating cytokine levels was

excluded in all participants. This let us assume that our results are the consequence of HPV infection.

Our results for changes within the peripheral cytokine network in cervical dysplasia and invasive cancer are not always in accordance with published data for individual cytokines [12-20]. In a study by Niwa et al. IFN-γ levels in plasma of patients with cervical cancer and of controls were similar, while in a study by Lebrecht et al. IFN-γ concentrations were below the detection limit in all groups [12,13]. Our results for plasma concentrations of TNF receptor I were in accordance with the results of Sheu et al. who found significantly lower serum levels of sTNFRI in patients with cervical cancer when compared with women with a benign disease, uterine myoma [17]. However, they differ from results of Malejczyk et al. [18]. In the latter study patients with HPV-16 associated CIN I/II and CIN III lesions and with HPV-16/18 associated squamous cervical cancer were compared with healthy controls. There were no significant differences in receptor levels between women with CIN lesions and controls, but both receptor concentrations increased significantly in patients with cervical cancer. Important factors that contribute to differences in results compared with earlier studies include selection criteria for controls and patients.

In our study we selected patients and controls according to their state of infection with hrHPV. The single presence of lrHPV type 70 in one woman with cervical carcinoma could be explained by hrHPV-DNA integrated via the L1 region in the cellular genome, which can be missed by the GP5+/6+ PCR. With the exception of the studies of Jacobs et al. and Maleczky et al. in none of the above- mentioned studies was the presence of HPV investigated [18,20]. Moreover, stratification for leukocyte number might be an explanation. Immunocompetent leukocytes are the main producers of circulating cytokines. In contrast to all other studies we calculated our results per leukocyte count and not per ml plasma. Finally, analytical methods for quantification of cytokines may differ. Some studies used plasma, others serum, a choice that influences quantitative outcome (unpublished results). All studies used enzyme-linked immunoassays (ELISAs), which were produced by different manufacturers. This implies the use of different antibody systems for cytokine binding and quantification with different sensitivities.

Our results indicate a change to a Th2 cytokine pattern already manifest in women with CIN III lesions. It provides the theoretical base of the clinical policy to treat all women with CIN III lesions. Furthermore, it might indicate that therapeutically vaccinations in cervical cancer patients should be directed on reversal of the Th2 response into a Th1 response and may be useful already in women with CIN II lesions.

In conclusion: Our study showed characteristic changes within the peripheral cytokine network during the course of hrHPV infection through different stages of dysplasia to invasive carcinoma: a significant decrease in circulating IFN-γ and sTNFRII and an increase in IL-10 concentrations. This indicates a shift to a Th2-type immune response in the development of cervical cancer. The changes in cell-mediated immunity become manifest in patients with CIN III, which is in line with the clinical observation that most CIN III lesions do not spontaneously regress but may ultimately progress into invasive carcinoma if not properly treated[32].

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Chapter 5.2

The role of CIN III in cytokine response modification during carcinogenesis of cervical cancer, investigated in PHA-stimulated whole blood cultures

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Submitted

ABSTRACT

Aims: The aim of the study was to investigate the effect of HR-HPV infection on the capacity of the cytokine network in whole blood cultures of women with different stages of cervical intraepithelial neoplasia (CIN). Of special interest were the effect of HR-HPV infection in women without clinical manifestation of dysplasia, possible changes in severe dysplasia (CIN 3), and a possible revival of inflammatory cytokine activity in cervical carcinoma.
Materials and Methods: Thirty-nine women with moderate dysplasia, severe dysplasia, cervical carcinoma or without dysplasia formed the study group. The control group consisted of 10 hrHPV negative women without CIN. Whole blood cultures were stimulated with phytohemagglutinin (PHA) and concentrations of tumour necrosis factor α (TNFα), interferon γ (IFNγ), interleukin 2 (IL-2), interleukin 12 (IL-12), interleukin 4 (IL-4) and interleukin 10 (IL-10) were determined by ELISAs.
Results: A significant increase in cytokine release was detected in HR-HPV positive women without dysplasia.
After initial infection, minimum release of IFNγ, TNFα and IL-12 was observed in CIN III and IL-2 in invasive carcinoma; IL-4 and IL-10 reached a maximum in patients with CIN III and decreased significantly in patients with carcinoma. Most Th1-type/Th2-type ratios decreased form CIN II to CIN III, and increased from CIN III to invasive carcinoma.
In women with cervical cancer, release of IFNγ and IL-12 was of the same magnitude as in HR-HPV positive women without clinical manifestations, but release of TNFα, IL-2, IL-4 and IL-10 was significantly lower.
Conclusions: Our study suggests: 1. Infection with HR-HPV without expression of cervical dysplasia induces activation of the cytokine network;
2. A fundamental change in the immuno-competence of women with cervical dysplasia occurs in CIN III;
3. Manifestation of a malignant tumour induces a second immune response that seems to be deregulated and incompetent.

INTRODUCTION

It is well established that high-risk (HR) human papillomavirus (HPV) types are causative for the development of cervical cancer [1-3]. The majority of HPV infections are cleared without further consequences for the host, but some infections with HR-HPV types may give rise to high-grade cervical intraepithelial neoplasia (CIN III) and cervical cancer [4-6]. There is evidence that cell-mediated immune responses of the host, both systemic and local, are important determinants for the course of the infection [7].

Cell-mediated immune responses are regulated by T lymphocytes [T-helper (Th) lymphocytes and cytotoxic lymphocytes (CTLs)] in cooperation with antigen-presenting cells (APCs) [monocytes (MCs) and dendritic cells (DCs)]. These cells all release cytokines that can influence one another's synthesis and actions in the setting of an immuno-regulating cytokine network. Cytokines in immune responses to infection are often classified as immuno-stimulating (tumour-suppressing) Th1-type cytokines and immuno-inhibitory (tumour-promoting) Th2-type cytokines. Th1-type cytokines such as interferon γ (IFNγ), tumour necrosis factor α (TNFα), interleukin 2 (IL-2), and IL-12 are produced mainly by lymphocytes, APCs and natural killer cells (NK-cells). They induce and exhibit cell-mediated immunity. Th2-type cytokines (IL-4, IL-5, IL-6, IL-8, Il-10), produced by lymphocytes and MCs, are immuno-inhibitory for cell-mediated responses and predominantly induce humoral immunity [8,9].

Qualitative and quantitative analyses of cytokine profiles have been used to characterize the immune response in HPV-related CIN. These were performed with peripheral blood mononuclear cells (PBMCs) [10-12] or with T-cell fractions isolated from PBMCs [13-16] and occasionally with whole blood cultures [17] after stimulation with several antigens. Selective cytokines, mostly IFNγ [11,12,14-18], IL-2 [10-14] and occasionally the APC-derived IL-12 [17] or TNFα [14] were measured together with one or two of the typical Th2-type cytokines IL-4, IL-5 and IL-10 [11,12,16,17]. Generally a shift from a Th1-type to a Th2-type cytokine response was observed when healthy controls or women with low grade squamous intraepithelial lesions (LSIL) were compared with cases of high grade SIL (HSIL) or cervical carcinoma [7,11,17,19].

We previously observed manifestation of a Th2-type cytokine pattern in plasma of HR-HPV positive women during carcinogenesis of cervical cancer at the stage of CIN III [20]. Recent studies with isolated T-cell fractions stimulated with HPV16-derived oncopeptides described a reactivation of an inflammatory response in patients with carcinoma [12,15].

In the present study we used whole blood cultures from HR-HPV negative controls, HR-HPV positive women without cervical dysplasia and HR-HPV positive patients with different grades of CIN and cervical cancer to investigate possible

changes in the capacity of circulating immunocompetent leukocytes to release cytokines in response to a mitogenic challenge. Of interest were the effect of HR-HPV infection without clinical manifestations, the special position of CIN III with a Th-2 type cytokine response and a possible revival of inflammatory cytokine activity in cervical carcinoma.

MATERIAL AND METHODS

Patients and controls

Inclusion took place at the outpatient clinic of the Gynaecology Department of the Erasmus University Medical Centre Rotterdam between July 2000 and August 2002. Our selection of patients for this study was based on the presence of HR-HPV and the grade of cervical intraepithelial neoplasia. HPV sampling and a cervical biopsy were carried out on all participating women. Histology results were defined as no dysplasia, mild dysplasia (CIN I), moderate dysplasia (CIN II), severe dysplasia (CIN III) or (micro) invasive cancer. An experienced pathologist revised all histological samples. Women with CIN I lesions (mild dysplasia) were excluded since more than fifty percent of our patients with CIN I turned out to be HR-HPV negative.

Healthy women who attended the outpatient clinic for a regular sterilisation procedure were recruited as HR-HPV negative controls after sampling for histology and HPV. Exclusion criteria for all participants were (anamnestic required): postmenopausal state, pregnancy at time of sampling, chronic diseases (diabetes, allergy, auto-immune), presence of sexually transmitted diseases (STDs) and infection with human immunodeficiency virus (HIV), signs of acute infection at time of sampling, and an immune-compromised state. With the exception of oral contraceptives no participant used medication on a regular base. No participant had used pain-medication (including NSAID's) for at least two weeks prior to sampling. The study protocol was approved by the Ethics Committee of the Erasmus Medical Center and all women voluntarily gave signed informed consent.

HPV-sampling and determinations

Cervical scrapes for HPV detection and typing were taken using a cervical biosampler (Accellon Combi® Medscand Medical, Sweden). HPV testing was performed with the consensus GP5+/GP6+ PCR enzyme immunoassay (EIA) using a cocktail probe covering all (probably) hrHPV types, as previously described[21]. This test is clinically validated[22]. We used β-globin PCR to identify sampling errors and to monitor for PCR inhibitors. Additionally, reverse line blot (RBL) analysis was performed on PCR-EIA positive cases to identify individual HPV types.

Blood sampling

For the preparation of whole blood cultures peripheral venous blood samples were collected between 08.00 and 12.00 hours a.m. in sterile endotoxin-free vacutainers (Endo Tubes Chromogenix AB, Mölndal, Sweden) coated with Na-heparin as anti-coagulant, and immediately processed.

For a leukocyte count peripheral venous blood samples, collected between 8 and 12 a.m., were drawn into endotoxin-free vacutainers (Becton-Dickinson, Meylan) with ethylene-diaminetetra-acetic acid (EDTA) as anticoagulant and leukocyte counts performed with a Sysmex XE-2100.

Whole blood cultures

For preparation of whole blood cultures blood was diluted 1:10 with RPMI 1640 culture medium with 25 mM Hepes, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 4 mM l-glutamine (medium and supplements from Life Technologies BV, Breda, The Netherlands). Diluted blood was distributed in cell culture plates and incubated with phytohemagglutinin (PHA) (Sigma, St.Louis, MO) dissolved in RPMI medium to a final concentration of 10 µg/ml blood culture, for 96 hours at 37°C and 5% CO_2. Blood cultures without PHA were run as controls. All cultures were sampled at 0, 24, 48, 72 and 96 hours, centrifuged for 10 min at 4°C and 1500 g, and culture supernatants kept at –80°C until analysis.

Cytokine determinations

All samples were analysed by commercially available enzyme-linked immunoassays (Biosource Europe, Nivelle, Belgium) for the cytokines TNFα, IFNγ, IL-2, IL-4, IL-10 and IL-12 [23]. The detecting antibody in the immunoassay for IL-12 recognized the bioactive heterodimeric (p40+p35) cytokine as well as the subunit p40 monomer or homodimer. According to the manufacturer the minimal detectable concentrations (MDCs) and intra- and inter - assay coefficients (CVs) of variation were as follows: TNFα: MDC, 3 pg/ml; CVs, <6 and <10%; IFNγ: MDC, 2 pg/ml; CVs, <5 and <10%; IL-2: MDC, 7 pg/ml; VCs, <6 and <10%; IL-4: MDC, 2 pg/ml; CVs, <5 and < 7%; IL-10: MDC, 1 pg/ml; CVs, <5 and <10%; IL-12+p40: MDC, 1.5 pg/ml, CVs, <10 and <10%.

Statistical analysis

Preliminary Komolgoroff-Smirnov tests showed a non-normal distribution of cytokine values in PHA-stimulated whole blood cultures. Accordingly, cytokine data are presented as medians with ranges unless stated otherwise. The non-parametric Kruskal-Wallis test (K.W. test) and Mann-Whitney's U-test were used as appropriate to assess differences in cytokine levels between groups. Levels of statistical

significance were adjusted for the number of comparisons according to Bonferroni's method, as indicated in the graphics. Differences in patient characteristics between groups were evaluated by one-way ANOVA and unpaired two-tailed T-tests. Spearman's correlations were used to investigate possible relations between age at time of sampling and released cytokines.

RESULTS

The study groups

Thirty-five patients with different grades of CIN were selected. Five of them were excluded because of diabetes (n=1), allergy (n=2), auto-immune disease (n=1) or acute infection at time of sampling (n=1), leaving 30 women eligible for inclusion: 10 women with moderate dysplasia (CIN II), 10 women with severe dysplasia (CIN III) and 10 women with cervical carcinoma (8 squamous cell carcinoma, 2 adenocarcinoma). All women of this group revealed a positive GP5+/6+ HR-HPV PCR test. We selected 22 healthy women with normal histology. Three of them were excluded because of the presence of allergy (n=2) or acute infection at time of sampling (n=1), leaving 19 healthy women without cervical dysplasia. Nine women had a positive HR-HPV test, 10 women tested negative for HR-HPV, forming the control group. Baseline characteristics of the study groups are summarized in table 1.

Table 1. Baseline characteristics of study groups

	CIN 0 HPV neg	CIN 0 HPV pos	CIN II	CIN III	CA	Statistical significance
	n=10	n=9	n=10	n=10	n=10	
Age at time of sampling*	36.9 (6.1)	27.4 (6.9)	32.5 (5.4)	31.9 (4.5)	34.6 (6.9)	p=0.019
STD in history	0/10	3/9	4/10	3/10	1/10	p=0.172
Use of OCs	5/10	6/9	5/10	6/10	7/10	p=0.139
Smoking at time of sampling	3/10	5/9	6/10	9/10	4/10	p=0.073
HR-HPV types (n)						
16		0	5	7	2	
18		0	2	1	5	
31		4	2	2	0	
other		10	4	3	3	
multiple infections		3	2	3	0	

*years; mean (standard deviation), one-way ANOVA

STD=sexually transmitted disease

OCs=oral contraceptives

The mean age of HR-HPV positive women without cervical dysplasia is significantly lower than in the other groups. This could be expected since first infection without clinical manifestation is frequently observed in young sexually active women. Spearman's correlations between age at time of sampling and released cytokines over the whole group of patients and controls were not significant (data not shown). The changes in immune-competence in our study are not related to age.

The cytokine response

The results of cytokine assays were calculated per 10^6 leukocytes, in order to stratify for possible different numbers of cytokine-producing leukocytes between study subjects [20,24]. Preliminary experiments were carried out on all investigated cytokines to determine the time of peak production in response to PHA stimulation of our whole blood culture system (data not shown). Cytokine concentrations from 0 to 96 hours stimulation time were analysed in at least six randomly chosen study subjects for each stage of CIN. Peak time for TNFα, IFNγ and IL-12+p40 production was 72 hours, for IL-2 48 hours of cultivation time. Maximum release for IL-4 and IL-10 varied between 48 and 72 hours. In general our data of maximum cytokine release are in accordance with kinetic studies of PBMC's [25].

On the basis of these results IL-2 release was determined after 48 hours, release of TNFα, IFNγ and IL-12+p40 after 72 hours, and of IL-4 and IL-10 after 48 and 72 hours of cultivation. For calculations of the latter two cytokines values of maximal release were chosen.

A significant difference in cytokine release was observed between the two groups of women without dysplasia: with the exception of IL-12 all investigated cytokines were significantly increased in HR-HPV positive women. The results are summarized in table 2.

In HR-HPV infected women, release of Th1-type cytokines IFNγ, TNFα and IL-2 decreased with increasing grades of CIN. IFNγ increased again from CIN III to carcinoma. IL-12 reached a maximum in CIN II and decreased in CIN III and carcinoma.

Table 2. Influence of HR-HPV infection on the cytokine network in women without cervical dysplasia

GROUP	N	IL-12+p40*	IFN-γ *	TNF-α*	IL-2 *	IL-10*	IL-4*
HR-HPV(-)	10	25 (13-170)	2406 (364-10795)	433 (68-1714)	32 (3-192)	58 (11-140)	3 (0.4-14)
HR-HPV(+)	9	32 (11-106)	6378 (3064-30452)	723 (504-2244)	183 (55-378)	15 (86-221)	9 (4-89)
statistical significance		**p =** 0.87	**0.001**	**0.006**	**0.009**	**0.027**	**0.034**

*pg/10^6 leukocytes, values median (range)

But the differences between groups were statistically not significant (K.W.test: p=0.068 for IL-12+p40, p=0.264 for IFNγ, p=0.077 for TNFα and p=0.071 for IL-2). The Th2-type cytokines IL-4 and IL-10 behaved differently. Release reached a maximum for IL-10 and IL-4 in patients with CIN III and decreased significantly for both cytokines in patients with invasive carcinoma. (K.W.test: p=0.019 for IL-10 and p=0.033 for IL-4).The results are summarized in figure 1.

In order to characterize a possible Th1-type / Th2-type shift we calculated the ratios of Th1-type cytokines IL-12, IFNγ, TNFα and IL-2 to Th2-type cytokines IL-10 and IL-4 in HR-HPV infected groups. (Results of K.W.tests: IL-12 / IL-10 p=0.005, IL-12 / IL-4 p=0.01, IFNγ / IL-10 p=0.013, IFNγ / IL-4 p=0.015, TNFα / IL-10 p=0.303, TNFα / Il-4 p=0.096, IL-2 / IL-10 p=0.642, IL-2 / IL-4 p=0.251). There was a significant decrease in Th1-type / Th2-type ratios between with CIN II and CIN III for IL-12 / IL-4 and IL-12 / IL-10. IFNγ / IL-4, and IFNγ / IL-10 showed a similar though statistically not significant trend as demonstrated in figure 2. Values increased again in invasive carcinoma compared to CIN III. This increase was significant for IL-12 / IL-4, IL-12 / IL-10, IFNγ / IL-4 and IFNγ / IL-10.

In order to characterize a possible Th-1 type cytokine pattern after establishment of an invasive carcinoma we compared cytokine levels in PHA-stimulated blood cultures of patients with invasive carcinoma with levels in HR-HPV positive women without dysplasia. There was no difference between levels of IL-12+p40 and IFNγ between both groups, but release of TNFα and IL-2 as well as of IL-10 and IL-4 was significantly lower in patients with carcinoma. The results are summarized in table 3.

Table 3. Cytokine levels in PHA-stimulated blood cultures of CIN (-) women with HR-HPV infection and women with invasive cervical carcinoma

GROUP	N	IL-12+p40*	IFN-γ*	TNF-α*	IL-2*	IL-10*	IL-4*
CIN(-) HR-HPV(+)	9	32 (11-106)	6378 (3064-30452)	723 (504-2244)	183 (55-378)	115 (86-221)	9 (4-89)
Carcinoma	10	43 (7-170)	6975 (509-13004)	497 (110-1127)	50 (8-220)	63 (20-342)	4 (0.3-13)
statistical significance	**p =**	0.37	0.33	**0.011**	**0.009**	**0.011**	**0.011**

*pg/10^6 leukocytes, values median (range).

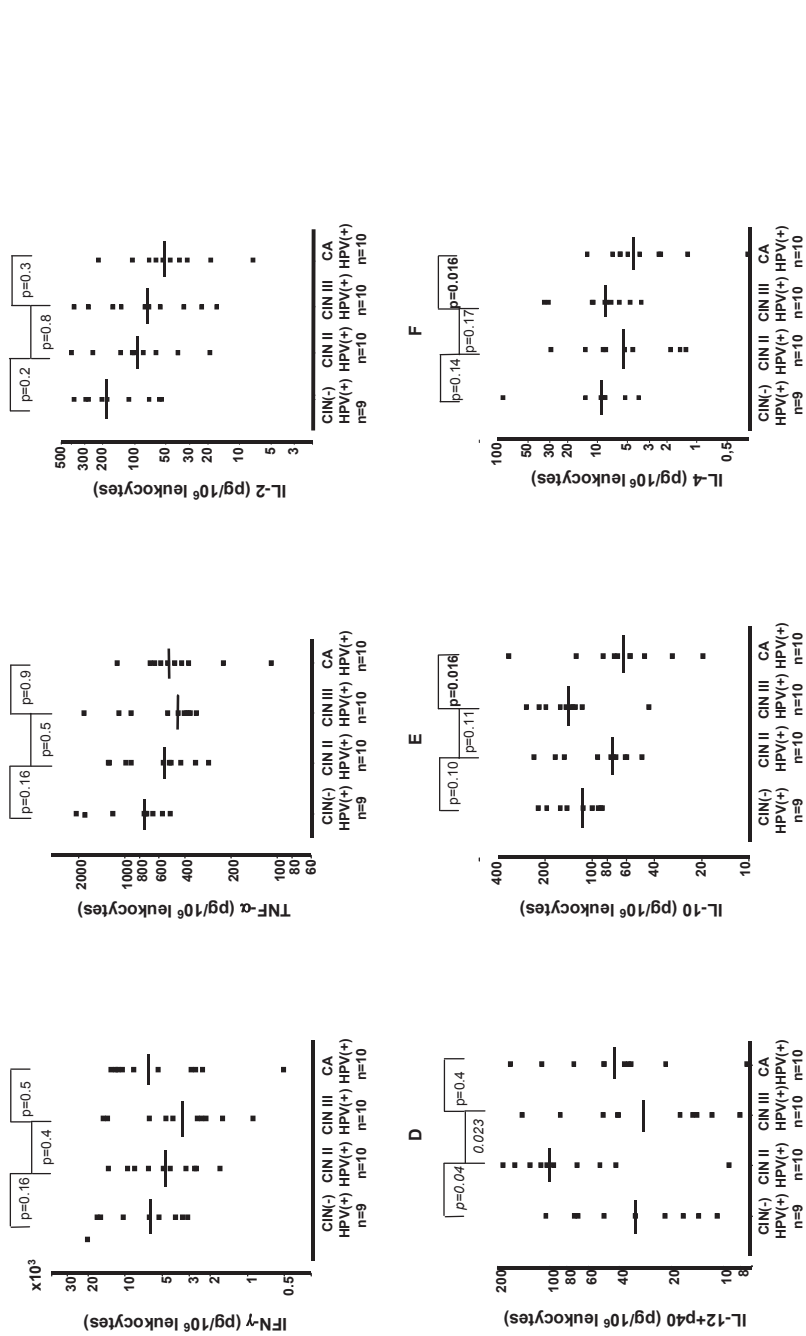

Fig 1. Release of cytokines in full blood cultures of HR-HPV positive women without and with cervical dysplasia or cervical cancer. Blood cultures were stimulated with 10 μg PHA per ml. Logarithmic scale. A horizontal line indicates median values. Comparisons are significant with p<0.017. A: IFNγ, B: TNFα, C: IL-2; D: IL-12+p40; E: IL-10; F: IL-4.

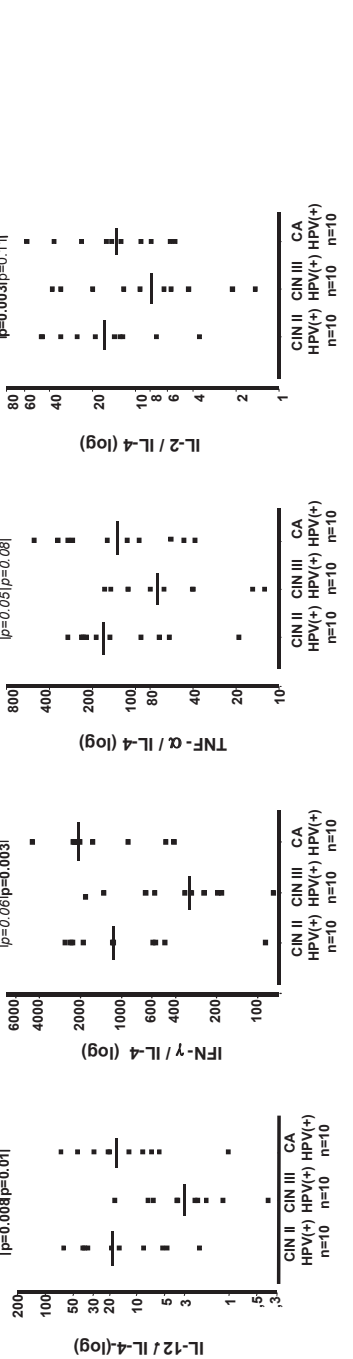

Fig 2. Cytokine ratios in full blood cultures of HR-HPV positive women with cervical dysplasia and cervical cancer. Blood cultures were stimulated with 10 µg PHA per ml. Logarithmic scale. A horizontal line indicates median values. Comparisons are significant with p<0.025. A: IL-12 / IL-4; B: IFNγ / IL-4; C: TNFα / IL-4; D: IL-2 / IL-4; E: IL-12 / IL-10; F: IFNγ / IL-10; G: TNFα / IL-10; H: IL-2 / IL-10.

DISCUSSION

The significant increase in Th1-type as well as Th2–type cytokines in our HR-HPV positive women with normal histology suggests viral activation of the systemic cytokine network and induction of cell-mediated immunity after initial HR-HPV infection (Table 2). To our knowledge this is the first description of activation of the systemic cytokine network in HR-HPV positive women without dysplasia.

Cytokine release changed in women with CIN III to an anti-inflammatory, tumour-promoting pattern with increased IL-4 and IL-10 secretion. This result is in agreement with earlier studies showing a shift from Th1-type to Th2-type cytokines during carcinogenesis[11,17]. Clerici et al.[11] observed decreased IFNγ and IL-2 and increased IL-4 and IL-10 in mitogen-stimulated cultures of PBMCs isolated from women with CIN III when compared with cultures from HR-HPV negative women. Jacobs et al.[17] described increased IL-10 and decreased IL-12 release in whole blood cultures of patients with HSIL. Tsukui et al.[10] stimulated PBMCs of patients with cervical dysplasia and carcinoma with HPV-16 peptides. They found decreasing IL-2 release with increasing severity of the disease, which is in agreement with our results for IL-2. The observed minimium for IFNγ release in CIN III but not in invasive carcinoma, differs from the observations of an earlier study by Mori et al.[18] where PHA-stimulated IFNγ release from PBMCs in cases of invasive carcinoma was significantly decreased when compared with data from healthy women. In the study of Mori et al. however, the presence of HR-HPV was not investigated, which might explain the difference in results with our study.

A shift to a Th2-type cytokine pattern in CIN III becomes more obvious when the ratios between Th1-type and Th2-type cytokines (Figure 2) are evaluated. They show a tumour-promoting change in cytokine balance, significant for IL-12 / IL-4 and IL-12 / IL-10 and a trend for IFNγ / IL-4, IFNγ / IL-10 and TNFα / IL-4.

The course of IL-12 secretion in our study groups merits consideration. IL-12 is one of the first cytokines released during an innate immune reaction and stimulates a Th-1 type cytokine response in cell-mediated immunity. Our HR-HPV positive women with normal histology demonstrated significantly increased Th1- and Th2-type cytokine release, with the exception of IL-12 which was low. Our observation of high secretion of IL-12 in CIN II might be explained by an observation made by Moscicki et al.[26]. These authors report high levels of IL-12 in cervical mucous in HSIL and hypothesize that high IL-12 levels could represent a defence mechanism in turning on a Th1-type anti-tumour response and, as IL-12 is known to inhibit angiogenesis, preventing growth of a tumour.

It was our goal to study the state of immunocompetence in HR-HPV-infected women representing various stages of cervical carcinoma development by means of their cytokine network. The cytokine network is probably best represented in a whole blood culture. The use of whole blood cultures for determination of mitogen-stimulated cytokine release by immunocompetent leukocytes has distinct advantages over cultures of isolated leukocytes or lymphocytes. It permits interaction between different leukocytes, preserves concentrations of stimulatory and inhibitory mediators, and avoids activation and changes in cell ratios associated with procedures of isolation and purification[23]. For stimulation of the cytokine network we chose the mitogen PHA. PHA activates mainly lymphocytes and induces rapid cell proliferation together with release of inflammatory and immune cytokines. Endotoxin (LPS) as used in Jacobs' study[17], induces mainly inflammatory cytokines but almost no lymphocyte-derived interleukins[27].

The significant increase of the four cytokine ratios between CIN III and carcinoma seems to indicate that the presence of a tumour with an inflammatory reaction and exposure of viral antigens (high viral load) eventually induces a T-cell response. However, this response remains incomplete and finally ineffective, as shown in our cytokine data as presented in table 3. Values of IFNγ and IL-12 release in cervical carcinoma are comparable to data obtained after initial HR-HPV infection; all other cytokine levels remain significantly lower. These results are in agreement with observations of de Jong et al. and Steele et al.[12,15]. Steele et al.[15] studied T-cell responses to HPV 16 oncoproteins by measuring IFNγ release in women with low and high grade CIN and cervical carcinoma and found higher levels of T-cell responses in carcinoma patients compared to high grade CIN cases. A similar observation was made by de Jong et al. who investigated HPV16 positive women[12]. This study reports a higher frequency of HPV16-specific CD4+ T-cell responses in patients with cervical carcinoma than in women with CIN III lesions.

The increase in the IFNγ / IL-4 ratio found in our study was not observed by de Jong et al.[12] when T-cell cultures were stimulated with PHA. In part, this discrepancy might be owing to differences in HR-HPV types within the study groups since De Jong et al. only selected patients infected with HPV 16. Correlations between specific HPV types and IFNγ release, possibly influenced by IFNγ gene polymorphisms, are suspected but not yet fully investigated[28].

Most studies dealing with cytokine patterns in HR-HPV-related cervical neoplasia and cancer concentrate on infections with HPV 16 (the most frequently observed oncogenic HPV type in Caucasian population)[4]. In contrast to these studies we did not select our patients for particular HR-HPV types. The small sample size of our study groups did not permit us to correlate cytokine response with specific HR-HPV-types.

Further studies with enlarged numbers of participants are needed to investigate the individual impact of different HR-HPV-types on the cytokine network.

In conclusion: Our study suggests that infection with HR-HPV in women without cervical dysplasia induces activation of the cytokine network. A fundamental change in the immuno-competence of women with cervical dysplasia is reached in CIN III. These immunological findings are supported by clinical observations: Many CIN I or II lesions usually regress without treatment, whereas CIN III lesions mostly will develop into invasive cancer if not properly treated[29]. Manifestation of a tumour induces a second deregulated and incompetent immune response.

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Chapter 6

General discussion and conclusions

6.1 INTRODUCTION

Development of cancer of the human cervix uteri is preceded by a long period of well-defined premalignant stages. These precursor lesions are easily detectable by cervical cytology, and this has led to the organisation of nation-wide cervical cancer screening programs. These programs have resulted in a substantial reduction in mortality and morbidity of cervical cancer[1-4]. However, due to sampling- and screening errors, false positive and false negative cytology results lead to an excess of diagnostic procedures or a delay in the diagnosis of cervical cancer, respectively[5-7]. Since infection with hrHPV is necessary for development of cervical cancer, it has been postulated that screening might be made more efficient by using a combination of cytology and hrHPV testing[8-10].

HPV testing can be incorporated into screening strategies in a number of ways in order to reduce the risk of detection failure. The HPV test can identify women with cervical disease as well as those who are at risk for developing cervical neoplasia within the next 3 to 10 years[11-13]. Although the majority of HPV infections are transient[14-16], HPV testing can be useful in primary cervical cancer screening to identify women with a persistent infection. Sensitivity for the detection of premalignant lesions may increase[17-19]. The high negative predictive value of HPV testing means that screening intervals can be prolonged and less referrals are necessary[20-24]. Different approaches are possible for the introduction of HPV testing into cervical screening, including combined cytology and hrHPV testing, or primary screening by hrHPV with cytology tests performed only in those women who test positive for hrHPV (= reflex testing). Randomised population based screening trials are on the way to establish more efficient primary cervical screening strategies[9,12,25,26].

6.2 TRIAGE OF BORDERLINE AND MILD DYSKARYOSIS

The population-based screening program in The Netherlands comprised in 2003 over 530,000 cervical smears[27]. Approximately 2% showed borderline or mild dyskaryosis (BMD), and in 31% of these patients the repeat smear was abnormal. For these cases referral was advised. Severe abnormalities were present in 0.5% of the total population screened; these women were referred directly to the gynaecologist.

A high-grade CIN lesion is present in only 10% of women with a repeat BMD smear, and therefore the majority regress spontaneously[22,28,29]. Addition of hrHPV testing could improve the efficiency of triage. We demonstrated that for women with a repeat BMD smear and absence of hrHPV, referral to the gynaecologist is not indicated and follow-up can take place according to the 5-yearly screening interval. Other studies on triage for low cytological abnormalities confirmed these results,

although most of these studies were based on single borderline or ASUS/LSIL cytology results[9,30-33]. For women with a repeat BMD smear who tested hrHPV positive, an additional HPV test after 6 months was recommended. Referral for colposcopy is subsequently only required if persistence of hrHPV is confirmed. Women who clear the hrHPV infection are no longer at risk and further surveillance can take place within the screening program at the protocollized interval of 5 years.

In July 2006, Dutch National guidelines for Pathology were updated in accordance with these data. The Dutch Pathology Association (NVVP), in agreement with general practitioners (NHG) and gynaecologists (NVOG), now advise that only women with a repeat BMD smear positive for hrHPV are to be referred to the gynaecologist[34]. In contrast, the ALTS trial concluded that HPV testing was not of value in the management of women with single LSIL cytology, since over 80% were found to be hrHPV positive. This might be due to the younger age of the women included. The ALTS trial described women with a mean age of 25-29 years, whereas in our study the mean age was 39 years which is representative of our screened population (between 30-60 years). The American Society for Colposcopy and Cervical Pathology (ASCCP) recommend that all women with single LSIL cytology undergo colposcopy instead of HPV testing[29]. For women with ASC-US cytology a consensus management guideline includes repeated cytological assessment, immediate colposcopy and HPV testing as options[35].

Although the inclusion of HPV testing as part of guidelines for women with low grade cytology varies, there is no disagreement about the negative predictive value of HPV testing. All studies show where hrHPV is absent women are not at risk for development of high-grade CIN lesions. Triage with HPV testing early in the management of women with low cytological abnormalities can reduce referrals by more than 50%. As a consequence, the average length of follow-up and the costs decrease. We analysed costs and side effects of HPV triage strategies for women with a repeat BMD smear. We found that HPV triage can decrease the average follow-up time by six months, and reduce the associated costs by a third. However, compliance of general practitioners with more complex diagnostic guidelines, as well as possible difficulty for women in accepting a wait-and-see approach need to be taken into account. Nevertheless, adding hrHPV testing where there is a repeat BMD smear considerably reduces the referral rate and the total costs of management.

6.3 HPV-TESTING FOR FOLLOW-UP AFTER TREATMENT FOR HIGH-GRADE CIN

Ablative or excisional techniques for treatment of premalignant cervical lesions are generally very effective, more than 90% cure rates are reported. However, in

approximately 5-15% the precursor lesions will persist or re-occur, requiring close follow-up and even re-treatment once residual or recurrent high-grade CIN has been established[36]. Although the risk of developing invasive cervical cancer after conservative treatment is low, it is still about five times greater for at least 8 years than that among the general population[37]. Several studies have suggested post treatment CIN strategies incorporating hrHPV testing. According to our review combined testing with HPV and cytology yielded the best test characteristics. This has been confirmed by other studies, where HPV testing alone has been suggested to be more reliable than colposcopy and cytology[38,39]. These findings were obtained in small studies, with short follow-up periods, and warranted further study of implementation of hrHPV testing in post treatment management. In this context, we conducted the first randomised trial for post treatment CIN, whereby combined testing with hrHPV and cytology was compared to conventional cytology. Our study required colposcopic examination when abnormal cytology and hrHPV were detected. Consequently, hrHPV positive cases without cytological cervical changes, or cases with abnormal cytology without a hrHPV infection did not need additional investigation. Combined testing turned out to be more specific and equally sensitive compared to cytology alone. Although the total health-care costs increased when both hrHPV and cytology were used, this outcome was improved by a modified strategy. Women with normal cytology and absence of hrHPV 6 months after treatment, showed no increased risk for development of residual or recurrent CIN within 18 months. Therefore, follow-up 12 months after treatment can be omitted for this group, limiting follow-up to 24 months. In our opinion this represents the best follow-up strategy, total health-care costs decrease because unnecessary examinations are avoided. This strategy might have a positive influence on quality-of-life, on the other hand a longer period without control might reduce compliance. These points remain to be evaluated.

It has been suggested that clearance and progression rates in cervical screening are influenced by presence or absence of particular hrHPV types[40-43]. In addition, an increased risk for development of residual/recurrent CIN was found for HPV16[44]. In these studies close surveillance of women with hrHPV16 is advised. Data for HPV18, HPV31 and HPV33 remain inconclusive. In our study, we could not establish an increased risk of development of post treatment CIN for HPV16 positive women. Although the majority of women who had residual/recurrent CIN were positive for HPV 16, we were unable to demonstrate a statistically significant increase. This might be because of the small numbers of patients with residual/recurrent CIN in our study. To justify a follow-up strategy based on hrHPV type-specific adjustments, these data need to be extended.

6.4 SELF-SAMPLING WITH HRHPV TESTING FOR NON-RESPONDERS

The proportion of women who participate in cervical screening is important for the effectiveness of the program[45]. In 2003 the coverage rate in The Netherlands (proportion of women at risk between 30 and 64 years of age with at least one smear in the preceding 5 years) was 77%, whereas the attendance rate (proportion of women at risk who responded to the screening invitation) was 66%[27]. Consequently, approximately one third of the women at risk do not participate in cervical screening. These so-called non-responders are at high risk for development of cervical cancer, since at least 50% of women diagnosed with cervical cancer have no previous history of participation in cervical screening[12,45,46]. Women who do not respond to invitations for conventional smears may respond to a self-sampling method[47-50]. Although cytology results on self-sampled vaginal material have poor sensitivity than conventional cytology, the material is representative for detection of hrHPV[48-52].

Self-sampling has previously only been studied in outpatient clinic populations. A pilot-study was conducted to investigate whether actual non-responders to the national screening program are willing to perform a self-sampling test[53]. 200 non-responders were selected, 100 of them received a self-sampling package, while 100 were invited for conventional cytology. The results of this study showed a 3-times greater response using self-sampling, compared to regular smears (39% vs. 11%, respectively). Owing to the ethnic diversity in the investigated area (Rotterdam, The Netherlands, with 46% of women of non-Dutch origin, predominantly Turkish (8%) and Moroccan (6%))[54], special attention was paid to women without knowledge of the Dutch language. Since Turkish women formed the majority of the non-Dutch participants, an information letter with explanations translated into Turkish, was included in the self-sampling invitation. This increased the motivation in Turkish women to participate in self-sampling, compared to Turkish participation in conventional cytology.

We implemented self-sampling for HPV testing in non-responders for a larger area of the Dutch screening program (Amstelland, de Meerlanden and Kennemerland) where 2546 women received a self-sampling package and 284 were invited for cytology. We invited all women in Dutch, since only 4% of the population in this area is of Turkish origin. The response to self-sampling was significantly higher than the response in the control group, who received a second reminder for participation in conventional cytology (34% vs. 17%). This indicates that self-sampling can motivate women who are otherwise not willing to participate in cervical screening. When presence of hrHPV was determined, women were invited for additional investigation at the gynaecological outpatient clinic. Although sampling of a conventional smear taken by a general practitioner was previously not acceptable for these non-responders, the majority (86%) was willing to visit the gynaecologist after being informed about the presence of a hrHPV infection.

In our study, the hrHPV prevalence was similar in women who responded to the request for self-sampling compared to women participating in screening. Yet, the yield of high-grade CIN lesions was higher for self-sampling responders, indicating the higher risk for cervical cancer in women who do not participate in regular screening. Moreover, self-sampling for hrHPV for these women improves the effectiveness of the regular screening program at nearly the same costs per detected high-grade CIN lesion. To optimise screening improvements by self-sampling, a distinct follow-up strategy needs to be formulated (i.e. reflex-cytology to prevent unnecessary colposcopy for hrHPV positive women without any cytological abnormality). This improvement is now under investigation in an enlarged study with 44,000 non-responders.

6.5 IMMUNOLOGICAL OBSERVATIONS

Infections with hrHPV are frequent in sexually active young women. Most of these infections are transient and clear within 8 to 14 months without further impact on the host [14,21,55]. However, in a certain percentage of infected women failure of the immune system leads to persistence of viral infection and progression of the clinical symptoms from pre-malignant CIN to cervical cancer [14,56-59]. Studies of hrHPV infection in immune-suppressed women have shown that cell mediated immunity (CMI) is of importance for success or failure of the host's immune response [60,61].

It is well documented that hrHPV develops mechanisms to avoid recognition by the innate immune system [62,63]. But, recent studies with virus-like particles (VLPS) show evidence that in cervical cancer at least certain effector cells of the innate immune system like plasmacytoid dendritic cells (pDCs) are involved in the natural immune response against HPV16 [64,65].

Mediators of an effective innate and adaptive immune response are cytokines released by the effector cells of both systems and influencing each other within a complex cytokine network. A possible deregulation of the cytokine network was evaluated in women with different stages of CIN and correlated to the presence or absence of hrHPV. Circulating cytokine levels were determined in hrHPV positive women with CIN 2/3 and cervical carcinoma and were compared to levels in hrHPV positive and hrHPV negative women without cervical lesions.

Representative immunostimulating Th1-type (IL-12, IFN-γ, TNF-α and IL-2) and immunosuppresive Th-2 type (IL-4 and IL-10) cytokines in the peripheral circulation were determined. The results demonstrate a shift towards a Th2-type tumor-promoting response in women with CIN III lesions.

A more detailed insight into characteristic changes of the immune response to hrHPV was gained by investigation of the capacity of immunocompetent leucocytes

to release cytokines. This was investigated in mitogen stimulated whole blood cultures of women with different stages of cervical dysplasia. A significantly increased Th1-type and Th2-type cytokine release in hrHPV positive women without CIN, showed that hrHPV infection itself already induces activation of an inflammatory response.

An important change in the immunocompetence of women with cervical dysplasia is reached during progression of CIN 2 to CIN 3. It is expressed in a significant change of Th1-type to Th2-type cytokine ratio's. These results are in line with the clinical observation that most CIN III lesions do not spontaneously regress but may ultimately progress into invasive carcinoma if not properly treated. In contrast, CIN 2 lesions only progress in 20-45% of cases.

Of interest was the observation that there is a decrease in Th2-type cytokines IL-4 and IL-10 in women with cervical carcinoma. This results in a renewed increase of Th1-type/Th2-type ratio's. It confirms and extends observations already earlier reported suggesting an induction of a second deregulated and incomplete immune response in cervical carcinoma [66,67].

The observations reported in this study implicate specific targets for possible treatment options. They confirm the clinical policy of treating women with CIN 3, since this stage of cervical dysplasia is characterised by a tumor-stimulating immune response.

6.6 CONCLUSIONS

The use of HPV testing can reduce the risk of detection failure in cervical screening, the studies described in this thesis confirm this in clinically validated settings.

HPV testing improves the selection of women at risk for development of (pre) malignant cervical lesions amongst those with a persistent BMD smear. Referral to the gynaecologist is not indicated in the absence of hrHPV and as a consequence unnecessary referral, treatment and costs can be prevented.

Identification of those women at risk of recurrence after treatment for high-grade CIN can be improved if hrHPV testing is combined with cytology in the follow-up protocol. In our randomised clinical trial we provided an enhanced follow-up policy with addition of hrHPV testing, without an increase in costs.

Women who do not respond to an invitation for the cervical screening program can be motivated to participate by using self-sampling for hrHPV testing. This leads to risk reduction for the women involved.

Observations on the immunological background demonstrate activation of the cytokine network in hrHPV positive women without CIN and a shift to a tumor-promoting kind pattern in CIN 3. This confirms current treatment policies for CIN 3 lesions.

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SUMMARY

Development of cervical cancer is preceded by well-defined premalignant lesions. These lesions are classified as cervical intraepithelial neoplasia (CIN) and are detectable by cervical cytology, resulting in nationwide cervical cancer screening programs. Although these programs have led to a substantial reduction in mortality and morbidity of cervical cancer, there are many drawbacks including limited accuracy of cytology, unnecessary screening rounds, and over-treatment. There is therefore a strong need for a more accurate screening tool.

A persistent infection with high risk Human Papillomavirus (hrHPV) is necessary for development, maintenance and progression of CIN lesions. HPV testing, as an adjunct to cytology, can lead to a better selection of women at risk for development of cervical cancer and consequently to more efficient cervical cancer screening strategies. A general introduction on the development and characteristics of CIN, the role of HPV infection in the carcinogenesis of cervical cancer, and immunological changes during hrHPV infection is presented in **Chapter 1**.

The introduction of a clinically validated HPV test, reduces the risk of detection failure in cervical screening. In The Netherlands 2% of cervical smears in the cervical cancer screening program are read as borderline or mildly dyskaryotic cytology (BMD smear). Women with a persistent BMD smear are referred to the gynaecologist. Only 10% of these women have a high-grade CIN lesion (CIN 2/3) and need to be treated. Therefore, for the majority of women referral is unnecessary.

In **Chapter 2.1** triage with hrHPV testing is studied to identify women at risk for development of high-grade CIN lesions after a persistent BMD smear. In almost half of the women with a repeat BMD smear hrHPV is negative at baseline. None of these women showed high grade (progressive) CIN lesions. They are not at increased risk for development of cervical cancer. Women with a persistent BMD smear without presence of hrHPV can be followed by their general practitioner with 5-yearly screening according to the protocol.

The majority of hrHPV positive women has a low-grade lesion. In almost half of these women the hrHPV infection cleared within one year. Referral and treatment are only required if persistence of hrHPV is established.

The conventional treatment strategy for persistent BMD smears with direct referral to colposcopy leads to a considerable number of unnecessary referrals and costs. In **Chapter 2.2** costs, (for example referral, treatment, and follow-up visits) are assessed for different HPV triage strategies. Adding a HPV test when persistence of a BMD smear is established, can reduce the referral rate by 50% and consequently

the costs. Analysis of different strategies favours implementation of HPV testing after establishment of a persistent BMD smear.

In July 2006, based on the outcome of these data, the Dutch association of Pathology (NVVP), together with the Dutch association of Gynaecology (NVOG) and the Dutch association of General Practitioners (NHG), adjusted the current guidelines for referral of women with a persistent BMD smear. Referral of women with a persistent BMD smear is now only required in case of presence of hrHPV.

According to the current guidelines in most western countries, women treated for CIN 3 are followed by cytology for at least 2 years after treatment. Approximately 20% of these women demonstrate abnormal cytology, yet less than half of them turn out to have residual/recurrent CIN. HrHPV testing could be used to improve monitoring of these women. In the review in **Chapter 3.1** literature concerning the implementation of hrHPV testing in monitoring after treatment for CIN is summarised. The studies comprise observational and retrospective data. The combination of hrHPV and cytology testing proved to be the most sensitive and specific strategy.

These results formed the basis of the first randomised clinical trial of post treatment implementation of hrHPV testing, as described in **Chapter 3.2**. In this study, addition of hrHPV-testing to cytological follow-up after treatment for high-grade CIN is evaluated to establish a better selection of women at risk for residual/recurrent CIN. Different strategies of combined testing (hrHPV and cytology) are investigated, including considerations of costs. Using cytology in combination with hrHPV testing improves the specificity, with unaltered sensitivity. These results have led to new recommendations: follow-up after treatment for high-grade CIN should consist of combined cytology and hrHPV testing. Women without abnormalities at the 6-month visit (normal cytology, absence of hrHPV) may omit the 12-month visit. This results in cost reduction.

It is known that women who do not take part in cervical screening programmes have a 3-times greater risk of developing cervical cancer. Availability of self-sampling devices like cervico-vaginal brushes for hrHPV testing may encourage participation in the screening program by non-responders. This is investigated in **Chapter 4**.

More women respond if approached for self-sampling for hrHPV, than if invited for conventional cytology performed by the general practitioner (34.2% vs 17.6%; $p<0.001$). HrHPV prevalence was similar in self-sampling women and the general population, but high-grade CIN cases were more frequent in self-sampling responders. The costs per detected high-grade CIN lesion with self-sampling are comparable to costs with conventional cytological screening. These results show that availability

of self-sampling for hrHPV testing can contribute to increased efficiency of the population based screening, by increasing participation of women who would not otherwise respond and are known to be at increased risk.

The competence of the immune system is of influence on clearance or persistence of HPV infection. Important mediators of the immune response are certain proteins, cytokines. Changes in circulating cytokines during carcinogenesis are described in **Chapter 5.1**. Cytokine levels in the circulation were determined in different stages of cervical disease in relation to hrHPV infection. Representative immuno-stimulating Th1-type (IL-12, IFN-γ, TNF-α and IL-2) and immuno-suppresive Th-2 type (IL-4 and IL-10) cytokines were studied. The results demonstrate a shift towards a tumor-promoting response in women with CIN 3 lesions.

The influence of hrHPV infection on the capacity of cytokine release by immunocompetent leucocytes is described in **Chapter 5.2.** Blood cultures from women with different stages of cervical dysplasia were stimulated to secrete cytokines, which were then measured. Women with a hrHPV infection without cervical dysplasia show an activated cytokine network.

An important change in the cytokine-balance of women with cervical dysplasia is observed in CIN 3. Manifestation of a tumour seems to induce a second deregulated and incompetent immune response.

These results indicating important immunological changes in women with CIN 3 are supported by clinical observations: most CIN 1 or 2 lesions regress without treatment, whereas CIN 3 lesions will more often develop into invasive cancer if not properly treated.

Chapter 6 provides a general discussion based on the results as described in this thesis. New guidelines and recommendations are formulated.

SAMENVATTING

De ontwikkeling van baarmoederhalskanker wordt voorafgegaan door welomschreven premaligne stadia. Deze stadia zijn geclassificeerd als CIN-laesies (cervicale intraepitheliale neoplasie) en zijn detecteerbaar met behulp van een uitstrijkje (cervicale cytologie). Dit heeft het nationale screenings onderzoek naar baarmoederhalskanker mogelijk gemaakt, waardoor deze ziekte in een vroeg stadium ontdekt kan worden. Hoewel deze bevolkingsonderzoeken hebben geleid tot een substantiële vermindering in mortaliteit en morbiditeit van baarmoederhalskanker, zijn nadelen als beperkte testeigenschappen van cytologie, onnodige screenings ronden, en overbehandeling aanwezig. Dit impliceert de noodzaak voor verbetering van de screeningsmethode.

Voor de ontwikkeling en progressie van CIN-laesies is een persisterende infectie met hoogrisico Humaan Papillomavirus (hrHPV) noodzakelijk. Met een aanvullende test op hrHPV, naast de gebruikelijke cytologie, kan men vrouwen met een verhoogd risico op de ontwikkeling van baarmoederhalskanker selecteren en daarmee de doelmatigheid van het screeningsprogramma verbeteren.

Een algemene introductie over de ontwikkeling en karakteristieken van CIN, de rol van HPV-infectie in de carcinogenese van baarmoederhalskanker en immunologische veranderingen bij een hrHPV-infectie worden beschreven in **hoofdstuk 1**.

Sinds de introductie van een klinisch gevalideerde HPV-test kan verbetering van het risicoprofiel van vrouwen met CIN-laesies worden onderzocht. Van alle uitstrijkjes in het bevolkingsonderzoek op baarmoederhalskanker in Nederland zijn 2% licht afwijkend: Pap 2/3A1 (borderline of milde dyskaryose (BMD)). Indien deze afwijking een tweede keer wordt geconstateerd, zal verwijzing plaatsvinden naar de gynaecoloog. Slechts bij 10% van de vrouwen met een persisterende Pap 2/3A1 wordt een hooggradige CIN-laesie (CIN 2/3) vastgesteld die moet worden behandeld. Voor de meerderheid is verwijzing overbodig.

In **hoofdstuk 2.1** wordt toepassing van triage met een hrHPV-test beschreven om vrouwen te identificeren met een verhoogd risico op ontwikkeling van baarmoederhalskanker bij een persisterende Pap 2/3A1. Bij ongeveer de helft van de vrouwen met een persisterende Pap 2/3A1 is bij aanvang van deze studie hrHPV-infectie niet aantoonbaar. Bij deze vrouwen werden geen (progressieve) hooggradige CIN-laesies aangetroffen. Zij hebben geen verhoogd risico op de ontwikkeling van baarmoederhalskanker. Bij afwezigheid van hrHPV kunnen vrouwen met een persisterende Pap 2/3A1 onder controle blijven bij de huisarts volgens het 5-jaarlijkse protocol van het bevolkingsonderzoek.

Bij hrHPV-positieve vrouwen wordt meestal een laaggradige laesie (CIN 1) aangetroffen. Bij ongeveer de helft van deze vrouwen verdwijnt de hrHPV-infectie binnen een jaar. Verwijzing en behandeling zijn alleen geïndiceerd indien een persisterende hrHPV-infectie wordt aangetoond.

Het nationale beleid om alle vrouwen uit het screenings programma met een persisterende Pap 2/3A1 uitstrijk te verwijzen naar de gynaecoloog leidt tot een aanzienlijk aantal overbodige verwijzingen en daarmee gepaard gaande kosten. In **hoofdstuk 2.2** worden kosten en bijkomende gevolgen (verwijzing, behandeling, tijdsduur) voor verschillende HPV-triagestrategieën beschreven. Het toevoegen van een HPV-test bij vrouwen met een persisterende Pap 2/3A1 leidt tot 50% minder verwijzingen en daarmee tot reductie van de kosten. Na analyse van verschillende strategieën lijkt het toepassen van een HPV-test bij vaststelling van een persisterende Pap 2/3A1 optimaal.

In juli 2006 heeft de Nederlandse Vereniging voor Pathologie (NVVP), in samenwerking met de Nederlandse Vereniging van Obstetrie en Gynaecologie (NVOG) en de Nederlandse Huisartsen Vereniging (NHV), op basis van de beschreven studieresultaten de richtlijn voor verwijzing van vrouwen met een persisterende Pap 2/3A1 aangepast. Verwijzing naar de gynaecoloog is nu alleen geïndiceerd indien bij een persisterende Pap 2/3A1 ook hrHPV aanwezig is.

Volgens de huidige richtlijnen in de meeste westerse landen worden vrouwen die behandeld zijn voor een hooggradige CIN-laesie tenminste 2 jaar gecontroleerd met een uitstrijkje. Bij ongeveer 20% van deze vrouwen wordt een afwijkend uitstrijkje geconstateerd, waarvan minder dan de helft daadwerkelijk een residu/recidief CIN-laesie vertoont. Het gebruik van een hrHPV-test kan deze follow-up verbeteren. In het overzicht in **hoofdstuk 3.1** wordt de literatuur over toepassing van een hrHPV-test na behandeling van CIN samengevat. Deze studies bestaan uit observationele en retrospectieve waarnemingen. Hieruit worden aanwijzingen verkregen dat het gecombineerde gebruik van een hrHPV-test en cytologie de meest betrouwbare testuitkomsten geeft.

Deze resultaten vormden de basis voor de eerste gerandomiseerde klinische studie naar toepassing van HPV-diagnostiek na behandeling van CIN, zoals beschreven in **hoofdstuk 3.2**. In dit onderzoek wordt nagegaan of aanvulling van een hrHPV-test naast de cytologische follow-up na behandeling van hooggradige CIN-laesies kan leiden tot een betere selectie van vrouwen met een verhoogd risico op de ontwikkeling van residu/recidief CIN. Variaties in de strategie van gecombineerd testen (hrHPV en cytologie) worden met elkaar vergeleken, waarbij ook het kostenaspect

wordt meegewogen. Bij gebruik van cytologie in combinatie met een hrHPV-test verbetert de specificiteit (een negatieve testuitslag in afwezigheid van een afwijking) met gelijkblijvende sensitiviteit (een positieve testuitslag in aanwezigheid van een afwijking).

Deze uitkomsten hebben geleid tot nieuwe aanbevelingen: follow-up na behandeling van een hooggradige CIN-laesie zou moeten plaatsvinden met behulp van cytologie en een hrHPV-test. Vrouwen zonder afwijkingen tijdens de controle van 6 maanden (normaal uitstrijkje, geen hrHPV) kunnen de controle op 12 maanden overslaan. Dit leidt tot vermindering van de kosten.

Het is bekend dat vrouwen die niet reageerden op de oproep voor het landelijke bevolkingsonderzoek een 3-maal verhoogd risico hebben op ontwikkeling van baarmoederhalskanker. In **hoofdstuk 4** wordt beschreven of het aanbieden van een zelf-test op hrHPV deze vrouwen alsnog kan motiveren deel te nemen aan het bevolkingsonderzoek.

Meer vrouwen reageren op het verzoek om een zelf-test op hrHPV uit te voeren dan op een herhaald verzoek om een regulier uitstrijkje bij de huisarts te laten maken (34.2% vs. 17.6%, $p<0.001$). Bij vrouwen die een zelf-test hebben uitgevoerd is de hrHPV-prevalentie nauwelijks hoger dan de prevalentie in de algemene bevolking. Het aantal hooggradige afwijkingen (CIN 2/3- laesies) is wel hoger. De kosten om een hooggradige CIN-laesie te detecteren met behulp van een zelf-test zijn gelijk aan de kosten in het huidige bevolkingsonderzoek. Uit deze resultaten blijkt dat het aanbieden van een zelf-test op hrHPV een bijdrage kan leveren tot verbetering van de effectiviteit van het bevolkingsonderzoek op baarmoederhalskanker.

Het immuun systeem is van invloed op het verdwijnen of het persisteren van een hrHPV-infectie. Het wordt beïnvloedt door de werking van bepaalde eiwitten, cytokines. Veranderingen in circulerende cytokines tijdens de carcinogenese van baarmoederhalskanker worden beschreven in **hoofdstuk 5.1**. Concentraties van cytokines in de circulatie zijn gemeten in vrouwen met verschillende stadia van premaligne cervicale afwijkingen in relatie tot een hrHPV-infectie. Representatieve Th1-type cytokines (IL-12, IFN-γ, TNF-α en IL-2) die voornamelijk afweerstimulerend/tumorremmend werken en Th2-type (IL-4 en IL-10) cytokines met afweerremmende/tumorstimulerende werking zijn bepaald. De resultaten demonstreren een verschuiving naar een tumorstimulerend cytokine respons bij vrouwen met een CIN 3-laesie.

De invloed van een hrHPV-infectie op de capaciteit van de cytokine productie door immuuncompetente leukocyten is onderzocht in **hoofdstuk 5.2**. Bloedculturen van

vrouwen met verschillende stadia van cervicale dysplasie zijn gestimuleerd tot productie van cytokines en deze laatste zijn vervolgens gemeten. Bij vrouwen met een hrHPV-infectie zonder CIN blijkt het cytokine netwerk geactiveerd te zijn.

Een belangrijke verandering in de cytokine balans bij vrouwen met CIN wordt geobserveerd bij CIN 3. Aanwezigheid van baarmoederhalskanker lijkt een tweede maar incompetente afweerreactie te induceren.

Deze immunologische waarnemingen in vrouwen met CIN 3 ondersteunen bekende klinische observaties: CIN 1- en CIN 2-laesies gaan meestal in regressie, dit in tegenstelling tot CIN 3-laesies die zonder behandeling zich vaak ontwikkelen tot baarmoederhalskanker.

Hoofdstuk 6 geeft een algemene beschouwing gebaseerd op de uitkomsten van de beschreven studies. Nieuwe richtlijnen en aanbevelingen worden geformuleerd.

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CURRICULUM VITAE

Aagje Bais was born on the 17th of November 1972 in Middelburg, The Netherlands. In 1991 she finished secondary school at the Sint Willibrord College in Goes. In 1991 she started Medical School at the University of Antwerp, Belgium and continued Medical School at the Erasmus University in Rotterdam, The Netherlands in 1992, where she was registered as Medical Doctor in 1998.

From 1998-1999 she worked as a researcher at the department of Experimental Surgery at the University Medical Center Utrecht. In 1999 she started with research activities at the department of Obstetrics and Gynaecology of the Erasmus Medical Centre Rotterdam, under supervision of prof.dr. ThJM Helmerhorst. Activities during the first years were concentrated on medical education, especially the development of a renewed Intern program for medical students at the Erasmus University. The following years she extended her research activities as presented in this thesis and became an expert colposcopist with outpatient clinics in the Erasmus MC and Albert Schweitzer Hospital in Dordrecht.

In 2005 she started her residency of Gynaecology and Obstetrics at the Reinier de Graaf Gasthuis in Delft (under supervision of dr. JC Kuijpers and dr. WA ter Harmsel), continuing in 2007 at the Erasmus Medical Centre Rotterdam (under supervision of prof.dr. CW Burger).

She received the first price of best research at the WMJ Schellekens symposium in 2006 and she won the Prof.dr. JC Birkenhäger award at the Erasmus Medical Center Rotterdam in 2007 for her self-sampling project.