PREVENTION OF CERVICAL CANCER IN THE NETHERLANDS
STUDIES ON CYTOLOGY AND HPV INFECTIONS

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CONTENTS

CHAPTER 1.

INTRODUCTION TO THIS THESIS

Cervical cancer is a relatively infrequently occurring type of cancer in the Netherlands. In 2003, 584 women were diagnosed with invasive cervical cancer, and 214 women died in the same year. The chance of a Dutch female to be diagnosed with cervical cancer before she reaches the age of 75 is 0.46%, and 0.13% to die of the disease (1). In contrast, cervical cancer is the second cause of female cancer-related death worldwide. In developing countries, cervical cancer is one of the leading causes of female cancer deaths.

In the 1940's Papanicolaou published the results of a technique to investigate cervical cells, and during the following decades cytology screening for cervical cancer was slowly introduced in Western countries (2;3). Concurrently, human papillomavirus was identified as the putative causative agent in cervical cancer in 1977 by zur Hausen(4). During the 1980's and 1990's techniques to establish the presence of HPV were developed, and in a collective effort, the undispubtable role of HPV as the single most important necessary causal factor for the development of cervical cancer was confirmed in a landmark paper by Walboomers *et al.* (5).

Cervical cancer is considered an important public health problem in the Netherlands since secondary prevention is possible, and a screening programme has been instated (6). Quality-controlled cervical cancer screening using a population-based call-and-recall system has proved its effect on the incidence of cervical cancer (7). Unfortunately, in spite of the presence of such a cervical cancer screening programme in the Netherlands since 1988, cervical cancer has not been eradicated completely (8). The main reasons for the lack of eradication despite a screening programme are considered to be a low adherence of invited women to the screening programme, and a relatively high false-negativity rate of cervical cytology (9;10). Not yet implemented in the Dutch screening programme, high-risk human papillomavirus (HPV) testing in addition to conventional cervical cancer screening may improve the effectiveness of the screening programme since high-risk HPV is causally associated with cervical cancer.

In this thesis, prevention of cervical cancer will be addressed from a combined pathologic and epidemiological point of view in order to expand the knowledge on the prevention of cervical cancer and its precursors and to evaluate the implications of HPV testing for cervical cancer screening. Firstly, I provide an overview on HPV biology, histological and epidemiological aspects of cervical cancer and screening in **CHAPTER 2**. The issues addressed in the subsequent chapters include the following.

The occurrence of cervical cancer in the Netherlands, and the effects of screening on the incidence and survival of cervical cancer have not been addressed recently. Information concerning the epidemiology of cervical cancer and its mortality is essential for evaluation of Dutch screening practice in order to effectuate a more cost-effective screening programme. I designed two ecological studies on aspects of cervical cancer incidence, as described in **CHAPTER 3**. The first study (3.1) aimed to evaluate incidence and survival patterns of cervical cancer by histological subtype. The second study (3.2) was designed to investigate whether age-specific trends in cervical

cancer incidence can be discerned, and whether trends may be attributed to cervical cancer screening.

At present, the cervical cancer screening programme as employed in the Netherlands uses cytology only as screening tool. In **CHAPTER 4**, I describe the cervical cytology coding classification used in the Netherlands. I evaluated the effects of the adaptation of the classification system in 1996 to investigate whether the proforma reporting improve the quality of the cervical screening programme. The change resulted in a approximately 80% decrease in prevalence of borderline abnormalities.

Since high-risk types of HPV are the cause of cervical cancer, a main focus of this thesis is the possible role of testing for HPV in the prevention of cervical cancer in the Netherlands (**CHAPTER 5**). We investigated whether certain high-risk types confer a preferential risk for the development of cervical cancer compared to the other high-risk types. On this subject I have conducted two studies. Firstly, I investigated whether there is a difference in distribution of high-risk HPV types detected in women with normal cytology compared to high-risk types of cases with different subtypes of cervical cancer (5.1). In the second study, I compared cases with (invasive) squamous cell carcinoma with cases of cervical intra-epithelial neoplasia grade 2 and 3 (CIN2/3) for a difference in distribution of high-risk HPV types (5.2).

Finally, I investigated the role of high-risk HPV testing in the detection of high-grade lesions in cervical cancer screening (**CHAPTER 6**). I studied whether a type-specific HPV test better identifies women at risk for cervical precancer than cytology, first evaluating the effect of HPV typing retrospectively in 6.1 and subsequently evaluating combined testing prospectively in 6.2.

CHAPTER 7 and **CHAPTER 8** provide a discussion of some epidemiological aspects of the studies presented in this thesis and a summary.

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CHAPTER 2.

SCREENING FOR CERVICAL CANCER AND POTENTIAL FOR HPV TESTING

Characteristics of the human papillomavirus

Papillomaviruses are a group of more than 120 different double-stranded DNA viruses that infect epithelial cells. The papillomavirus genome encodes 8 open reading frames (ORFs). Proteins encoded by E6 and E7 genes are supposed to generate an optimal cellular environment for viral replication by intereference with cell cycle and apoptosis control mechanisms. Another four viral genes (*i.e.*, E1, E2, E4 and E5) are also directly or indirectly involved in viral replication, whereas two viral genes (*i.e.*, L1 and L2) encode structural proteins composing the viral capsid. Different HPV *types* have more than 10% difference in the L1 ORF. A *subtype* of HPV is defined by a 2-10% difference in homology in the L1 ORF, whereas variants have less than 2% difference. Papillomaviruses are classified as *cutaneous* or *mucosal*, depending on the type of epithelium they infect. Mucosal types that infect genital epithelium are subdivided into low-risk HPVs and high-risk HPVs. Low-risk HPVs are not associated with cervical cancer, whereas high-risk HPVs are associated with cervical cancer and lesions that may progress to cancer.

High-grade cervical neoplasia and invasive cervical cancer arise in women who cannot resolve an infection with high-risk HPV and consistently virus activity is maintained during a prolonged period of time following initial exposure. Although at least 40 HPV types can infect the cervix, 15 are considered high-risk HPV and have the potential to cause invasive cervical cancers (1). Three more HPV types are probably associated with invasive cervical cancers. Generally, these HPVs belong to the phylogenetically determined α9 and α7 groups, HPV16 and HPV18 being the most prevalent types, respectively (2;3). HPVs, especially HPV16, may also cause cancer of the anal canal, vulvae, penis and oropharynx (4).

HPVs infect the basal cells of squamous epithelium and replicate in differentiating epithelial cells. Most HPV infections result in *productive lesions* resulting in infectious *virion* production. In productive infections of the cervix, histologically recognisable as CIN1 and part of CIN2 lesions (see further), HPV DNA is *episomal*. Separate phases of the infection with different viral proteins playing distinctive roles can be distinguished. An initiating event involves substantial E6 and E7 expression of the virus in non-dividing differentiating keratinocytes. E7 associates with pRb, which results in a loss of regulatory pRb function. This results in reactivation of the cellular DNA-replication machinery in a differentiation-dependent manner and an S-phase environment is created supporting viral DNA replication in differentiating keratinocytes. E6 associates with p53, resulting in loss of ubiquitin-dependent degradation of p53, and this complements the function of E7 by ensuring cell survival. In conjunction with the activities of other viral genes, the activity of these genes forces infected differentiating keratinocytes to produce infectious virions. E6 and E7 are also referred to as viral oncogenes given their indispensable role in cervical carcinogenesis.

In *transforming infections*, the production of virions has been distorted, resulting from an aberrant pattern of viral gene expression with an increased cancer risk. Transforming infections may be diagnosed histologically as CIN2 and CIN3 lesions (see **CHAPTER 2.2**). Here, E6 and E7 genes are expressed in dividing basal cells in an uncontrolled manner. The exact cause of deregulation of E6 and E7 expression is not completely known, but integration of the virus in the cellular genome and/or altered epigenetic control mechanisms have been suggested as possibilities. Integration may lead to the deregulation of E6/E7 expression as the control of oncogene expression provided by E2 may be lost by disruption of the HPV genome with concomitant loss of E2 expression. Deregulation of E6 and E7 will result in chromosomal instability and provides the driving force for further progression. However, expression of E6 and E7 alone is not sufficient for a malignant phenotype to occur. Papillomavirus-mediated oncogenesis requires the accumulation of additional genetic and epigenetic changes that may occur over time following initial infection with high-risk HPV (5). These changes result in, amongst other, immortalization of HPV-infected cells and once the capacity to invade the basal membrane has been acquired, cancer has occurred (6).

Cervical cancer and its precursor lesions

Squamous pre-cancerous lesions are histologically graded as cervical intra-epithelial neoplasia (CIN). Mild (CIN1), moderate (CIN2), or severe (CIN3 including carcinoma *in situ*) lesions can be distinguished. These lesions are also referred to as squamous intraepithelial lesions. Once the basal layer has been invaded, squamous cell carcinoma has occurred.

Histologic classification of biopsies depends on judgement of the degree of mitotic activity and nuclear atypia, and of extension into the epithelium of these characteristics (7;8). CIN categorisations are moderately reproducible (9-12). About 20% of cervical cancer cases is not of squamous origin, but arises from glandular cells in the endocervical canal (13). These adenocarcinomas do not have a well-defined spectrum of gradually worsening intra-epithelial neoplasia. Only adenocarcinoma *in situ* has been claimed to be a histologically and cytologically reliably recognisable precursor lesions of invasive adenocarcinoma (14).

Despite the subjectivity of diagnosing CIN lesions, clinically relevant distinctions between the different grades of CIN can be made. Lesions graded as CIN1 display a high regression rate. A high regression rate is also displayed by CIN2 lesions, but women with CIN2 do experience an estimated risk of cervical cancer of around 40% (15). Finally, CIN3 lesions often persist or even progress to invasive cervical cancer, regression being less common (16). Invasive cervical cancer has not been demonstrated ever to regress to a noninvasive phenotype.

Aspects of cervical cancer development

In contrast to most forms of cancer that occur at older ages, the peak of cervical cancer incidence is before middle age (13;17). Women who do not involve in sexual activity are highly unlikely to develop cervical cancer (18). Therefore, it has been recognized since the nineteenth century that occurrence of cervical cancer is associated with the practice of sexual intercourse. Indeed, the sexual transmissibility of HPV has been proven beyond any doubt (19). The more sexual partners a woman (or her male partner) has had, the higher the risk of cervical cancer (20;21). Of note, not having had multiple sexual partners is not associated with an absence of cervical cancer risk. More than half and up to 80% of sexually active women has been infected by one or more genital HPV types at some point throughout their life. In most countries, HPV prevalence is highest in young women shortly after their sexual debut, and a high incidence has been demonstrated in young, sexually active women who were HPV negative previously (22-24). HPV prevalence reaches a relatively stable level after age 30 in most populations studied, as most infections are transient and risk behavior minimizes over time (25). HPV prevalence in men is at least equal to the prevalence in women (24;26). Changes in sexual behavior in populations have occurred during the 20th century. The overall pattern suggests an increasing risk of cervical cancer over successively born generations, presumably linked to an increasing risk of becoming infected with HPV by generational changes in sexual activity (27;28).

It has often been postulated that cancer development after HPV infection involves a continuum of progression through different stages of lesion severity (*i.e.*, CIN1 to CIN2 to CIN3), finally resulting in cervical cancer in women who did not clear the virus, either spontaneously or after ablative treatment (29). However, HPV infections often result in nothing more than a slightly increased proliferation of the epithelium and most women developing an cervical intraepithelial lesion do clear the lesion (and the infection) spontaneously (16;30-32). Thus, it is highly likely that low-grade cervical lesions represent a productive HPV infection process without an inherent progression risk.

High-grade lesions are considered the true precursor lesion of cervical cancer. The notion of high-grade lesions being a separate entity from low-grade lesions is supported by studies showing that high-grade lesions may occur within a very short interval after a new infection with high-risk HPV (23;33-35). Still, long-term presence of high-risk HPV is the main risk factor for the development of high-grade lesions as the development of high-grade lesions and invasive cancer is linked to persistence of the virus. The more likely a HPV type is to persist, the more prevalent it is in the population (2). Clearance rates vary between HPV types, but high-risk types seem to clear less effectively. Some high-risk types have an elevated risk of progression given persistence and especially HPV16 confers an elevated risk of progression to high-grade lesions and cervical cancer (2).

After a high-grade lesion has developed, the progression of the lesion to invasive cancer does seem to be a process that requires years (16;36). This notion is supported

by the fact that peak prevalences of lesions are highly age dependent. In general, for low-grade abnormalities the most affected age groups are women aged 20-30 years, for high-grade dysplasia or carcinoma in situ 30-40 years, and 40-60 years for invasive cancer (25).

Prevention of cervical cancer by vaccination

Immunity to HPV infections is conferred by both cell-mediated and humoral immunity. However, HPV quite effectively evades the human immune system as HPVs are not accompanied by inflammation, and the immune system is not easily alerted to the presence of the virus (37-39).

HPVs cause no viraemia, display a strictly epithelial tropism and hardly activate an immune response since differentiated keratinocytes are inherently destined for cell death and desquamation. The emergence of antibodies to HPV seems to be secondary to prolonged exposure and high viral load. Moreover, there is no definite relationship between immune responsiveness and infection clearance (38).

The humoral immune response is directed at epitopes on the papillomavirus L1 protein displayed on the outer surface of the virion. In the absence of viral DNA, the L1 proteins can assemble into virus-like particles geometrically and antigenically almost identical to real infectious virions. Virus-like particles induce generation of high titers of serum neutralizing antibodies when the immune system has not come into contact with the HPV type in question previously. As virus-like particles are noninfectious and nononcogenic, these particles have been used to develop prophylactic HPV vaccines (40). Indeed, humoral antibodies to HPV are transsudated into the mucus covering the epithelial surface of the vagina and the cervix (39). Prophylactic HPV vaccines prevent a primary infection with the virus by binding of the antibodies induced by virus-like particles to native virus.

Prophylactic vaccination will be especially important for women of younger ages as the age of sexual debut in a population decreases while the age limits of the population-based screening programme may not. Indeed, two of these prophylactic vaccines have been developed commercially. A bivalent vaccine aimed at HPV16 and HPV18, and a quadrivalent vaccine that includes the low-risk types HPV6 and HPV11 as well have been marketed (41-43). HPV16 is the most commonly found type in cervical cancer, high-grade CIN lesions and low-grade CIN lesions. HPV16 and HPV18 are both associated with an increased ratio of cancer versus low-grade lesions, and of cancer versus high-grade lesions (44-48). It is estimated that types HPV16 and HPV18 are the causative factor of approximately 70% of all cases of cervical cancer (49). The response to the two vaccines has been studied during a period of about five years. Vaccination induced a type-specific immune response, with a low level of cross-reactivity of HPV16 antibodies to HPV33, and HPV18 antibodies to HPV45. Generation of type-specific antibodies resulted in prevention of new infections with that type, and in a decreased incidence of high-grade lesions caused by the types vaccinated against. Expectations are that vaccinated women will not develop invasive

cancer as new infections with HPV are prevented. Unknown as yet is the duration of protection of type-specific immunity, as waning of the immunological response may occur, and, since most high-risk types are not included in the vaccine at present, the phenomenon of type replacement has not been excluded.

In contrast to prophylactic vaccination, therapeutic vaccinations are directed at treating HPV-induced cervical lesions by the annihilation of HPV-infected cells. Hereby, the histologic sequelae of HPV infection are treated. These vaccines targeting non-structural early viral antigens of HPV, such as E6 and E7, are still under development (50).

Prevention of cervical cancer by screening

Methodological aspects of screening tests. Screening test results can be either negative (*i.e.*, the test indicates that disease is absent) or positive (*i.e.,* test indicates that disease is present). These results can be either *true-negative* or *true-positive* (the test result reflects the true condition) or test results can be *false-negative* or *false-positive*. False-negative results indicate the absence of disease while in reality, disease was present. However, false-negative results may also be the result of disease that was present, but undiagnosable, or disease may have developed afterwards (so-called *interval disease*). False-positive results indicate the presence of disease, while in reality, disease is absent. In screening, false-positive results are usually much more common than true-positive results, because of the low pre-test probability of disease (*i.e.*, *prevalence*) of preclinical disease in screening-eligible individuals.

Test results in a screening population can be converted into so-called predictive values. Predictive values depend strongly on the prevalence of disease in the population studied, and are not intrinsic aspects of the test under study. The *positive predictive value* (PPV) is the proportion of people with a positive test who have the disease tested for. A low PPV indicates that a high proportion of costs and efforts are being wasted on the detection and diagnostic evaluation of false positives. However, since the prevalence of preclinical disease in a screened population is usually low (<1%), the PPV is usually low. In contrast, the *negative predictive value* (NPV) is the proportion of people with a negative test who do not have the disease in question.

Another way to express test accuracy is test *sensitivity* and test *specificity*. Sensitivity is the probability that a test correctly classifies people with preclinical disease as positive. A test is called positive in a person with preclinical disease if a manifestation of the disease is detected and the degree of change exceeds the criterion of positivity. A screening test with a high sensitivity will result in a low number of false-negative results. Specificity is the probability that a test classifies people who are not diseased as negative. The specificity of a test determines whether or not the frequency of false-positives will be low enough for a screening programme to be feasible. A screening test with a high specificity will result in a low number of false-positive results. The reliability of a test is its capacity to give the same result (either positive or negative),

whether correct or incorrect, on repeated application in a person with a given state of disease.

As mentioned before, screening aims to detect disease in its preclinical stages in asymptomatic people. However, the inherent absence of symptoms of disease detected through screening (otherwise, a person would not have been eligible for screening) may result in several biases that hinder the evaluation of effectiveness of screening. Firstly, screening may lead to the diagnosis of disease that would never have resulted in symptomatic disease in the absence of screening. Also, screening may result in a seeming improvement in patient management based on *lead time bias*. Lead time bias is the amount of time by which the diagnosis is early, without resulting in a true improvement of diagnosis. As screening inherently advances the time of diagnosis, survival time is a biased measure. Another bias that may occur is *length time bias*. Length time bias pertains to comparisons of screening effectiveness that are not adjusted for the rate of disease progression. Indeed, the probability that a case will be detected by screening is directly related to the length of its detectable preclinical phase, which is inversely related to the rate of progression. Thus, slowly progressive disease processes will be diagnosed through screening more often than rapidly progressive disease.

The evaluation of screening effectiveness is difficult. Besides the previously mentioned biases, participants in screening may be a highly selected segment of the population at large, with a better life expectancy even without participation in screening. Since screening aims at preventing the demise of patients, mortality, both disease-specific and all-cause mortality, is the most appropriate outcome measure of in the evaluation of screening effectiveness. Histological diagnosis of (pre)malignant lesions is an intermediate measure of screening effectiveness. Thus, randomized controlled trials of screening using mortality as outcome measures instead of measures of disease prevalence are the true proofs of screening effectiveness. Unfortunately, screening procedures aiming at invasive cancer, with a very low prevalence in the population, make proving effectiveness of screening difficult.

Screening for cervical cancer with cytology. The era of cervical cancer screening commenced with the development of Pap smear cytology in the 1940's and the realization that cytological abnormalities in cervical smears are a measure of the different histological degrees of abnormality of the cervix (52). Cytological screening programmes were first introduced in the 1960's, and gradually opportunistic screening was replaced by population-based screening. In the Netherlands, cervical cancer death rates started to decline in the 1950's, and cervical cancer screening commenced in the beginning of the 1960's. The first organized screening programmes were introduced in the 1970's, and nationwide screening was introduced in 1988 for women aged 35-54 with a 3-year screening interval. In 1996 the cytological screening programme was restructured and screening ages were extended to women aged 30-60, and the screening interval widened to 5-years (59). Throughout this period, cervical cancer mortality death rates

have continued to decline. In 2003, 584 women were diagnosed with invasive cervical cancer, and 214 women died in that same year. Nowadays, the chance of a Dutch female to be diagnosed with cervical cancer before she reaches the age of 75 is 0.46%, and 0.13% to die of the disease (53).

Since the introduction of cervical cancer screening programmes preceded the development of the randomized controlled trial, screening effectiveness has not been investigated with what is nowadays considered the golden standard of scientific evidence (51). Instead, the effect of screening was investigated by comparing cervical cancer incidence or disease-specific mortality in countries by comparing rates before and after screening, and comparing data in opportunistic screening situations to data obtained in programme-based screening. As these comparisons showed a positive effect of screening, soon screening was so widespread that a formal evaluation of cervical cancer screening was deemed unethical (54).

All evidence of cervical cancer screening, albeit obtained in less than perfect studies, provide confirmation of a protective effect of having had a negative Pap smear (actually, the Pap smear itself does not offer protection, but the absence of cervical dysplasia is an indicator for the absence of risk). The highest benefit seems to be obtained by having had multiple negative smears (thus, participating in screening regularly) (31). Indeed, the main problem with cytological cervical cancer screening is its poor performance in terms of single screening. Its test characteristics are rather inadequate, as the low sensitivity and the low specificity show. A second important objection against cervical cancer screening by Pap smear cytology is that, given the fact that cervical cancer is a very rare disease in Western-Europe, the test characteristics give rise to substantial overtreatment and burden of disease for participating women (55;56). Especially the low specificity results in high absolute numbers of women who are treated for (non-existing) disease based on a false-positive test result. Therefore, further research of cervical cancer screening procedures is essential to prevent unnecessary referrals and treatment.

Conventional cytology. Cytology screening has been the mainstay of cervical cancer prevention. Screening by conventional cytology within a high-quality programme may reduce cervical cancer incidence (31). In order to reach this reduction, several consecutive smears have to be taken as sensitivity and specificity of single screening are rather low (10;11).

For a cytological diagnosis, cells are scraped from the cervix with a sampling tool, such as a Cervex brush. The exfoliated cells are placed on a glass slide, fixated and coloured. The Papanicolaou technique is used, as published in the 1940's of the previous century, and hence, the expression 'Pap smear' is derived (52). Cytotechnologists read the smears for the presence of abnormal cells, that are graded based on the subjective interpretation of the degree of abnormality (see **CHAPTER 4**).

Several classifications exist to judge the degree of abnormality of a cervical smear, such as the Australian and British classifications. The American Bethesda classification

is most commonly used in internationally published studies on cervical cancer screening (57;58). In the Netherlands, the KOPAC-B classification is used (*a.k.a.,* CISOE-A) (59;60).

The CISOE-A uses a 5-tier system to formalize the evaluation of all components of the smear by a rating system including information on specimen composition, inflammatory characteristics, and adequacy of the smear. The letters C (composition), I (inflammation), S (squamous), O (other and endometrium), and E (endocervical cylindrical epithelium) are used to indicate the composition and morphology of the smears. The letter A (adequacy) provides information on the adequacy of the smear. Squamous, columnar, and other cells are graded for the presence of dyskaryosis (dysplasia), and these values determine the interpretation of the smear. The CISOE-A classification is more detailed than the Pap terminology.

Conversion between classifications can be made. CISOE-A cytology results can be categorised as Pap 0 or "inadequate" (A3), Pap 1 or "normal" (S1, O1–2, E1–2), Pap 2 or "equivocal" or borderline (S2–3, O3, E3), Pap 3a1 or mild dyskaryosis (S4, E4, A4), and Pap 3a2 and over or moderate dyskaryosis or worse (S≥5, O≥5, E≥5). The normal group corresponds to the "within normal limits" category of the Bethesda classification. Borderline and mild dyskaryosis corresponds to the ASC-US, ASC-H and LSIL categories, and the moderate dyskaryosis or worse categories translate into HSIL (57).

Liquid-based cytology. Over the past decades, a new technique called liquid-based cytology has been introduced to overcome shortcomings of conventional cytology. Whereas conventional cytology transfers cervical cells directly from the cervical os to the slide, liquid-based cytology involves rinsing the sampling tool into a vial of liquid to produce a suspension of cells, from which a concentrated monolayer of cells is prepared. It has been claimed that smears will be better gradable as the effect of obscuring substances and faulty smear processing of the smear taker will be decreased. Slides produced in this way can be read more quickly than slides prepared with the conventional technique, and the liquid sample can be used for other tests, such as HPV DNA testing. Disadvantages include the lower number of cells available for interpretation on the slide and the loss of structural integrity of cell groups (61). Cell groups may aid the diagnosis of high-grade lesions in particular.

Liquid-based cytology has been employed quite extensively in recent years (62;63). A recent meta-analysis showed that an improvement of cytology reading using liquid-based cytology instead of conventional cytology could not be proven since both sensitivity and specificity of liquid-based cytology did not show an advantage over conventional cytology (64-66). The often proclaimed improvement in inadequacy rate could not be established firmly. This meta-analysis showed a substantial effect of study quality, with lesser quality studies displaying more advantage of liquid-based cytology over conventional cytology than medium and high quality studies. Since the study design aimed to demonstrate the absence of superiority of liquid-based cytology,

both techniques may be equivalent in effectiveness. Other as yet unproven characteristics, such as greater reproducibility, lower costs, or the capacity of HPV DNA testing, could make liquid-based cytology as desirable as conventional cytology in a screening programme.

HPV tests. Originally, insensitive methods such as immunofluorescence and *in situ* hybridisation have been developed to detect the presence of HPV DNA in cells. In clinical practice more sensitive HPV DNA detection methods are used. The most commonly used test for HPV is based on a method that uses signal amplification of DNA-RNA hybrids (Hybrid Capture II method, Digene). In epidemiological studies, the presence of HPV is usually established by PCR methods. As several different types may be the cause of cervical cancer, general primer PCR systems have been developed that test for several types concurrently. Commonly used highly sensitive general primer PCR methods are PGMY09/11, SPF10 and GP5+/6+ PCR (67-69). However, these PCR methods do vary substantially in sensitivity, leading to widely different estimates of HPV prevalence. With increasing severity of HPV-associated lesions, prevalence estimates by the different methods become indistinguishable. The GP5+/6+ method used in the research presented in this thesis combines excellent sensitivity for the detection of disease (*i.e.*, clinical sensitivity with a high analytical precision (70).

In order to detect the presence of individual HPV types as well as the presence of HPV in general, type-specific amplification methods may be used (71). Since type-specific amplification is a laborious method to detect a broad spectrum of individual HPV types, read-out systems using type-specific oligonucleotide probes such as enzyme immunoassays (EIAs), reverse line blot assays or bead-based technologies to detect multiple types have been developed for most general PCR systems (72-74). In the research described in this thesis, we used the GP5+/6+ PCR-EIA combined with a reverse line blot with specific oligonucleotide probes for 14 high-risk types to diagnose the presence of individual types (73).

As a drawback of the previously mentioned general primer PCRs, most general primer systems target fragments of DNA that sometimes cannot be amplified after the virus has integrated into the host genome due to loss of integrity of the integrated fragment. However, for a virus to sustain its malignant phenotype upon integration, the E6 and E7 ORFs must remain intact and therefore, integrated HPVs may be detected by type-specific amplification based on fragments of the E7 gene. E7-specific PCR has been used as an additive test for the studies on CIN2/3 published in this thesis.

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CHAPTER 3

CERVICAL CANCER IN THE NETHERLANDS

3.1 Incidence and Survival Rate of Women with Cervical Cancer in the Greater Amsterdam Area

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Abstract

To evaluate the effect of population-based cervical cancer screening on the occurrence of cervical cancer in the Netherlands, we investigated the incidence and survival of cervical cancer registered by a cancer registry in the Greater Amsterdam area.

The incidence rate of squamous cell carcinoma decreased significantly from 9.2/100.000 women in 1988 to 5.9/100.000 in 2000 (P <0.001). The incidence rate of adenocarcinomas remained stable. After adjustment for age, stage and lymph node involvement, the relative risk of death was 1.6 times higher for patients with adenocarcinomas than for patients with squamous cell carcinoma (95%CI 1.2-2.1). The decreased survival was related to histological type, as the effect remained significant after correction for confounding factors. Over time, the prognosis of women with squamous cell carcinoma increased significantly. No significant change was observed for women diagnosed with adenocarcinoma.

These results suggest that the screening programme in the Netherlands as executed in the Greater Amsterdam area is associated with a decreased incidence and increased survival of patients with squamous cell carcinoma, but fails to detect (pre-) malignant lesions of adenocarcinoma. Since more than 92% of adenocarcinomas and its precursors contain high-risk HPV, adding HPV testing to cytologic screening might improve the present screening programme in detecting adenocarcinoma and its precursor lesions.

Introduction

Population-based cervical cancer screening has led to a decrease in the incidence of cervical cancer (1;2). However, recent data suggest that the decrease in incidence is caused by a decrease of squamous cell carcinoma, while the incidence of adenocarcinoma of the cervix shows no change or sometimes even an increase (3-6).

It has been suggested that the unchanged or even increased incidence of adenocarcinoma of the cervix is the result of systematic underscreening of cervical smears for (pre-)malignant changes of adenocarcinoma of the cervix (7-9). Moreover, patients with adenocarcinomas of the cervix are considered to have a decreased survival compared to patients with squamous cell carcinomas (10-13). It has been suggested that this decreased survival is associated with higher stages of disease with which patients with adenocarcinomas are detected.

Here, we report on the incidence and survival rates of cervical cancer cases registered by a cancer registry in a large geographically defined region of the

Netherlands, the Greater Amsterdam area. Special attention was paid to trends in incidence and survival rates for cases of squamous cell carcinoma and adenocarcinoma.

Materials and methods

Data collection. The cancer registry of the Comprehensive Cancer Centre Amsterdam (CCCA, 'Amsterdam Cancer Registry') is a population-based cancer registry since 1988, and part of the nationwide Netherlands Cancer Registry as of 1989. It covers 2 out of 12 Dutch provinces: Noord-Holland and the major part of Flevoland. The population of the CCCA region increased from 2.50 million on January 1st, 1988 to 2.80 million on January 1st, 2001. All malignant tumours were registered in all 20 hospitals in the region, comprising 2 university hospitals and a specialized cancer hospital, where cancer patients are treated.

Clinical information and pathology data were extracted from the medical records. Apart from demographic data, data were collected on tumour site, morphological classification (according to the International Classification of Diseases for Oncology) and stage of the tumour. The fourth edition of the TNM-classification was used whenever applicable (14). In 1988-1993 the FIGO-stage was registered separately, and after 1993 the FIGO stage was derived from TNM-stage. Data concerning participation in cervical cancer screening programmes was not available.

For this study, all cervical cancer cases diagnosed between 01-01-1988 and 31-12-2000 were selected from the cancer registry. The following tumours were excluded: non-invasive tumours and tumours diagnosed in patients living outside the CCCA-region. Patients diagnosed in a hospital outside the region, but living in the CCCA-region were included. Information on the vital status of all patients was collected in the hospitals and from general practitioners. However, the majority of the information on vital status was obtained from record linkage of computerized data on all deceased persons in the study period which were made kindly available by 51 of the 74 municipalities in the CCCA region (covering more than 85% of the population). Inquiries about the vital status of patients living in the other 23 municipalities were made at the municipal population registers and at the Central Office for Genealogy of the Netherlands, The Hague. Less than 1% of the cases were lost to follow-up. In the survival analyses, cases diagnosed in 1998-2000 were excluded, because of the short period of follow-up. Tumours first diagnosed at autopsy, second (or third, etc.) tumours and non-carcinomas were also excluded from the survival analyses. Follow-up of the patients diagnosed in 1988-1997 was complete until at least January 1st , 1999.

Population data of the Netherlands were obtained from Statistics Netherlands (CBS, Voorburg/Heerlen, the Netherlands). Data from Statistics Netherlands were also obtained concerning the survival of the general Dutch population.

Statistical Analysis. Incidence of cervical cancer was calculated per 100,000 person years. Direct standardisation was used for age adjustment with respect to the European standard population, and the European Standardised Rates (ESRs) were calculated. Trends in the incidence of the ESR were investigated by calculating the estimated annual percent change (EAPC) (15). For the analyses, cases were divided into squamous cell carcinoma, adenocarcinoma and other histological type. Differences in distribution over stage and age categories were assessed with Chi-square statistics.

Relative survival and 95% confidence intervals (CI's) were calculated as a measure of disease-specific survival (16). The relative survival is the ratio between crude and expected survival and is close to disease-specific survival. We did not calculate disease specific survival, because the cause of death was not available as linkage with the death registry in the Netherlands is not possible.

Cox multivariate regression analysis for survival was performed to investigate survival. In these analyses, age categories 15-29 and 30-44 years were analysed jointly, based on the low number of cases in the category 15-29 years. Associations were examined for all cases, and for cases of squamous cell carcinoma and adenocarcinoma separately. In the analyses, the FIGO classification was used to adjust for stage. Lymph node status is not considered in the FIGO classification for cervical carcinoma, and therefore it was introduced in the analyses as a separate variable. For cases diagnosed in 1988, TNM was not registered, so these cases were classified as "nodal involvement unknown". Age, stage and nodal involvement were divided into categories and entered into the model as dummy variables. P values of 0.05 or less were considered statistically significant. Using STATA 6.0 for Windows, hazard ratios and 95% CI's were calculated.

Results

From 1988 up to 2000, 1921 patients were diagnosed with invasive cervical cancer (Table 3.1). The annual number of incident cases of cervical cancer decreased from 157 patients registered in 1988 to 133 patients in 2000. The ESR decreased from 11.8/100,000 women in 1988 to 8.2/100,000 women in 2000. The total ESR decreased with 3.5% annually ($p=0.001$). This decrease in incidence was mainly caused by a decrease in the incidence of squamous cell carcinoma cases, as the EAPC in ESR for squamous cell carcinomas was –3.2% ($p<0.001$). For adenocarcinomas, there was no statistically significant trend in the incidence (EAPC –1.2%, $p=0.51$). The incidence of other cervical malignancies decreased with 8.5% annually ($p=0.04$). However, this concerned only a small number of cases annually (range 2-14 cases). During the study period, the contribution of adenocarcinomas to the total number of malignancies increased from 16% in 1988-90 to 18% in 1998-2000.

The median age of patients with adenocarcinoma was 3 years below the median age of patients with squamous cell carcinoma, that is 45 years (range: 18-92) and 48 years (range: 19-98), respectively ($p<0.05$). The median age of patients with other tumour types was 42 years (range: 24-89). Age-specific incidence was highest in age groups 35-39 years and 70-84 years for squamous cell carcinoma patients, and in age group 35-49 years for adenocarcinoma patients (Figure 3.1).

Younger patients were more often diagnosed in early stages of cancer than older patients (Figure 3.2). Of women 15-29 years, 82% were diagnosed with FIGO stage I disease, while only 15% of patients 75 years and over were diagnosed with FIGO stage I disease. Only 2% of the patients in the age category 15-29 years were diagnosed in stage IV, while 14% of the oldest patients were diagnosed in this stage. Figure 3.3 shows that 55% of squamous cell carcinomas, and 65% of patients with adenocarcinomas were diagnosed in FIGO stage I ($p=0.003$). However, adenocarcinomas were diagnosed less often in the micro-invasive (*i.e.* IA) stage of the disease than squamous cell carcinomas (15% and 22%, respectively) ($p< 0.0001$) . The

	*Scc**		*AdCx†*		*Other*		Total§	
Year	*Cases*	*ESR‡*	*Cases*	*ESR‡*	*Cases*	*ESR‡*	*Cases*	*ESR‡*
1988	122	9.2	22	1.6	13	1.0	157	11.8
1989	117	8.6	24	1.8	14	1.1	155	11.5
1990	120	8.5	29	2.2	9	0.6	158	11.3
1991	106	7.2	20	1.2	8	0.6	134	9.0
1992	133	9.5	28	1.9	7	0.5	168	11.9
1993	123	8.7	30	2.1	5	0.4	158	11.1
1994	113	7.4	26	1.8	5	0.3	144	9.6
1995	117	7.6	25	1.7	6	0.4	148	9.7
1996	103	6.6	19	1.3	10	0.6	132	8.5
1997	115	7.3	32	2.1	4	0.3	151	9.6
1998	117	7.4	33	2.1	2	0.1	152	9.6
1999	106	6.6	17	1.1	8	0.4	131	8.2
2000	97	5.9	26	1.6	10	0.6	133	8.2
EAPC§		-3.2%		-1.2%		-8.5%		-3.5%
p value		<0.001		0.51		0.04		0.001

Table 3.1 Incidence of cervical cancer in the Greater Amsterdam area, the Netherlands, 1988-2000 *: SCC indicates squamous cell carcinoma.

†: AdCx indicates adenocarcinoma; ‡: ESR indicates European Standardised Rate; §: EAPC indicates Estimated Annual Percent Change

percentage of cases diagnosed in FIGO-stage IV did not differ statistically significantly between squamous cell carcinomas (7%) and adenocarcinomas (6%) (p=0.71). During the study period, there were no statistically significant changes in distribution over the stages.

During 1988-97, there were 1441 patients with cervical carcinoma in the region of the CCCA, 480 of whom died (33.3%). The median follow-up time was 56 months (range 0-165 months). The overall 5-year relative survival of cervical carcinoma was 71% (Table 3.2). Relative survival decreased from 91% for women 15-29 years, to 41% for women of 75 years and over. Patients with FIGO stage I had a relative survival of 91% decreasing to 16% for tumours diagnosed in FIGO stage IV. Relative survival of squamous cell carcinomas was 72% (95% CI: 69-75%), and somewhat lower for adenocarcinomas (66%, 95% CI: 59-73%).

Table 3.2 Relative survival of patients with cervical carcinoma in the Greater Amsterdam area, the Netherlands in 1988-1997

Variable	*Category*	*Cases*	*5-year survival*	*95% CI**
All cases		1441	71 %	68-74 %
Age	15-29	90	91%	82-95%
	30-44	575	84%	80-87%
	45-59	321	72%	66-77%
	60-74	285	54%	47-60%
	75+	170	41%	31-52%
FIGO-stage	I	831	91%	88-93%
	II	304	57%	50-64%
	III	168	35%	27-43%
	IV	99	16%	9-24%
	unknown	39	44%	27-61%
Histology	SCC†	1169	72 %	69-75 %
	AdCx‡	201	66 %	59-73 %
	Mixed/other carcinoma	71	61 %	48-72 %

*: CI denotes confidence interval; †: SCC indicates squamous cell carcinoma; ‡: AdCx indicates adenocarcinoma

Table 3.3 Relative risk of death for patients in the Greater Amsterdam area, the Netherlands, with cervix carcinoma diagnosed in 1988-1997 (n=1441).

Factor			Univariate		Multivariate†	
		Cases	Hazard ratio	95% CI‡	Hazard ratio	95% CI‡
FIGO-stage	I	831	1	Reference	1	Reference
	II	304	5.0*	3.9-6.4	3.4*	2.6-4.4
	III	168	10*	8.0-13	6.4*	4.7-8.6
	IV	99	19*	14-25	11*	7.5-15
	Unknown	39	9.2*	5.9-15	5.6*	3.5-9.0
Nodal involvement	No	563	1	Reference	1	Reference
	Yes	668	3.2*	2.5-4.1	2.1*	1.6-2.8
	Unknown§	210	1.6*	1.3-2.0	1.4*	1.1-1.8
Morphological type	SCC‖	1169	1	Reference	1	Reference
	AdCx¶	201	1.1	0.9-1.5	1.6*	1.2-2.1
	Mixed/other carcinoma	71	1.4	1.0-2.1	1.1	0.7-1.5
Year of diagnosis	1988/1990	451	1	Reference	1	Reference
	1991/1993	442	0.7*	0.6-0.9	0.7*	0.6-0.9
	1994/1997	548	0.8	0.7-1.1	0.8*	0.6-1.0

*: $p < 0.05$; †: Adjusted for age category and all other factors in the table; ‡: CI denotes confidence interval; §: Cases diagnosed in 1988 were all classified as unknown; ‖: SCC indicates squamous cell carcinoma; ¶: AdCx indicates adenocarcinoma

Table 3.3 displays the results of the multivariate analysis of survival for all types of cervical cancer. Compared to FIGO stage I, all other stages had significantly increased hazard ratios. Lymph node status is not considered in the FIGO classification for cervical carcinoma. For cases diagnosed in 1988, TNM was not registered and these cases were classified as "nodal involvement unknown". However, nodal involvement appeared to be associated with an increased risk of death (HR 2.1, 95% CI 1.6-2.7). Tumour histology was investigated with squamous cell carcinoma cases as reference category. Univariately, the hazard ratio of adenocarcinomas was slightly increased (HR 1.1), while mixed/other carcinomas were associated with a significant increase in risk. The increased risk for mixed/other carcinomas disappeared with multivariate adjustment, indicating that the increase in risk was caused by confounding by age and stage. After adjustment, the hazard ratio for adenocarcinomas was significantly increased (HR 1.6, 95% CI 1.2-2.1). Over time, the prognosis of patients with cervical cancer improved, as the multivariate hazard ratios were significantly decreased for the periods 1991-93 and 1994-97 as compared to the reference period 1988-90.

Squamous cell carcinoma cases and adenocarcinoma cases were also analysed separately (Table 3.4). Both for squamous cell carcinomas and adenocarcinomas survival decreased with increasing age, higher FIGO stage and positive lymph nodes at diagnosis (data not shown). The improvement in survival of women with cervical cancer during the period 1988-1997 was associated with cases of squamous cell carcinoma only. Survival of women with cervical adenocarcinoma did not improve during the study period (Table 3.4).

Table 3.4 Relative risk of death for patients in the Greater Amsterdam area, the Netherlands, with cervix carcinoma diagnosed in 1988-1997 (n=1441).

Factor		Univariate		Multivariate†	
		Hazard ratio	95% CI‡	Hazard ratio	95% CI‡
Year of diagnosis; AdCx§	1988/1990	1	Reference	1	Reference
	1991/1993	0.9	0.5-1.6	0.7	0.3-1.4
	1994/1997	0.8	0.5-1.4	1.2	0.7-2.2
Year of diagnosis; SCC¶	1988/1990	1	Reference	1	Reference
	1991/1993	0.7*	0.6-0.9	0.8*	0.6-1.0
	1994/1997	0.8	0.6-1.0	0.8*	0.6-1.0

*: $p<0.05$; †: Adjusted for age, stage and nodal involvement; ‡: CI denotes confidence interval; §: AdCx indicates adenocarcinoma; ¶: SCC indicates squamous cell carcinoma

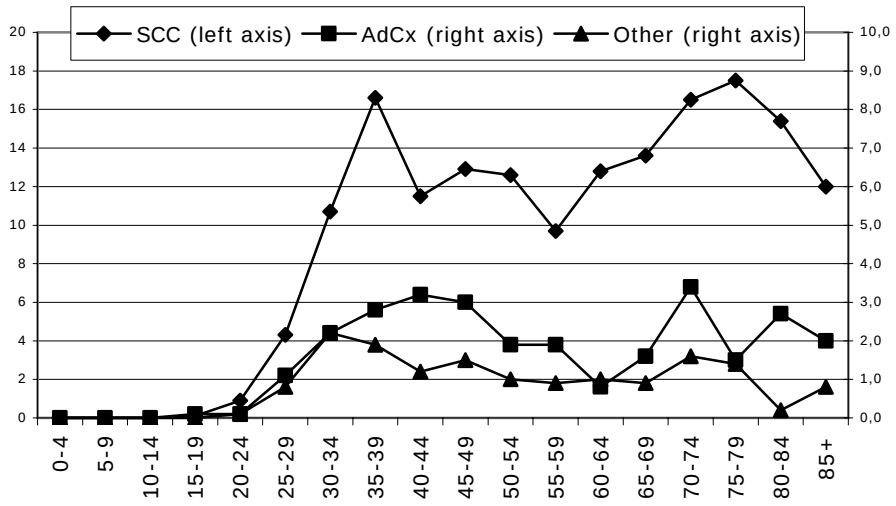

Figure 3.1 Age-specific incidence rates by histologic type of patients in the Greater Amsterdam area 1988-2000.

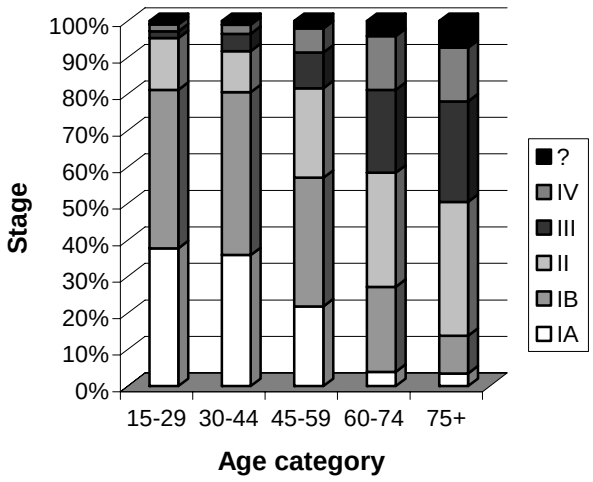

Figure 3.2 Stage at diagnosis by 15-year age category of patients in the Greater Amsterdam area, the Netherlands, 1988-2000

Discussion

Our results show that the incidence of cervical cancer has decreased significantly during the period 1988-2000, and that this decrease is caused by a decrease in the incidence of squamous cell carcinomas. In multivariate analyses, survival for patients diagnosed with adenocarcinoma of the cervix was significantly lower than survival for patients with squamous cell carcinoma. This indicates that women with adenocarcinoma of the uterine cervix have an intrinsically increased risk of death compared with women with squamous cell carcinoma independent of stage, age and nodal involvement.

We studied patients with cervical cancer who were all diagnosed within a geographically defined region in the Netherlands: the Greater Amsterdam area. As data were obtained by the Regional Cancer Registry, we were able to study an unbiased population of women with different histological types of cervical cancer for factors associated with survival. Nationally, the histological verification rate for cervical cancer is 99.7%, indicating a high accuracy rate of the Dutch Cancer Registries (17).

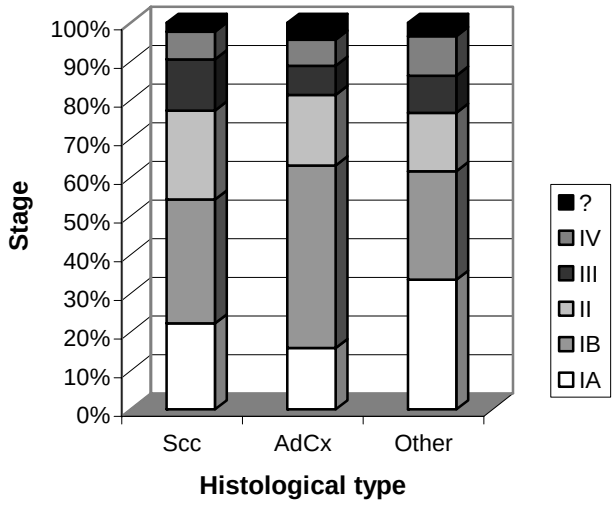

Figure 3.3 Stage at diagnosis by histologic type of patients in the Greater Amsterdam area, the Netherlands, 1988-2000

In this study, knowledge of participation in cervical cancer screening preceding the diagnosis of cancer may have been relevant. We did not have data on either individual Pap smear taking or participation in the nationwide screening programme in this group of women with cervical carcinoma. Cytological screening on an individual basis has been available for women in this region of the Netherlands since the 1970's. A nationwide screening programme aimed at specific age categories was initiated in 1988. Between 1988-96, women aged 34-54 years were screened triannually, and from 1996 onwards, women aged 30-60 are screened every 5 years. Overall, the coverage of cervical cancer screening activities over a period of 5 years is approximately 80% (18). However, regionally participation in each screening round of the population-based programme is lower (60-70%). Even without data on screening participation, some findings do suggest the efficacy of screening in this region of the Netherlands. The incidence of cervical squamous cell carcinoma decreased significantly, while no statistically significant change in the incidence of adenocarcinoma was found. Previous studies have reported that cervical adenocarcinoma and its pre-invasive stages are diagnosed less efficiently by Pap smear screening than squamous cell lesions (7;8). In some countries, not only the absence of a decrease in incidence (19;20), but even increases in the incidence of cervical adenocarcinoma have been described in the presence of a screening programme (4-6). One study even suggested a rise in incidence especially in younger women (3), whereas older women were not affected by rises in the incidence rate of cervical cancer. Our data do not support this trend. Our findings suggest a positive effect of the screening programme as shown by the decrease in cervical carcinoma incidence. Increasing screening participation to obtain an even higher degree of coverage will most likely increase the efficacy of the screening programme in decreasing the incidence of cervical cancer.

In our analyses, patients with cervical adenocarcinoma had a worse prognosis than patients with squamous cell carcinoma after correction for confounders such as age, stage and nodal involvement. Previous studies either usually lacked sufficient numbers of patients with adenocarcinomas (21), or were not population-based (12;13), or did not make direct comparisons between patients with squamous cell carcinoma and adenocarcinoma (22). Some studies attributed the decreased survival of women with adenocarcinoma to differences in histological type (10;11). In our study, patients with adenocarcinoma presented with more advanced stage I tumours than patients with squamous cell carcinomas. Still, patients with cervical adenocarcinoma were slightly younger at diagnosis than patients with squamous cell carcinoma, 48 years and 50 years, respectively. As the association between adenocarcinomas and a worse prognosis increased after correction for confounding factors, this indicates that women with cervical adenocarcinoma have an inherently worse prognosis than women with squamous cell carcinoma. The main causal factor for the development of both squamous cell carcinoma and adenocarcinoma of the uterine cervix is infection with high-risk types of the human papillomavirus (hrHPV) (23;24). There are substantial differences with respect to the exact type of hrHPV and the histological

diagnosis. Adenocarcinomas are more often associated with HPV type 18 than squamous cell carcinomas (23;25-27). Moreover, HPV 18 has been shown to be associated with a worse prognosis than other HPV types (28;29), but this finding has been challenged (21). An intrinsic difference between neoplasically converted squamous and cylindrical epithelium might also be the cause of the difference in prognosis, independently of HPV infection.

The survival of patients with squamous cell carcinoma increased during 1988-97, while the survival of patients with adenocarcinomas did not change significantly. During the study period, there were no large changes in treatment of patients with cervical cancer in any stage of the disease. The nationwide screening programme was introduced in 1988. Theoretically, an increase in prognosis of patients with squamous cell carcinomas might have been caused by a major change in stage at diagnosis during the study period. We did not observe such a change in stage at diagnosis over time (data not shown). However, we cannot exclude an effect of subtle changes within broad stages, such as a shift from FIGO 1B to FIGO 1A. Screening, leading to an earlier detection of cervical cancer even without significant changes in broad FIGO stage distribution, may contribute to such an effect. We were not able to find a trend over time in histological lymph node positivity over all stages of disease, as lymph node sampling is dependent on stage at diagnosis. In this study, in 31% of women with adenocarcinoma and 43% of women with squamous cell carcinoma, lymph node status was not determined histologically. Stage migration due to improved staging procedures might also have contributed to the increased survival.

The overall 5 year survival rate was 71%. Compared to other countries in Europe, this is a high survival rate, with only the Nordic countries having comparable survival rates (30). Other studies have also shown that within Europe, the Netherlands have a very low incidence, and a very high survival compared with other countries (31). It is reasonable to attribute these findings to the presence of a nationwide screening programme, as similar effects on incidence have been described in other countries with a screening programme (1). Whether the effect on survival in the absence of changes in treatment can be exclusively attributed to the cervical cancer screening programme, remains a question of debate (32;33).

In conclusion, in the Greater Amsterdam area the incidence of squamous cell carcinomas has decreased while there were no changes in the incidence of adenocarcinoma of the uterine cervix. Cases of adenocarcinoma of the uterine cervix are associated with a decreased survival rate compared to patients with squamous cell carcinoma. This decreased survival is related to tumour histology itself, since after correction for factors such as age, stage and lymph node status, the survival of adenocarcinoma patients is still lower compared with squamous cell carcinoma patients. Our study suggests that the present screening programme for cervical cancer is efficient in detecting (pre-) malignant stages of squamous cell carcinoma, but fails to detect (pre-) malignant stages of adenocarcinoma. Since more than 92% of the adenocarcinomas and its precursors contain hrHPV, adding hrHPV testing to

conventional cytological screening might improve the present screening programme in detecting adenocarcinoma and its precursor lesions.

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3.2 Cervical Cancer in the Netherlands 1989-1998. Decrease of Squamous Cell Carcinoma in Older Women, Increase of Adenocarcinoma in Younger Women

International Journal of Cancer. 2005;113(6):1005-9.

Abstract

Cervical cancer is a preventable disease, occurring in relatively young women. In the Netherlands, population-based cervical screening aims at women aged 30-60 years. We performed a population-based study of the incidence of invasive cervical cancer in the Netherlands to evaluate trends with emphasis on age at time of diagnosis. Of all women residing in the Netherlands diagnosed with invasive cervical cancer between 1-1-1989 and 31-12-1998, histological diagnosis was retrieved from the Netherlands Cancer Registry. In this ten year period, the incidence rate of squamous cell carcinoma decreased significantly from 7.1/100.000 women to 6.1/100.000 ($p<0.001$), with the highest decrease in women aged 60-74 (-5.5%). While the overall incidence rate of adenocarcinomas remained stable, it increased in women aged 15-29 (+15.8%) and in women aged 30-44 (+2.5%), although the number of cases was small. For squamous cell carcinoma, the incidence of stage II at diagnosis decreased most (-2.7%). There was no change in stage at diagnosis for adenocarcinoma. Most cases of cervical cancer, 60.5%, were detected between ages 30-60 years, *i.e.*, the Dutch screening age interval. Cervical cancer in women below age 30 contributed 5.0% to the total incidence of cervical cancer, with 3.0% occurring between ages 27-29.

Thus, the introduction of screening for cervical cancer in the Netherlands is associated with a decrease in the incidence of squamous cell carcinoma. Adenocarcinoma incidence seems to be increasing in younger women.

Introduction

The Netherlands has one of the lowest incidences of cervical cancer in Europe. In Dutch women, cervical cancer is only the 10th most common manifestation of invasive cancer (1). Possibly, the low incidence is due to the presence of cervical cancer screening programmes since the early 1970's.

Nowadays, the Dutch population-based cervical screening aims at women between the ages 30-60 years with an interval of five years between screening rounds (2;3). Since cervical cancer is a rare and late complication of infection with high-risk Human Papillomavirus (hrHPV) (4) and because women initiate sexual activity at increasingly younger ages (5), attention should be paid to the question whether the population-based screening programme for cervical cancer should decrease its lower age limit. In the Western countries with a screening programme, some programmes advocate screening to commence at age 30, but also at age 25, or even younger (6).

We evaluated the incidence and trends in incidence and stage at diagnosis over time of the different histological types of cervical cancer in the Netherlands. Special attention was given to the incidence of cervical cancer in relation to age at diagnosis, and age at which to commence cervical screening.

Materials and methods

Data collection. Data concerning the incidence of cervical cancer were obtained from the Netherlands Cancer Registry (NCR) (7). Nationwide cancer registration in the Netherlands started in 1989. The NCR consists of nine regional registries of the Comprehensive Cancer Centres in the Netherlands, combined into one national registry. The NCR collects data on all malignant neoplasms in the Dutch population.

For this study, all cases of cervical cancer diagnosed between 1-1-1989 and 31-12-1998 were selected from the cancer registry. Of the cervical cancer diagnoses registered by the NCR, 99.7% has been verified histologically (8). In the NCR, carcinoma *in situ* of the cervix is not registered and accordingly, only cases of invasive cervical cancer were included in this study. Clinical information and pathology data were extracted from the medical records. Data were collected on tumour site, morphological classification (according to the International Classification of Diseases for Oncology) and stage of the tumour (9). The fourth edition of the TNM-classification was used whenever applicable. If pathological TNM was not available, clinical TNM was used. The NCR registers stage using the TNM classification, and thus TNM stage was used and not FIGO stage. Data concerning participation in the cervical screening was not available. Mortality was not included in this study as mortality of cervical cancer is relatively low, and trends in mortality may be less clearly observed.

Population data of the Netherlands were obtained from Statistics Netherlands (CBS, Voorburg/ Heerlen, the Netherlands). During the study period, the total population of the Netherlands increased from 14.8 to 15.7 million persons. The average population size was 15.3 million.

Cytological screening on an individual basis has been available for women in the Netherlands since the 1970's, with population-based screening programmes occurring in a number of regions of the Netherlands. A nationwide screening programme aimed at specific age categories was initiated in 1988. Between 1988-96, women aged 34-54 years were screened triannually, and from 1996 onwards, women aged 30-60 are screened every 5 years. Overall, the coverage of cervical screening over a period of 5 years is approximately 80% (2). However, participation in each round of the population-based screening programme is lower (60-70%). In 1996, approximately one-third of all smears registered of women in all age groups were opportunistic (2). Between 1990 and 2000, the number of smears taken outside the screened age categories had decreased from 41.4% to 6.8%, the main decrease of which occurred in women under the age of 30 (10).

Statistical Analysis. Incidence of cervical cancer was calculated per 100,000 person years. Incidence rates were age-adjusted, using direct standardization to the European Standard Population, and European Standardized Rates (ESRs) were calculated (11).

Trends in incidence rates were examined by calculating the estimated annual percent change (EAPC) (11). For the analyses, cases were divided into squamous cell carcinoma, adenocarcinoma (including adenosquamous carcinoma) and other histological types. Age was categorised into 15 years age strata as follows, ages 15-29 years, 30-44 years, 45-59 years, 60-74 years and ages 75 and above. Differences in distribution over age were assessed using Chi-square statistics. Associations between variables were examined for all cases, and for cases of squamous cell carcinoma and adenocarcinoma separately. Cases with "other" histology were excluded from further analyses. Two-sided p values of 0.05 or less were considered statistically significant.

Results

Between January 1st, 1989 and December 31st 1998, 7,312 patients were newly diagnosed with invasive cervical cancer in the Netherlands (Table 3.5). The annual number of incident cases of cervical cancer increased from 719 patients registered in 1989 to 733 patients in 1998. The ESR decreased from 9.1/100,000 women in 1989 to 8.2/100,000 women in 1998. The total ESR decreased with 1.6% annually ($p<0.001$). This decrease in incidence was mainly caused by a decrease in the incidence of squamous cell carcinoma cases, as the EAPC in ESR for squamous cell carcinomas was –1.6% ($p<0.001$). For adenocarcinomas, there was no statistically significant trend in the incidence rate (EAPC 1.2%, $p=0.71$). The incidence of other and not specified cervical malignancies decreased with 8.0% annually ($p=0.002$). During the study period, the contribution of adenocarcinomas to the total number of cervical neoplasms ranged from 15.9% in 1989 to 21.0% in 1998 (statistically not significant).

Table 3.6 displays the number of cervical malignancies per age category. Overall, 5.0% (362/7312) of cases were diagnosed below age 30. Women aged 60 years and over, contributed 34.4% (2521/7312) to the overall number of cases. When evaluating age at diagnosis separately for squamous cell carcinoma and adenocarcinoma, the proportion of adenocarcinoma is higher at younger than at older ages. Below age 30, 4.3% (235/5432) of squamous cell carcinoma cases versus 5.9% (83/1402) of adenocarcinoma cases were diagnosed (X^2 6.38; $p=0.01$). In women over 60 years of age relatively more squamous cell carcinoma cases were diagnosed than adenocarcinoma cases, respectively 36.0% (1956/5432) versus 30.7% (431/1402), (X^2 13.60; $p<0.001$).

In this nationwide study, cervical cancer was rare in young women in the Netherlands. During the study period, no cases of cervical cancer were diagnosed at all under age 15, and only 5 cases of cervical cancer were diagnosed in patients less than 20 years of age during a study period of 10 years. Between 20 and 30 years, the number of all cervical carcinomas increased exponentiallywith increasing age, and overall the distribution of both adenocarcinom and squamous cell carcinoma was

bimodal (Figure 3.4). While 5.0% of all cases of cervical cancer were diagnosed under the age of 30, most of these (3.0%) were diagnosed between ages 27-29.

Trends in incidence rates were analysed over the age categories. Overall, from 1989 to 1998, there were no statistically significant changes in incidence rate for younger women aged 15-29 and 30-44 years. The incidence rate in women aged 45-59 years decreased with 1.2% annually (p=0.050), and with 5.1% annually for women aged 60-74 years (p< 0.001). For women aged 75 years and over, the incidence rate decreased albeit not statistically significantly (EAPC -0.9%, p=0.327) (data not shown). When analysing for squamous cell carcinoma cases separately, similar trends were observed (Table 3.7a). However, the decrease in incidence rate for women aged 45-59 years was not statistically significant. For women with adenocarcinoma, no significant decreases in incidence rates were observed (Table 3.7b). In contrast, a statistically significant increase in incidence rate was observed in women aged 15-29 (EAPC 15.8%, p=0.041) and in women aged 30-44 years (EAPC 2.5%, p=0.029). However, the absolute annual number of cases in women aged 15-29 years was very small (range 2-11 cases).

Trends in stage at diagnosis were analysed over time. Overall, there was a decrease in stage I and II cases (p=0.034 and p=0.012, respectively), whereas the incidence rates of higher stage cases did not change during the study period. (data not shown). Similar trends were observed when analysing for squamous cell carcinoma separately,

Table 3.5 Incidence of cervical cancer in the Netherlands, 1989-1998

	*Scc**		*AdCx†*		*Other*		*Total*	
Year	*Cases*	*ESR‡*	*Cases*	*ESR‡*	*Cases*	*ESR‡*	*Cases*	*ESR‡*
1989	556	7.1	114	1.4	49	0.6	719	9.1
1990	561	7.0	144	1.8	55	0.7	761	9.5
1991	537	6.4	135	1.7	61	0.8	737	8.9
1992	556	6.8	139	1.6	53	0.6	752	9.1
1993	523	6.3	143	1.7	53	0.6	722	8.6
1994	539	6.2	131	1.6	45	0.5	715	8.3
1995	547	6.4	141	1.7	34	0.4	725	8.5
1996	532	6.1	136	1.6	47	0.5	719	8.2
1997	535	6.1	157	1.8	37	0.4	729	8.3
1998	546	6.1	154	1.7	29	0.3	733	8.2
EAPC§		-1.6%		1.2%		-8.0%		-1.6%
p		0.001		0.71		0.002		<0.001

*: Scc indicates squamous cell carcinoma; †: AdCx indicates adenocarcinoma; ‡: ESR indicates European Standardised Rate;§: EAPC indicates Estimated Annual Percent Change

but only the decrease in stage II remained statistically significant (Table 3.8a). There were no statistically significant trends in stage at diagnosis of adenocarcinoma cases (Table 3.8b).

When analysing cases of adenocarcinoma excluding adenosquamous carcinoma, results did not change substantially (data not shown).

Discussion

Between 1989-1998, the incidence of cervical cancer decreased significantly in the Netherlands. About 1 in every 20 cases of cervical cancer is diagnosed before age 30. The decrease in incidence is caused by a decrease in the incidence of squamous cell carcinoma, and occurred mainly in women aged 60-74 years. In contrast, the incidence of cervical adenocarcinoma does not show any trend overall, but in women aged 15-44 years an increase in incidence was observed.

Some methodological issues of our study need to be addressed. This study includes all cases of cervical cancer in the Netherlands as registered by the NCR, so the population we studied was unbiased. The histological verification rate of registered cervical cancers is 99.7% (8). However, the use of the national registry has its disadvantages, as neither individual Pap smear taking nor participation in the nationwide screening programme preceding the diagnosis of cervical cancer is registered. Had we been able to restrict our study to women who had participated in the cervical screening programme, the effect of cervical screening on trends in incidence of cervical cancer would probably more clearly demonstrable. Overall, the coverage of cervical screening activities is approximately 80% (2). Still, a recent study showed that for half of the women with cervical cancer a screening smear, either opportunistic or programme-based, was not registered in the nationwide network and registry of histo- and cytopathology (PALGA), indicating half the cases of cervical cancer occur in the 20% of women who do not participate in cervical screening (12). Most likely, increasing the coverage of population-based screening will further decrease the incidence rate of cervical cancer, as has been shown in England (13).

In the Netherlands, nationwide registration of all cases of cancer started in 1989, and only a relatively short period of registration could be included in this study. Even though for this reason we were not able to study cohort effects in an age-period-cohort analysis, we believe that our analyses give meaningful estimates of trends over time in incidence rates of cervical cancer. In our study, the trends in incidence are in concordance with other studies on cervical cancer incidence after the introduction of cervical screening (15;16).

Table 3.6 Distribution of cases per age category, the Netherlands, 1989-1998.

Age category	*Scc** *N (%)*	*AdCx†* *N (%)*	*Total* *N (%)*
15-29 Years	235 (4.3)	83 (5.9)	362 (5.0)
30-44 Years	2006 (36.9)	564 (40.2)	2781 (38.0)
45-59 Years	1235 (22.7)	324 (23.1)	1648 (22.5)
60-74 Years	1239 (22.8)	263 (18.3)	1575 (21.5)
75+ Years	717 (13.2)	168 (12.0)	946 (12.9)
Total	5432	1402	7312

*: Scc indicates squamous cell carcinoma; †: AdCx indicates adenocarcinoma; Percentages do not add to 100 because of rounding

Table 3.7a. Incidence of squamous cell carcinoma of the cervix by age category in the Netherlands, 1989-1998

SCC*	15-29 yrs		30-44 yrs		45-59 yrs		60-74 yrs		75+ yrs		Total	
Year	N	ESR†	N	ESR†	N	ESR†	N	ESR†	N	ESR†	N	ESR†
1989	22	1.2	189	11.0	127	10.8	149	11.2	69	12.9	556	7.1
1990	29	1.6	203	11.6	110	9.3	145	10.3	74	14.3	561	7.0
1991	27	1.5	203	11.5	97	7.8	135	9.6	75	13.4	537	6.4
1992	18	1.0	209	11.8	128	10.0	135	9.0	66	11.9	556	6.8
1993	25	1.5	193	10.9	121	9.1	120	8.7	64	11.9	523	6.3
1994	19	1.1	180	10.0	133	9.5	118	7.9	89	15.8	539	6.2
1995	26	1.6	216	11.9	127	9.0	114	8.5	64	11.5	547	6.4
1996	17	1.1	215	11.8	120	8.5	104	6.7	76	13.0	532	6.1
1997	23	1.5	202	11.0	139	9.4	107	6.4	64	10.8	535	6.1
1998	29	1.9	196	10.6	133	8.8	112	7.2	76	12.1	546	6.1
EAPC‡		1.4%		-0.5%		-0.9%		-5.5%		-1.5%		-1.6%
P		0.572		0.485		0.368		<0.001		0.241		0.001

*: SCC indicates squamous cell carcinoma; †: ESR indicates European Standardised Rate ; ‡: EAPC indicates Estimated Annual Percent Change

Table 3.7b. Incidence of adenocarcinoma of the cervix by age category in the Netherlands, 1989-1998

*AdCx**	*15-29 Years*		*30-44 Years*		*45-59 Years*		*60-74 Years*		*75+ Years*		*Total*	
Year	N	ESR†	N	ESR†	N	ESR†	N	ESR†	N	ESR†	N	ESR†
1989	2	0.1	46	2.7	20	1.7	34	2.4	12	2.2	114	1.4
1990	7	0.4	50	2.9	38	3.2	31	2.4	18	3.3	144	1.8
1991	8	0.5	51	2.9	32	2.6	28	1.5	16	2.8	135	1.7
1992	5	0.3	55	3.1	28	2.1	31	2.1	20	3.8	139	1.6
1993	11	0.7	62	3.5	32	2.4	16	1.1	22	4.0	143	0.7
1994	3	0.2	56	3.1	35	2.6	22	1.1	15	2.6	131	1.6
1995	16	1.0	59	3.3	34	2.5	18	1.0	14	2.3	141	1.7
1996	9	0.6	51	2.8	38	2.7	23	1.5	15	2.5	136	1.6
1997	11	0.7	69	3.7	33	2.2	30	1.6	17	2.8	157	1.8
1998	11	0.7	65	3.5	34	2.2	25	1.5	19	3.0	154	1.7
EAPC‡		15.8%		2.5%		0.3%		-5.4%		-0.4%		1.2%
P		0.041		0.029		0.889		0.124		0.862		0.71

* AdCx indicates adenocarcinoma; †: ESR indicates European Standardised Rate; ‡: EAPC indicates Estimated Annual Percent Change

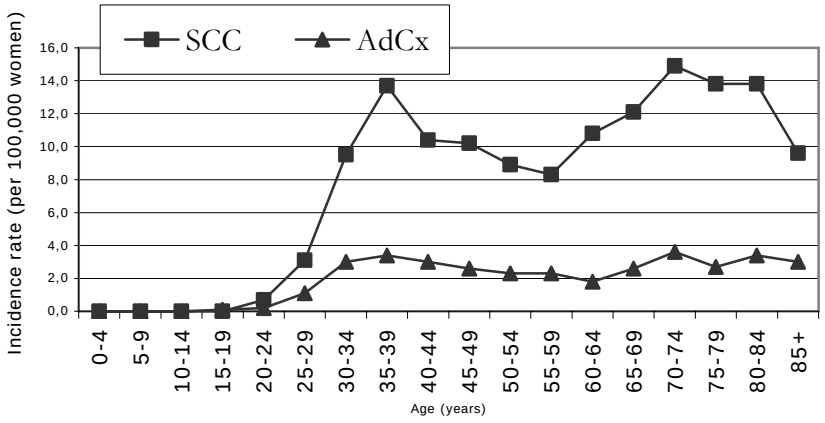

Figure 3.4. Age-specific incidence rate of cervical cancer in the Netherlands, 1989-1998

SCC indicates squamous cell carcinoma; AdCx indicates adenocarcinoma

Table 3.8a. Trends in incidence of squamous cell carcinoma according to TNM stage, the Netherlands, 1989-1998.

	Stage I		Stage II		Stage III		Stage IV		Stage X*		Total	
Year	N	ESR†	N	ESR†	N	ESR†	N	ESR†	N	ESR†	N	ESR†
1989	251	3.2	146	1.8	101	1.3	36	0.5	22	0.2	556	7.1
1990	270	3.4	125	1.5	113	1.4	41	0.5	12	0.1	561	7.0
1991	239	3.0	116	1.3	128	1.6	34	0.4	20	0.2	537	6.4
1992	269	3.4	137	1.6	104	1.6	35	0.4	11	0.1	556	6.8
1993	256	3.1	99	1.1	120	1.4	33	0.4	15	0.2	523	6.3
1994	247	2.9	104	1.1	127	1.5	46	0.5	15	0.2	539	6.2
1995	273	3.3	109	1.2	110	1.3	45	0.5	10	0.1	547	6.4
1996	269	3.1	116	1.3	102	1.3	40	0.5	5	0.1	532	6.2
1997	255	3.0	108	1.2	119	1.3	45	0.5	8	0.1	535	6.1
1998	277	3.2	99	1.0	113	1.3	44	0.4	13	0.2	546	6.1
EAPC‡		-0.5		-2.7		-0.9		0.6		-8.2		-1.6
P		0.404		0.019		0.277		0.547		0.094		0.001

*: Stage X indicates Stage unknown; †: ESR indicates European Standardised Rate; ‡: EAPC indicates Estimated Annual Percent Change

Table 3.8b. Trends in incidence of adenocarcinoma according to TNM stage, the Netherlands, 1989-1998.

	Stage I		Stage II		Stage III		Stage IV		Stage X*		Total	
Year	N	ESR†	N	ESR†	N	ESR†	N	ESR†	N	ESR†	N	ESR†
1989	62	0.8	14	0.2	23	0.3	10	0.1	5	0.1	114	1.4
1990	84	1.1	14	0.2	31	0.4	7	0.1	8	0.1	144	1.8
1991	78	1.0	19	0.2	23	0.2	10	0.1	5	0.1	135	1.6
1992	71	0.9	23	0.3	22	0.2	17	0.2	6	0.1	139	1.6
1993	88	1.1	18	0.2	27	0.3	6	0.1	4	0.0	143	1.7
1994	74	0.9	19	0.2	21	0.3	12	0.1	5	0.1	131	1.6
1995	80	0.9	17	0.2	28	0.3	12	0.1	4	0.0	141	1.6
1996	80	1.0	16	0.2	17	0.2	19	0.2	4	0.0	136	1.6
1997	95	1.1	24	0.2	26	0.3	9	0.1	3	0.0	157	1.8
1998	87	1.0	16	0.2	27	0.3	15	0.1	9	0.1	154	1.7
EAPC‡		1.2		2.1		-1.5		3.6		-7.6		1.2%
p		0.351		0.336		0.528		0.429		0.183		0.71

*: Stage X indicates Stage unknown; †: ESR indicates European Standardised Rate ; ‡: EAPC indicates Estimated Annual Percent Change

Overall cervical cancer incidence decreased most in women aged 45-74. As regional screening programmes have been available since the 1970's, and the population-based cervical screening programme started in 1988, these women have had the opportunity to participate in screening throughout their life (15). Presumably, removal of precursor lesions of cervical cancer has led to a decrease in incidence rate at mature ages. Cervical cancer incidence did not change in women below age 45. Indeed, women below age 30 are not screened in the Netherlands, and will only be diagnosed as they present with symptoms of invasive cancer. Women aged 30-44 years will mostly have been screened only once, and the trend in this age category is mainly based on prevalent cases. However, when analysing squamous cell carcinoma and adenocarcinoma separately, trends differ for adenocarcinoma. There are no statistically significant trends at mature ages. In contrast, an increase of adenocarcinoma at ages 15-29, and ages 30-44 is seen, even though these analyses are based on few cases in absolute numbers. The absence of an overall trend in adenocarcinoma incidence suggests that the screening programme may not prevent adenocarcinoma as effectively as squamous cell carcinoma. Adenocarcinomas do not have as well-defined precursor lesions cytologically as squamous cell carcinomas (17;18). We cannot exclude that the rise in adenocarcinoma at younger ages has been caused by the change in call-and-recall schedule as introduced in 1996. The extension of the screened ages (from 34-54 years to 30-60 years) may have led to an increased detection of prevalent cases of cervical cancer in the newly screened categories. However, the increase in incidence is not present in squamous cell carcinoma cases, pleading against this explanation. A rise in adenocarcinoma incidence especially in younger women has been described in several other countries with a cervical screening programme (15;16).

An effect of cervical screening on cervical cancer incidence is also supported by the fact that the stage at diagnosis decreased for both stage I and stage II, while the higher stage tumours (stage III and IV) did not change in incidence. An early effect of screening is a decrease in higher stage tumours, and a late effect would be a decrease in the lower stages (19;20). Indeed, the decrease in lower stage tumours as established in this study may well be attributed to screening, as women who are screened will be either diagnosed with pre-invasive lesions or early stage tumours, resulting in redistribution of stage II to stage I, and of stage I to cervical intraepithelial neoplasia grade III (CIN3). Unfortunately, we were not able to demonstrate a decreasing trend of CIN3, as the Netherlands Cancer Registry does not register premalignant lesions.

Cervical cancer is caused by infections with hrHPV (4). Infections with hrHPV are mainly acquired through sexual contacts, with hardly any genital hrHPV infections occurring before the first sexual contact (21;22). In several countries, including the Netherlands, women initiate sexual intercourse at decreasing age (5). A cohort effect in cervical cancer incidence will occur as women are infected by hrHPV at younger ages over time. Thus, decreasing trends in the age of initiation of sexual contacts should be monitored for future adaptations of the screening programme. Already,

women under the age of 30 years contribute 5.0% to the overall number of cases of cervical cancer in the Netherlands, and 3.0% are diagnosed shortly before the first screening round, between ages 27-29 years. Presumably, by introducing screening before the age of 30, these cases of cervical cancer will be diagnosed in earlier clinical stages or even be prevented. Based on Dutch modelling data, screening before age 27 would not be efficacious, but a first screening round at age 27 or 28, some years earlier than the current first screening round, could be cost-effective while preventing substantial morbidity (23). Also, adding an hrHPV test to the current screening programme, would increase the detection rates of precursors of cervical cancer for both squamous cell carcinoma and adenocarcinoma as both histological types of cervical cancer are caused by hrHPV (24;25). As transient hrHPV infections have a very high prevalence in women under the age of 30, additional viral tests, *e.g.*, viral load determination (26) or detection of E6/E7 transcripts (27), may be used to select those women with progressive squamous or glandular lesions from all women who test hrHPV positive. In contrast to the trends at younger ages, we expect that the number of cases in mature women will decrease over time as the incidence rate in women 60-74 years of age decreases most strongly, *i.e.*, in women who have had the opportunity to participate in programme-based screening (15). As no definitive evidence has been presented that introducing hrHPV-testing will reduce cervical cancer, cost-effectiveness studies will have to be performed to evaluate the effect of new tests.

In conclusion in the Netherlands, the incidence of squamous cervical cancer has decreased, but the incidence of adenocarcinoma does not display a decreasing trend. Since five percent of all cervical carcinoma cases are diagnosed before the age of 30 years, adding a test for hrHPV may decrease the incidence of especially adenocarcinoma in the future. Cost-effectiveness analysis should reveal whether the addition of hrHPV-testing to the cervical cancer screening programme is feasible.

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CHAPTER 4.

SCREENING CYTOLOGY IN THE NETHERLANDS

The Dutch CISOE-A Framework for Cytology Reporting Increases Efficacy of Screening upon Standardization Since 1996.

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Abstract

The aim of this study was to describe the effect of the introduction of the CISOE-A framework for reporting of cervical cytology results including changes in repeat and referral advices in the Netherlands on the efficacy of the screening programme. We report changes in distribution of cytologic results, detection rate of CIN lesions and detection rates of squamous cervical carcinoma.

In 7 laboratories in the greater Amsterdam area, we retrieved all results of gynecologic cytologic and histologic examinations as registered in the nationwide database for histo- and cytopathology (PALGA) from 1990-2000.

After the introduction of the CISOE-A classification, the percentage of cytologic results of equivocal diagnoses decreased statistically significant from 11.3 to 2.6%, without an increase in the percentages of moderate dyskaryosis or worse. During the study period, the detection rate of histologically diagnosed high-grade CIN lesions increased statistically significantly from 4.1 to 6.4/1000 smears, while there was no change in detection rates of low-grade lesions or invasive cervical cancer.

The introduction of the new CISOE-A classification resulted in a substantial decrease of equivocal results and repeat recommendations, without a decrease in the detection rate of high-grade lesions, making the screening programme more efficacious.

Introduction

Screening for cervical cancer and its precursor lesions as a method to reduce cervical cancer morbidity and mortality was gradually introduced in the Netherlands since 1970. Nationwide cytological screening was introduced in 1988 (1). However, a lack of a uniform and reproducible reporting procedure for cytomorphological findings resulted in a considerable number of repeat smears based on a so-called equivocal cytological result, *i.e.* smears coded as borderline or mild dyskaryosis (BMD). Especially the reading of smears with inflammation as 'borderline dyskaryosis' resulted in an extremely high percentage (14%) of equivocal results. This led to a high number of repeat and referral smears, burdening both pre-clinical and clinical capacities. In order to increase the efficacy of the programme, the new CISOE-A classification introduced in 1996 uniformly described cytomorphological results. Simultaneously, the nationwide screening programme was restructured to provide general practitioners and gynaecologists with clear guidelines and unambiguous repeat and referral advices and the age range of women invited for screening was extended.

In this paper, we describe the effect of the introduction of the CISOE-A classification by evaluating the percentage of equivocal results and by evaluating the effectiveness of the screening programme to detect squamous (pre)malignant lesions of the cervix (2). We used routinely collected data in the greater Amsterdam area of the Netherlands to compare the first five years of monitoring after the introduction of the CISOE-A classification in 1996 with the period preceding its introduction. As the screening programme aims at detection of squamous lesions, we excluded glandular lesions from this study.

Methods

The Dutch screening programme and coding of cervical cytology. Before 1996, women between the ages of 34 to 55 years were invited every three years, and from 1996 onwards women between the ages of 30 to 60 years are invited every five years (1). To be invited for screening, women have to reach the targeted age during that calendar year, meaning that the first screening round at age 30 actually includes women of both 29 and 30 years of age. From 1988, when nationwide screening was introduced, until 1996, repeat recommendations or referral for further diagnostic evaluation were based on the Pap classification of smears. The CISOE-A classification as introduced in 1996 has been described previously (3). Criteria have been distributed throughout all the laboratories around 1996 and 1997 on CD-rom, with clear images representing all the different grades of the CISOE-A nomenclature. Briefly, the CISOE-A classification interprets smears using a rating system including information on specimen composition, inflammatory characteristics and adequacy of the smear. The letters C (composition), I (inflammation), S (squamous), O (other and endometrium), and E (endocervical cylindrical epithelium) are used to indicate the composition and morphology of the smears (Table 4.1). The letter A (adequacy) is used to indicate the adequacy of the smear (three-tiered grading system) and, except for inadequate smears (A3), does not affect the advice. Squamous, columnar and other cells are graded for the presence of dyskaryosis (dysplasia) and these values determine the interpretation of the smear. The CISOE-A classification is more detailed than the previously used convention of reporting cervical smears in Pap terminology with descriptive diagnoses (Table 4.2). In short, the most important change in cytologic result reporting occurred in the criteria applied to the equivocal category. Using the CISOE-A classification, reactive changes such as induced by inflammation are diagnosed as normal, *i.e.,* non-neoplastic, where previously these changes were often diagnosed as equivocal. In conjunction with the introduction of the CISOE-A classification, a dedicated computer application of the local systems was introduced in the laboratories ("Cervical Registration and Information System version 3") to ensure uniform registration into local laboratory databases and to prevent 'illogical' CISOE-A combinations in the report. Description of the smears is performed by entering the CISOE-A scores in the program, which generates a report automatically with a clear conclusion concerning the advice for the clinician (for convenience, the Pap score is

included). Thus, CISOE-A interpretations can be 'translated' into Pap classifications or the Bethesda classification (Table 4.3) (4;5).

Data collection. Excerpts of all cervical smears and histological diagnoses are stored in the nationwide network and registry of histo- and cytopathology (PALGA). We investigated cervical screening data from seven laboratories in the greater Amsterdam area. From these laboratories, we included *all* cervical smears registered from 1-1-1990 to 31-12-2000 inclusive, as stored in PALGA without separating 'indication' from 'opportunistic' (*i.e.*, outside the national screening program) as registration of these categories for smear taking was either absent (prior to 1996) or not consistently reported (after 1996) (6). We included laboratories from the region that were connected with central PALGA for the entire duration of the study period, had examined a minimum of 5000 cervical smears yearly, and had consistently participated in the follow-up procedure of the quality assurance procedures. The selected laboratories covered approximately 90% of all screening smears perfomed in the region. Throughout the study period, there were no changes in the population referred to these laboratories for screening.

Since we retrieved data on all gynaecological cytology results from PALGA, more than one smear from one woman could be retrieved. We included all smears, unless a smear was taken for opportunistic reasons (*i.e.,* smear taken at an age *not* invited for screening). Repeat smears were excluded from the analysis whenever taken within a 2 year period after a smear included in the analysis. All histological reports of mild to high-grade cervical intra-epithelial lesions (CIN1-3) and squamous cell carcinoma (SCC) were included. Adenocarcinoma and its precursor lesions were excluded, since the changes in the classification aimed at improving the diagnosis of squamous lesions. If there was more than one histologically verified lesion reported after a smear (concerning one patient), the most relevant (*i.e.*, severe) histological diagnosis was chosen within a 1-year time frame after the index cytology. All data in this study were retrieved anonymously, without disclosure to the researchers of any identifying information such as name, date of birth, or address. Data from the 7 laboratories were pooled and were not allocated to individual laboratories.

Statistical Analysis. For the analyses, CISOE-A cytology results were categorised as 'inadequate'(A3), 'normal' (S1; O1-2; E1-2), 'equivocal' or borderline (S2-3, O3, E3), mild dyskaryosis (S4; E4, A4), and moderate dyskaryosis or worse (S≥5; O≥5; E≥5). To evaluate the effects of age on the efficacy of screening, we categorised age as follows, women who were never screened (≤28 years), women who were screened only after 1996 (age 29-33 and 54-61), women who were screened in both schedules (34-53 years) and mature women screened in neither schedule (≥62 years). Data on cervical histology were divided into CIN1, CIN2-3 and SCC. Detection rates of histological lesions were calculated per 1000 smears. Data were analysed for linear

trends over time by linear regression analysis. P values of 0.05 or less were considered statistically significant. All analyses were performed using SPSS 9.0 for Windows.

Results

Between 1990 and 2000, there was a statistically significant decrease in the percentage of equivocal results, from 11.3% in 1990 to 2.6% in 2000 ($p<0.001$) and was present in all age categories (Table 4.4 & Figure 4.1). The contribution of results >BMD did not change over time. Inversely related to the decrease in BMD results, the percentage of normal smears increased statistically significantly over time, from 87.1% in 1990 to 95.3% in 2000 ($p<0.001$). The category of inadequate smears showed a small increase from 0.7% to 1.2% ($p=0.004$).

During the study period, the total number of smears decreased from over 100,000 smears to approximately 80,000 smears (Table 4.5). The number of smears per age category was influenced by the change in repeat and referral schedule in 1996. For this reason a substantial proportion of women aged 34-53 years were invited with an interval less than 5 years to compensate for a too long interval in 1996 and 1997. Overall, the number of screening smears increased from 65,265 in 1990 (ages 34-53 years) to 72,892 smears (ages 29-61 years), while the number of smears outside the age categories targeted for screening decreased from 46,009 to 5,278.

The detection rate of histological lesions is displayed in Table 4.6. During the study period, the overall detection rate of CIN2-3 lesions increased from 4.1/1000 smears in 1990 to 6.4/1000 smears in 2000 ($p<0.001$), whereas there was no increase in either the detection rate of CIN1 lesions ($p=0.180$) or SCC ($p=0.508$). In the youngest age category, women below age 29 years, the detection rate of CIN2-3 lesions increased from 5.2/1000 smears to 11.6/1000 smears ($p<0.001$), also with an increased detection rate of CIN1 lesions ($p<0.001$). In women aged 29-33 years, the detection rate of CIN2-3 lesions increased from 6.4/1000 smears with opportunistic screening to 14.1/1000 smears with programme-based screening ($p<0.001$). In women aged 34-53 years, the detection rate of CIN2-3 lesions increased statistically significantly from 1.2/1000 smears to 5.0/1000 smears ($p=0.003$), without an increase in detection rate of SCC ($p=0.449$). There were no obvious linear trends in the detection rates of lesion in age categories 54-61 years or 62 years and over, except for a statistically significant decrease in the detection rate of SCC of 1.3/1000 smears to 0.5/1000 smears in women aged 54-61 years ($p=0.008$).

Table 4.1 Overview of the CISOE-A classification.

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

The smears are examined for 5 different aspects of the composition of the smear, and a score is assigned leading to the CISOE code.
"A" indicates adequacy of the smear, which is graded as 1-3; 1, adequate; 2, adequate but suboptimal (reason specified by cytotechnologist); 3, inadequate.

Table 4.2 Comparison of referral and repeat schedule before and after 1996 in the Netherlands.

Before 1996 Descriptive diagnosis	**Pap**	**Advice**	**1996 onwards** **Description with CISOE-A score**	**Pap**	**Advice**	
Normal	Pap1	Default screening advice: repeat after 3 years	Normal squamous (S1), endocervical cells (E1), other cells (O1), atrophy (O2), no endocervical cells (E2)	Pap1	Default screening advice: repeat after 5 years	Normal
Borderline	Pap2	Repeat at 12 and 24 months	Aypical cells atypical squamous (S2), endocervical cells (E3), squamous metaplasia (S3) atypical repair (O3)	Pap2	Repeat at 6 and 18 months	BMD
Mild/ moderate dyskaryosis	Pap3a	Repeat at 3 and 12 months	Mild dyskaryosis squamous (S4), endometrial cells (O4), endocervical cells (E4), moderate dyskaryosis of endocervical cells (E5)	Pap3a1		
			Moderate dyskaryosis* squamous cells (S5), endometrial cells (O5)	Pap3a2		
Severe dyskaryosis Carcinoma in situ Carcinoma	≥Pap3b	ColpoBx	Severe dyskaryosis, (adeno)carcinoma in situ, Carcinoma squamous (>S5), endometrial cells (>O5), endocervical cells (>E5)	≥Pap3b	ColpoBx	>BMD

*: Moderate dyskaroysis had no separate advice prior to 1996.
ColpoBx indicates colposcopy and/or colposcopically directed biopsies; BMD indicates borderline or mild dyskaryosis

Table 4.3 The CISOE-A classification compared to Bethesda 2001 and Pap classification.

S	O	E	Pap	Description	Bethesda 2001	
0	0	0	0	Inadequate	Unsatisfactory for evaluation	
1	1	1-2	1	Normal	Negative for intra-epithelial lesion or malignancy	
1	2	1-2	1	Normal	Atrophy, negative for intra-epithelial lesion or malignancy	
2-3	3	3	2	Borderline dyskaryosis	ASC-US/ASC-H	AGC
4	4	4	3a1	Mild dyskaryosis	ASC-H/LSIL	AGC favor neoplastic
5	5	5	3a2	Moderate dyskaryosis	HSIL	AGC favor neoplastic
6	6	6	3b	Severe dyskaryosis	HSIL	AGC favor neoplastic
7	-	7	4	Carcinoma in situ	HSIL	AIS
8-9	7-8	9	5	Carcinoma	Squamous cell carcinoma	Adenocarcinoma

Abbreviations: ASC-US: Atypical squamous cells of undetermined significance, ASC-H: Atypical squamous cells cannot exclude HSIL, AGC: Atypical glandular cells, LSIL: Low-grade squamous intra-epithelial lesion, encompassing CIN1, HSIL: High-grade squamous intra-epithelial lesion, encompassing CIN2-3, and AIS: Endocervical adenocarcinom *in situ*.

Table 4.4 Cytological diagnoses of cervical smears and trends, 1990-2000.

	Inadequate*	Normal†	BMD‡		>BMD§	Total
	Pap 0	Pap 1	Pap 2	Pap 3a1	≥Pap 3a2	
	N (%)	N (%)	N (%)	N (%)	N (%)	N
1990	818 (0.7)	96,929 (87.1)	10,875 (9.8)	1,693 (1.5)	959 (0.9)	111,274
1991	843 (0.8)	94,768 (86.6)	11,119 (10.2)	1,723 (1.6)	975 (0.9)	109,428
1992	922 (0.8)	94,935 (85.2)	12,555 (11.3)	1,898 (1.7)	1,093 (1.0)	111,403
1993	878 (0.8)	95,222 (85.1)	12,797 (11.4)	1,994 (1.8)	1,019 (0.9)	111,910
1994	921 (0.8)	98,681 (85.9)	12,367 (10.8)	1,918 (1.7)	1,038 (0.9)	114,925
1995	947 (0.9)	90,272 (87.0)	9,801 (9.5)	1,704 (1.6)	981 (0.9)	103,714
1996¶	951 (0.8)	100,668 (89.5)	8,452 (7.5)	1,480 (1.3)	967 (0.9)	112,518
1997	879 (0.8)	105,821 (91.4)	6,747 (5.8)	1,305 (1.1)	1,028 (0.9)	115,780
1998	888 (0.9)	93,038 (94.7)	2,531 (2.6)	960 (1.0)	875 (0.9)	98,292
1999	912 (1.0)	83,875 (95.2)	1,881 (2.1)	718 (0.8)	764 (0.9)	88,150
2000	956 (1.2)	74,477 (95.3)	1,506 (1.9)	537 (0.7)	694 (0.9)	78,178
p#	0.004	<0.001	<0.001	0.001	0.427	

*: Inadequate indicates Pap 0; †: Normal indicates negative for intra-epithelial abnormalities (Pap 1); ‡: BMD indicates borderline or mild dyskaryosis (Pap 2-3a1); §: >BMD indicates moderate dyskaryosis or worse (≥ Pap 3a2); ¶: In 1996, the CISOE-A classification with formal reporting and referral was introduced and, in addition, 3-yr interval screening for women 34-55 years was changed into 5-yr interval screening for women 30-60 years; #: P value for linear test-for-trend

Table 4.5 The number of smears taken per age category, 1990-2000.

Year	≤ 28 yrs	29-33 yrs	34-53 yrs	54-61 yrs	≥ 62 yrs	Total
	N	N	N	N	N	N
1990	22,290	16,179	65,265	4,599	2,941	111,274
1991	19,943	15,502	67,238	4,193	2,552	109,428
1992	20,965	16,291	66,319	4,839	2,989	111,403
1993	18,098	15,961	70,188	4,828	2,835	111,910
1994	16,814	15,439	74,044	5,642	2,986	114,925
1995	14,049	14,287	66,834	5,572	2,972	103,714
1996*	10,071	24,267	58,902	16,640	2,638	112,518
1997	6,880	22,244	67,575	16,722	2,359	115,780
1998	5,417	16,795	61,855	12,028	2,197	98,292
1999	4,482	14,896	52,456	14,177	2,139	88,150
2000	3,437	9,584	50,740	12,568	1,841	78,178

Shaded areas indicate age categories invited for the population-based screening programme. *: In 1996, the CISOE-A classification with formal reporting and referral was introduced and, in addition, 3-yr interval screening for women 34-55 years was changed into 5-yr interval screening for women 30-60 years.

Discussion

Our data show that as a result of the introduction of a uniform classification system, the number of cytologic BMD results decreased substantially, without decreasing the detection rate of high-grade CIN lesions or worse in the cervical cancer screening programme. The new repeat and referral schedule that was introduced in conjunction with the CISOE-A classification in 1996 strongly influenced the number of smears taken. One of the aims of the new schedule was to decrease the number of opportunistic smears. Outside the screened ages 29-61 years, the number of smears decreased substantially. Even though the decrease was present throughout the entire period studied, it was especially impressive in the youngest age category of women under 29 years of age. Also, within the screened age range, the number of smears decreased in women aged 29-33 and 34-53 years. In mature women screened, the number of smears more than doubled after 1996. Spontaneous screening is associated with a decreased participation rate in mature women (6;7). Thus, the introduction of the new schedule has led to a substantially increased coverage of mature women in the cervical cancer screening programme in our region.

The efficacy of the screening programme, as measured by the detection rate of high-grade lesions, has not decreased during the study period. In contrast, upon restructuring the national screening programme in 1996, detection rates of high-grade lesions increased significantly, from 1.5/1000 smears to 5.4/1000 smears. This shift toward higher rates was noted not only in the group that has been screened throughout 1990-2000, but also in women below age 29 (who were never in the official screening program) and women aged 29-33 (who only entered the programme after 1996). We propose several explanations for the increased detection rates of CIN2-3. Firstly, the detection rates of high-grade lesions increased because the efficacy of the screening programme increased by the more consistent application of the CISOE-A classification and its translation into repeat and referral advices. Since the CISOE-A classification and its referral policies were applied for cytology in younger ages as well, it may have contributed to higher detection rates in the younger group as well. Secondly, the observed increased detection rates may be related to a true rise in the incidence of premalignant cervical lesions, irrespective of the new classification and referral policy. However, we did not detect a significant rise in carcinoma incidence in all age groups (see Table 4.6). The most favoured explanation for a rise of high-grade lesions in the young category only, is based on increased prevalences of human papillomavirus infections caused by changes in sexual behaviour (8;9). Thirdly, an increase in detection rate of CIN2-3 may be a spurious finding caused by length-time bias, as the repeat and referral schedule changed. Because the interval between normal smears increased from 3 to 5 years, high-grade CIN lesions have more time to develop and thus be diagnosed. These CIN lesions could be progressive, but also regressive. Several studies indicate that regressive CIN

lesions are more frequent in younger women (10-12). Again, the stable detection rates of squamous cell carcinomas argue against this option.

We do not have evidence favouring the second and third mechanism as explanation for the observed rise in high-grade CIN lesions. However, since high-grade CIN lesions precede invasive cervical carcinoma by several years, and a more efficient cervical cancer screening programme will only lead to a decrease in cervical cancer incidence many years later (12;13), it may be too early to conclude that there is no effect on the detection rates of SCC. The group of mature women (aged 54-61 years) has been screened for a large part of their adult life, and therefore precursor lesions of invasive cervical cancer will have been diagnosed and treated more often than in the younger age categories. Thus, in this age group, a decrease in SCC detection rate may well be a consequence of successful screening. Although caution remains because of the small numbers of SCC cases involved, support from this theory comes from data on cervical cancer incidence of the Netherlands as the strongest decrease in incidence is observed in mature women, who have been screened throughout most of their adult life (Bulk *et al.*, unpublished data).

In conclusion, the introduction of the CISOE-A classification together with clear guidelines for its use has caused a substantial decrease in the percentage of equivocal results, and resulted in a substantial decrease in the number of repeat recommendations. At the same time, this effect has not been paralleled with a decrease in the detection rate of histological high-grade CIN lesions as might be expected in an inefficient screening programme. In contrast, the detection rate of histological high-grade CIN lesions increased in all age categories, indicating the efficacy of the screening programme.

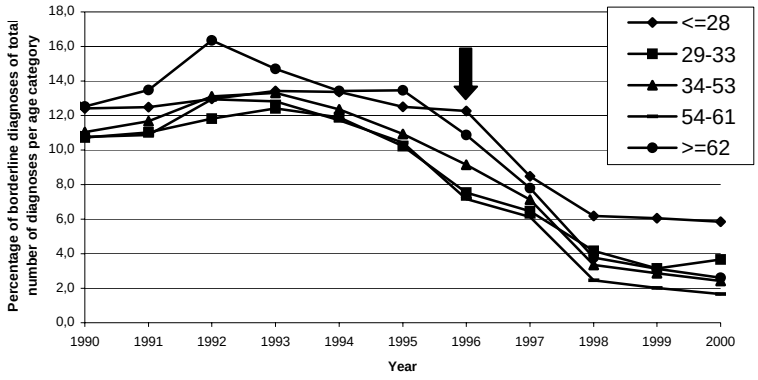

Figure 4.1 Decrease of equivocal cytologic conclusions over all age categories, 1990-2000.

Decrease of cumulative percentages of equivocal cervical smears results. The arrow denotes the introduction and implementation of the CISOE-A classification and transition from 3-yr interval screening to 5-yr interval screening

Table 4.6 Age-dependent effect of screening. Number of histologic lesions detected per 1000 cervical smears taken in the same calendar year, 1990-2000.

	≤28 yrs			29-33 yrs			34-53 yrs			54-61 yrs			≥62 yrs			All		
	CIN1	CIN2-3	SCC	CIN1	CIN2-3	SCC	CIN1	CIN2-3	SCC	CIN1	CIN2-3	SCC	CIN1	CIN2-3	SCC	CIN1	CIN2-3	SCC
1990	3.4	5.2	0.1	3.5	6.4	0.2	3.1	1.2	0.3	2.8	1.1	1.3	3.7	1.4	5.4	3.2	4.1	0.4
1991	3.5	5.1	0.2	3.9	5.3	0.3	2.3	1.1	0.3	2.1	1.9	1.0	7.1	1.2	4.7	2.9	3.7	0.4
1992	3.9	5.0	0.1	4.6	7.8	0.4	3.8	1.1	0.6	4.3	3.1	0.4	4.3	1.3	5.4	3.9	5.0	0.6
1993	5.9	6.3	0.1	5.9	7.0	0.7	4.0	0.8	0.4	5.8	3.5	1.2	4.9	3.2	5.3	4.6	5.0	0.6
1994	5.8	6.4	0.1	6.2	8.0	0.3	3.3	0.5	0.3	3.2	1.9	0.7	3.3	4.0	7.0	4.1	4.5	0.5
1995	6.5	6.8	0.3	5.8	9.5	0.5	4.0	1.9	0.5	3.9	3.1	0.7	5.0	2.0	6.4	4.6	5.7	0.6
1996*	6.8	9.7	0.2	3.7	7.1	0.2	3.5	2.2	0.5	1.1	1.2	0.1	3.4	2.7	6.8	3.5	4.9	0.5
1997	8.4	11.5	0.3	5.2	10.1	0.4	4.2	4.4	0.4	1.6	1.9	0.1	2.5	0.8	5.9	4.2	6.1	0.4
1998	9.2	10.5	0.6	6.6	12.7	0.4	4.2	4.8	0.3	1.8	1.7	0.2	3.6	2.7	5.0	4.6	6.9	0.4
1999	8.5	8.5	0.0	5.0	14.2	0.5	3.9	4.0	0.5	1.7	1.5	0.0	4.2	4.2	2.8	4.0	6.7	0.4
2000	9.3	11.6	0.6	7.2	14.1	0.3	3.5	5.0	0.4	1.8	2.1	0.5	2.2	3.3	8.7	3.9	6.4	0.6
p†	<0.001	<0.001	0.082	0.040	<0.001	0.569	0.080	0.003	0.449	0.101	0.531	0.008	0.057	0.096	0.610	0.180	<0.001	0.508

CIN indicates cervical intra-epithelial neoplasia, SCC indicates squamous cell carcinoma. Shaded areas indicate age categories invited for the population-based screening programme.
*: In 1996, the CISOE-A classification with formal reporting and referral was introduced and, in addition, 3-yr interval screening for women 34-55 years was changed into 5-yr interval screening for women 30-60 years. †: p value for linear test-for-trend

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CHAPTER 5

HIGH-RISK HPV TYPES IN THE DEVELOPMENT OF CERVICAL CANCER AND ITS PRECURSOR LESIONS

5.1 Preferential Risk of HPV16 for Squamous Cell Carcinoma and of HPV18 for Adenocarcinoma of the Cervix Compared to Women with Normal Cytology in the Netherlands

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Abstract

Both adenocarcinoma and squamous cell carcinoma of the uterine cervix are caused by infections with high-risk types of the human papillomavirus (HPV). The preferential risk conferred by the different high-risk HPV types for the development of histological subtypes of cervical cancer needs to be established more clearly. We present the type-distribution of high-risk HPV in women with normal cytology (n=1,467), adenocarcinoma *in situ* (n=61), adenocarcinoma (n=70), and squamous cell carcinoma (n=83). Detection of HPV was performed by typing for 14 high-risk types. Cervical adenocarcinoma and adenocarcinoma *in situ* were significantly more frequently associated with HPV18 (OR_{MH} 15.0; 95%CI 8.6-26.1 & 21.8; 95%CI 11.9-39.8, respectively) than normal cytology. HPV16 was only associated with adenocarcinoma and adenocarcinoma *in situ* after exclusion of HPV18 positive cases (OR_{MH} 6.6; 95%CI 2.8-16.0 & 9.4; 95%CI 2.8-31.2, respectively). For squamous cell carcinoma, HPV16 prevalence was elevated (OR_{MH} 7.0; 95%CI 3.9-12.4) compared to cases with normal cytology, and HPV18 prevalence was only increased after exclusion of HPV16 positive cases (OR_{MH} 4.3; 95%CI 1.6-11.6). These results suggest that HPV18 is mainly a risk factor for the development of adenocarcinoma whereas HPV16 is associated with both squamous cell carcinoma and adenocarcinoma.

Introduction

Cervical carcinomas are unfortunate complications of longstanding infections with high-risk types of human papillomavirus (hrHPV) (1). Testing for hrHPV types combined with cervical cytology becomes increasingly attractive as data accumulate that a combined test increases the efficacy of cervical screening programs and triage policies for women with both equivocal and normal cervical smears (2;3). Possibly, even more efficient screening strategies can be developed by selecting hrHPV types conferring a preferential risk for the development of cervical cancer, and treat these infections more aggressively. In order to assess the preferential risk for cervical cancer and its precursors, type-specific prevalence of hrHPV types in cancer cases should be compared to type-specific prevalence in women without cancer. In a meta-analysis of cervical squamous cell carcinomas (SCC) compared to high-grade squamous intraepithelial lesions (HSIL), HPV16, HPV18 and HPV45 appeared to display an elevated prevalence in cervical cancer (4). A second meta-analysis revealed that HPV16 and HPV18 are more prevalent in SCC than in low-grade SIL (LSIL) (5). However, a comparison with hrHPV prevalence in women with normal cytology was

not made, hampering the translation of these findings to implementation of type-specific hrHPV testing in population-based screening.

Recently in a cross-sectional study, we have demonstrated that amongst the hrHPV types, HPV16 and HPV33 were significantly more common in women with cervical intra-epithelial neoplasia grade 2 or more (≥CIN2) than in women with normal cytology. However, in that study cases of ≥CIN2 were retrieved from population-based screening, and consequently, the prevalence of invasive carcinomas as well as adenocarcinoma *in situ* (ACIS) was low.

In order to obtain a more comprehensive view on the change in distribution from hrHPV infections without cytological abnormalities to hrHPV prevalence in cervical cancer, we compared cross-sectional screening data of women with normal cytology to retrospectively collected cases of SCC, adenocarcinoma (AdCx) and ACIS.

Materials & Methods

Women with normal cytology. Women with normal cytology and a positive hrHPV test were recruited from the POBASCAM trial (POpulation-BAsed SCreening AMsterdam). The POBASCAM trial is a population-based randomised, controlled trial to evaluate the efficacy of screening using hrHPV testing in conjunction with conventional cytology versus cervical screening with conventional cytology only. This trial was conducted within the regular Dutch cervical screening programme in which women aged 30-60 years are screened cytologically with a screening interval of five years (6). In the POBASCAM trial, details of which have been described elsewhere, conventional smears were taken using either a Cervex brush or a cytobrush (7). After taking the smear, the brush was placed in a vial containing collection medium for hrHPV testing. Cervical smears were then classified according to the Dutch CISOE-A classification, which can be translated to the Bethesda system (8;9).

Detection of hrHPV was performed by GP 5+/6+ PCR enzyme immunoassay, using a cocktail of 14 high-risk types, *i.e.*, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, and HPV68 (10). All hrHPV positive samples were typed by reverse line blotting (11).

Between 1998 and 2002, a total of 44,102 women were included in the study. Of these women, 3.7% (n=1,576) were diagnosed with a normal Pap test and a positive hrHPV-test. Women who were positive for hrHPV by GP5+/6+-PCR but negative for reverse line blot were excluded (n=109), leaving 1,467 women for the analysis. All technicians were blinded to hrHPV status and cytological diagnosis of the samples.

Cases with cervical carcinoma (in situ). All cases of cervical carcinoma (*in situ*) were identified during the course of previous studies (12;13). Cases with ACIS and invasive AdCx of the cervix were enrolled from four pathology departments in the Netherlands. In the period 1996-2000, formalin-fixed specimens of 62 ACIS cases and 77 cases of AdCx were identified that were adequate for hrHPV PCR analysis. Cases with SCC were identified in a geographically defined region of the Netherlands.

Archival formalin-fixed material was collected of 91 SCC cases from the period 1981-1998.

HrHPV detection was performed by GP5+/6+ PCR as described above. In addition, all GP5+/6+ PCR negative samples were evaluated by E7 region type-specific PCR for the same 14 high-risk types in order to exclude that cases with hrHPV DNA integrated in the GP5+/6+ primer binding region were diagnosed as hrHPV negative (1). Laboratory personnel unaware of the histological diagnosis performed hrHPV determinations.

Of the cases with ACIS, one tested negative for hrHPV by both PCR tests, and four cases tested positive only by type-specific E7 PCR. Seven of the 77 AdCx cases tested negative for hrHPV by both tests, and 7 tested positive only for type-specific E7 PCR. Of the women with SCC 7 cases tested negative for hrHPV by both assays and 1 case tested positive by type-specific E7 PCR only, leaving 83 cases with SCC for analysis.

Statistical analysis. Differences in prevalence of hrHPV types for women with normal cytology compared to women with cervical carcinoma were examined using the Mantel-Haenszel common odds ratio (OR_{MH}). 95% Confidence intervals (95%CI's) were calculated. Statistical significance was tested by Cochran's Mantel-Haenszel test. Only OR_{MH} with values of 1.0 or higher are reported. Data were adjusted in 5-year age categories (*i.e.*, below 29 years, 29-33, 34-38, 39-43, 44-48, 49-53, 54-58, 59-61, and 61 years and over), matching with age categories in nationwide screening. The presence of an association between OR_{MH} and age was tested by Breslow-Day's test of homogeneity. Variations in the prevalence of single and multiple infections were assessed using Chi-square analysis. Analyses were performed separately for women with either single and multiple infections or single infections only. Because HPV18 prevalence heavily dominated in AdCx and ACIS, and HPV16 infections dominated in both normal cytology and SCC, we also calculated risk estimates after either discarding HPV16 positive cases, or HPV18 positive cases, or both.

A potential source of bias may be that women with normal cytology were matched with cases with cancer in the same 5-year age category. Because it takes at least 8-10 years to develop invasive carcinoma of the cervix after infection with hrHPV, we repeated the analyses matching cases with cancer with normal controls 10 years younger (12;14;15). For these analyses, we included women with normal cytology who were sampled during the enrolment phase of the POBASCAM trial, but did not meet the age criteria for population-based cervical screening (n=58).

Results

Study subjects. The mean age of women with normal cytology was 37.0 years (range 17-63 years). The mean age of women with ACIS was 37.1 years (range 23-55) and did not differ statistically from the age of women with normal cytology. Women with invasive AdCx and SCC had a mean age of 44.7 years (range 28-79 years) and 49.0 years (range 27-88 years), respectively. Both for women with AdCx ($p<0.01$) and SCC

($p<0.01$) the mean age was statistically significantly increased compared to women with normal cytology or ACIS.

Of the 1,525 women with normal cytology, 268 (17.6%) were diagnosed with a multiple hrHPV infection. Multiple infections were also common in women with ACIS (13/61, 21.3%). Women with invasive AdCx (5/70, 7.1%) and SCC (9/83, 10.8%) had a lower prevalence of multiple hrHPV infections than women with either normal cytology or a non-invasive lesion ($p=0.007$).

HrHPV type-specific prevalence in women with normal cytology. Table 5.1 displays the hrHPV type-specific prevalence in women with normal cytology compared with cases of cervical cancer (*in situ*). In women with normal cytology, HPV16 accounted for 30.0% of hrHPV positivity and HPV31 for 15.7%. Another 10.0% of hrHPV infections in women with normal cytology involved HPV18 and 8.0% HPV45. Type-specific prevalence did not substantially change when analysing women with single infections separately (Table 5.1).

HrHPV type-specific prevalence in women with cervical adenocarcinoma in situ. In women with ACIS, HPV18 infections (66.2%) dominated. Of the remaining hrHPV infections, HPV45 accounted for 6.2% of infections, HPV16 for 33.8% and HPV31 for 4.6% (Table 5.1). No other single hrHPV infections were observed in ACIS. When cases with single infections were analysed separately, type-specific prevalence did not change substantially.

When analysing for individual types, women with ACIS were statistically significantly more likely to carry HPV18 than women with normal cytology (OR_{MH} 21.8; 95%CI 11.9-39.8). The prevalence of HPV16 was comparable between women with normal cytology and women with ACIS. However, after excluding HPV18-positive cases from the analyses, prevalence of HPV16 was 9.4 times as frequently present in cases with ACIS compared to women with normal cytology (95%CI: 2.8-31.2). After excluding both HPV16 and HPV18 positive cases, prevalence of HPV45 was also statistically significantly increased in cases with ACIS (OR_{MH} 14.0; 1.3-150.9). When cases with single infections were analysed separately, type-specific prevalence did not change substantially. All analyses were repeated when matching ACIS cases with normal controls 10 years younger, but estimates were not affected (data not shown). For none of the HPV types, OR varied with age (data not shown).

HrHPV type-specific prevalence in women with cervical adenocarcinoma. In women with AdCx, HPV18 infections (57.1%) displayed the highest prevalence. Of the remaining hrHPV infections, HPV45 accounted for 11.4% of infections and HPV16 for 35.7% (Table 5.1). When cases with single infections were analysed separately, type-specific prevalence did not change substantially.

Results were comparable for women with ACIS and for women with invasive AdCx since HPV16 prevalence was not different between AdCx and normal cytology

(OR_{MH} 1.3; 95%CI 0.8-2.2) and women having AdCx were more likely to be infected by HPV18 than women with normal cytology (OR_{MH} 15.0; 95%CI 8.6-26.1). After exclusion of HPV18 positive cases, both HPV16 and HPV45 were statistically significantly associated with AdCx (OR_{MH} 6.6; 95%CI 2.8-16.0, and OR_{MH} 4.3; 95%CI 1.7-10.6, respectively). Results for single infections only were comparable (Table 5.1). All analyses were repeated matching cases with AdCx with normal controls 10 years younger, but estimates were not affected (data not shown). For none of the HPV types, OR varied with age (data not shown).

HrHPV type-specific prevalence in women with squamous cell carcinoma. Compared with cervical AdCx and its precursor ACIS, results were reversed for HPV16 and HPV18 in women with SCC. Women with SCC had an increased prevalence of HPV16 infections (69.9%) compared to HPV18 infections (12.0%). Compared to the cases with cervical AdCx, squamous cell carcinoma showed more diversity in types as only HPV51, HPV52, HPV59, HPV66 and HPV68 did not occur at all in the cases of SCC.

Women having SCC were significantly more likely to carry HPV16 than women with normal cytology (OR_{MH} 7.0; 95%CI 3.9-12.4). Since HPV16 dominated in cases of SCC, analyses were repeated after exclusion of HPV16. In these analyses women with SCC were more likely to carry HPV18 infections than women with normal cytology (OR_{MH} 4.3; 95%CI 1.6-11.6). Again, we investigated whether less prevalent types displayed type-specific increases in prevalence as well by excluding both HPV16 and HPV18 from the analyses. In multiple infections, both HPV 31 and HPV39 were more frequently present in SCC than in normal cytology after exclusion of both HPV16 and HPV18 co-infections (OR_{MH} 3.5; 95%CI 1.0-11.5, and OR_{MH} 5.4; 95%CI 1.3-21.7, respectively). However, the prevalence of these types was not increased in single infections. Results for single infections were comparable. After matching SCC cases with controls 10 years younger, none of the estimates were affected appreciably (data not shown). For none of the HPV types OR varied with age (data not shown).

Table 5.1 HrHPV type-specific prevalence in adenocarcinoma and its precursors and squamous cell carcinoma versus normal cytology - single infections and single and multiple infections combined.

Type	Normal	ACIS	AdCx	SCC	Normal	Normal	Normal
	N (%)	N (%)	N (%)	N (%)	~> ACIS	~> AdCx	~> SCC
*Multiple**	N=1,467	N=65	N=70	N=83			
16	440 (30.0)	22 (33.8)	25 (35.7)	58 (69.9)	1.3 (0.7-2.2)	1.3 (0.8-2.2)	7.0 (3.9-12.4)
18	146 (10.0)	43 (66.2)	40 (57.1)	10 (12.0)	21.8 (11.9-39.8)	15.0 (8.6-26.1)	1.3 (0.6-2.8)
31	230 (15.7)	3 (4.6)	-	6 (7.2)	-	-	-
33	88 (6.0)	-	-	3 (3.6)	-	-	-
35	74 (5.0)	-	-	2 (2.4)	-	-	-
39	70 (4.8)	-	1 (1.4)	4 (4.8)	-	-	1.5 (0.5-4.3)
45	118 (8.0)	4 (6.2)	8 (11.4)	5 (6.0)	-	1.6 (0.8-3.5)	-
51	98 (6.7)	1 (1.5)	-	-	-	-	-
52	92 (6.3)	-	1 (1.4)	-	-	-	-
56	134 (9.1)	-	-	2 (2.4)	-	-	-
58	93 (6.3)	-	-	2 (2.4)	-	-	-
59	43 (2.9)	-	-	-	-	-	-
66	100 (6.8)	1 (1.5)	-	-	-	-	-
68	24 (1.6)	-	-	-	-	-	-
Single	N=1,221	N=48	N=65	N=74			
16	344 (28.2)	14 (29.2)	22 (33.8)	55 (74.3)	1.0 (0.5-1.9)	1.3 (0.7-2.2)	9.2 (4.9-17.3)
18	108 (8.8)	31 (64.6)	36 (55.4)	8 (10.8)	69.4 (20.8-231.8)	15.8 (8.8-28.4)	1.5 (0.6-3.3)
31	170 (13.9)	1 (2.1)	-	3 (4.1)	-	-	-
33	66 (5.4)	-	-	1 (1.4)	-	-	-
35	48 (3.9)	-	-	-	-	-	-
39	37 (3.0)	-	1 (1.5)	1 (1.4)	-	-	-
45	81 (6.6)	2 (4.2)	6 (9.2)	4 (5.4)	-	1.5 (0.6-3.2)	-
51	60 (4.9)	-	-	-	-	-	-
52	57 (4.7)	-	-	-	-	-	-
56	91 (7.5)	-	-	-	-	-	-
58	60 (4.9)	-	-	1 (1.4)	-	-	-
59	31 (2.5)	-	-	-	-	-	-
66	59 (4.8)	-	-	-	-	-	-
68	9 (0.7)	-	-	-	-	-	-

Normal indicates normal cytology; ACIS indicates adenocarcinoma *in situ*; AdCx indicates adenocarcinoma; SCC indicates squamous cell carcinoma. Analyses are adjusted for age in 5-year strata.

*: Multiple and single infections combined.

Discussion

This study on the distribution of 14 hrHPV types revealed marked differences between cervical adenocarcinoma and its precursors, and SCC. The prevalence of HPV16 is increased in SCC compared to normal cytology, whereas HPV18 is more prevalent in adenocarcinoma and its precursor. However, when accounting for the distorting effect of extremely high prevalence types, HPV16 and HPV45 are also associated with adenocarcinoma and its precursors, and HPV18 is associated with SCC as well. These data suggest that within the group of high-risk types of which the association with cervical cancer has already been established beyond any doubt (16), infections with either HPV16, HPV18 or HPV45 confer a preferential risk to develop a malignancy of the uterine cervix.

Some methodological aspects of this study need to be discussed. Firstly, we compared cross-sectional data of women with normal cytology obtained from the POBASCAM trial with retrospectively collected cases of cervical cancer. This approach may have biased our estimates of risk associated with specific hrHPV types. Women with cervical cancer were on average older than the women with normal cytology, and age has been shown to be associated with hrHPV type-specific prevalence (17). We used two methods for to correct for the age difference. Firstly, we analysed our data stratified in age categories. Secondly, a potential source of bias may be that it takes at least 8-10 years to develop invasive carcinoma of the cervix (12;15). Women with cancer may have contracted an hrHPV infection 10 years before they were diagnosed with cancer. We repeated the analyses matching cases of cancer with normal controls 10 years younger, and our estimates were not affected, indicating the robustness of our data. Also, we defined women with normal cytology as women having a screening smear diagnosed as normal without either histologically or cytologically diagnosed lesions in the 2 years preceding the screening smear. In the Dutch screening programme, these women are considered to be free of cervical disease, and they will not be called for cervical screening for the next five years (6). However, our population with normal cytology may have contained a small number of women with either an underlying high-grade lesion or who may develop a high-grade lesion during follow-up. This diagnostic bias would have a diluting effect on the risk estimates obtained by our study, as hrHPV prevalence in the normal population would have resembled the cervical lesion cases more closely. A strong point of our cross-sectional approach is that we were able to use a reference group of women with normal cytology taken from the same geographic region as cases with cervical cancer. In contrast, other studies relating hrHPV prevalence to histological type have relied on pooling of data obtained from worldwide studies to provide estimates of hrHPV prevalence in cancer and its precursor stages and regional variations in type-specific prevalence may have affected comparisons in these studies (4;5;18).

Thirdly, we have performed a type-specific E7 PCR in women with carcinoma in order to diagnose integrated hrHPV infections, whereas women with normal cytology

were not evaluated using the type-specific PCR. This may have resulted in a confirmation bias in the diagnosis of hrHPV infections, as integrated hrHPV infections in women with normal cytology may not have been diagnosed. However, integration of the hrHPV occurs late in the progression from normal epithelium to carcinoma, and viral integration is extremely rare in a population of women with normal cytology (16). Therefore, we do not consider our approach biased.

In addition to other studies demonstrating that adenocarcinomas are more often HPV18 positive than HPV16 positive (18-23), we have now shown that when comparing invasive adenocarcinoma cases to cytologically normal controls, the OR_{MH} is only 1.3 for HPV16 and 15.0 for HPV18. Conversely, when comparing invasive SCC cases to normal controls, OR_{MH} is 7.0 for HPV16 and only 1.3 for HPV18. This suggests that HPV16 and HPV18 are associated with a preferential risk compared to the other high-risk types of hrHPV for the development of either SCC or adenocarcinoma. Combining our analyses both including HPV18 and excluding HPV18, our data suggest that HPV18 is mainly a risk factor for the development of adenocarcinoma whereas the highly aggressive HPV16 is associated with both SCC and adenocarcinoma. Alternatively, HPV16 and HPV18 might preferentially induce differentiation in either squamous or columnar direction respectively after infection of epithelial stem cells localized in the basal layer of the epithelium. The hrHPV types tested for in this study other than HPV16, HPV18 and HPV45, did not reveal an increased prevalence in either histological subtype of cervical cancer, suggesting that the other types all pose a similar relatively low risk of progression to cancer. This also includes HPV33, which is prevalent in lesions ≥CIN2. However, a plausible explanation might be that HPV33 has the potential to induce high-grade CIN lesions relatively easy, whereas its capacity to induce progression from high-grade CIN to invasive carcinoma might be relatively low (24).

What are the consequences of our findings for cervical screening? Recently, it was shown that both HPV16 and HPV18 infections in women with normal cytology are associated with an increased 10-year absolute risk for high-grade lesions and cervical cancer (3). However, two other recently published studies did not demonstrate an association of HPV18 with cytological abnormalities and high-grade histological lesions in either short-term follow-up or in a cross-sectional design (24;25). These data suggest that HPV18 infections, which we have shown to be preferentially increased in prevalence in cervical adenocarcinomas, either do not induce cervical lesions diagnosed as abnormal cytologically or that cervical lesions associated with HPV18 are not diagnosed as a result of sampling error, since these lesions are more often localized high in the endocervical canal (26). Whatever the cause, our results show that HPV18 has a preferential risk for AdCx and ACIS. Being aware of this association should warrant a less expectant attitude for women with persistent HPV18 infections and normal cytology to refer women to colposcopically-mediated biopsies and endocervical sampling in case of a normal transformation zone.

In conclusion, we have shown that HPV16, HPV18 and HPV45 display an increased prevalence in cervical cancer compared to cytologically normal smears. HPV16 confers the greatest risk for SCC and HPV18 for adenocarcinoma of the cervix. These data strongly argue for hrHPV type-specific risk stratification of women with normal cytology and a positive hrHPV test participating in cervical screening programmes.

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5.2 The Contribution of HPV18 to Cervical Cancer is Underestimated Using High-Grade CIN as a Measure of Screening Efficiency

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Abstract

In one geographical area, 14 hrHPV types in CIN2/3 (n=139) and cervical squamous cell carcinoma (SCC; n=84) were analysed. HPV18 was more prevalent in SCC than CIN2/3 (OR 9.8; 95%CI:2.5-39). Other high-risk types prevalences corresponded in CIN2/3 and SCC. Evaluations using CIN2/3 as a measure of efficiency underestimate the contribution of HPV18 to SCC.

Introduction

Human papillomavirus (HPV) infections are the cause of cervical cancer and its precursors lesions (1). So far, 15 HPV types have been classified as high-risk types, and 3 types have been classified as probable high-risk (2). Within the group of high-risk HPV (hrHPV) types, different types seem to confer different degrees of risk for cervical cancer and its precursor lesions (3). A markedly increased risk of cervical cancer has been attributed to HPV16 and HPV18, since prevalence studies have shown that 60-70% of cervical squamous cell carcinoma (SCC) cases are positive for HPV16 or HPV18 (2).

As a consequence, assessment of the additive value of HPV typing in cervical screening is the subject of many ongoing studies. However, screening evaluations generally use high-grade cervical intraepithelial neoplasia (CIN2/3) as intermediate endpoint of cervical cancer. This may lead to underestimation or overestimation of the contribution of certain HPV types in case the HPV type distribution between CIN2/3 and cervical cancer differs. Indeed, several studies suggest that HPV18 is underrepresented in CIN2/3 compared to SCC (4-6).

Here, we address type-distribution of different hrHPV types in CIN2/3 and SCC samples collected in one geographical area.

Materials and Methods

Study selection. From the archives of the department of pathology of Ziekenhuis Walcheren in the Netherlands, we identified 187 specimens of CIN2/3 (87 CIN2 and 100 CIN3) diagnosed after screening between 1996 and 2000, and 90 specimens of SCC from the period 1981-1998. The department of pathology serves the population of the Walcheren peninsula, where follow-up of screened women is approximately 90% complete. All histological samples were collected retrospectively and were subjected to revision. The Medical Ethics Committee of VU University Medical Center approved the study (2001/179).

HPV testing. Representative samples of the formalin-fixed biopsies were processed for PCR analysis (1;7). Amplification of a 209 base pair fragment of the β-globin gene was performed to test the integrity of DNA (8). Detection of hrHPV was performed by GP 5+/6+ consensus primer PCR enzyme immunoassay, using a cocktail of 21 low-risk types and 14 high-risk types. All HPV positive samples were typed by reverse line blotting (9). We tested for hrHPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 and lrHPV6, 11, 32, 40, 42, 43, 44, 54, 55, 57, 61, 70, 71, 72, 81, 83, 84, 85, 86, cp6108 and jc9710 (10). In addition, all GP5+/6+ PCR negative samples were evaluated by E7 region type-specific PCR for the same 14 high-risk types in order to exclude the presence of hrHPV in integrated form and/or loss of the GP5+/6+ primer binding region (1). Technicians performing PCR analysis were blinded for histological classification of samples.

Study material. Formalin-fixed archival material could not be retrieved for 17 cases with CIN2/3. Of the women with CIN2/3, 21 samples tested HPV negative and in 7 cases, only low-risk types could be identified. One case tested hrHPV positive without identifying a specific high-risk type, leaving 139 cases of CIN2/3 with known hrHPV type for statistical analysis. Of the women with SCC, 7 cases tested negative for HPV by both assays, leaving 83 cases with SCC and a known hrHPV type for statistical analysis.

Statistical analysis. Differences in prevalence of hrHPV types for CIN2/3 compared to SCC were examined using the Mantel-Haenszel common odds ratio (OR_{MH}) and 95% confidence intervals (95%CIs). Statistical significance was tested by Cochran's Mantel-Haenszel test. Data were stratified by age (i.e., below 29 years, 29-33, 34-38, 39-43, 44-48, 49-53, 54-58, 59-61, and 62 years and over) and whether infections were single or multiple. The presence of an association between OR_{MH} and age was tested by Breslow-Day's test of homogeneity. Analyses were repeated for women with single infections only.

The presented results may not be free of hidden bias since there is some evidence that HPV18-positive lesions are underrated by cytology (11;12). Therefore, it cannot be excluded that prevalence differences of certain hrHPV types in CIN2/3 compared to SCC is associated with cytological screening failures. We examined the influence of hidden bias by computing an adjusted p-value obtained under the assumption that among the hrHPV-positive CIN2/3 and SCC cases, the odds of observing a certain hrHPV type in SCC compared to CIN2/3 differed by a factor 2 (13).

Results

The mean age of women with CIN2/3 (n=139) was 37 years (range 26 to 57). Women with SCC (n=84) had a mean age of 49 years (range 27 to 88 years). The mean age of women with SCC was higher than the mean age of women with CIN2/3 ($p<0.001$).

Multiple HPV infections were present in 9.4% (13/139) of CIN2/3, and in 10.8% (9/83) of SCC (p=0.719).

Table 5.2 displays the hrHPV type-specific prevalence rates in women with CIN2/3 compared to SCC. In women with CIN2/3, 59.7% of hrHPV positivity was due to HPV16 and 12.9% to HPV31. Another 2.9% of hrHPV infections were HPV18 and 11.5% HPV52 positive. In women with SCC, 69.9% of hrHPV positivity was due to HPV16. HPV31 was present in 7.2% of cases, and HPV18 in 12.0%. No SCC cases tested positive for HPV52 in this study. Type-specific prevalences did not substantially change when analyzing women with single infections separately.

Women having SCC carried HPV16 more often than women with CIN2/3, although the estimated OR_{MH} was not statistically significant (OR_{MH} 1.5; 95%CI: 0.8-3.1). The prevalence of HPV18 was significantly higher in SCC than in CIN2/3 (OR_{MH} 8.3; 95%CI: 1.9-36). The other high-risk HPV types were not associated with increased risks individually, but when all hrHPV-positive infections without HPV16 and HPV18 were analysed as one group, the result was significant (OR_{MH} 0.4; 95%CI: 0.2-0.8). For none of the HPV types OR varied with age (data not shown). Results for single infections were comparable.

Even when assuming that the odds to detect an HPV18-associated SCC compared to an HPV18-associated CIN2/3 differed by a factor 2, HPV18 would still be increased in SCC compared to CIN2/3 (p=0.035).

Discussion

In this cross-sectional study, we compared hrHPV type-specific prevalence in CIN2/3 and invasive carcinomas in women identified through population-based cervical screening in a geographically restricted area. We demonstrated that HPV18 prevalence is increased in SCC compared to pre-malignant lesions. In addition, this study underlines the previously recognized importance of HPV16 for the development of SCC. Other types did not show a prevalence difference.

The increased prevalence of HPV18 in cervical cancer compared to CIN2/3 as found in our study is in agreement with a recent meta-analysis and data from Australia, South-Africa and the United States (14-16). The sample size of our study was slightly low, because CIN2/3 and SCC cases were collected from one restricted geographical area. Nonetheless, we were able to demonstrate effects of HPV18 in SCC. Our study cannot differentiate between increased progression risks of HPV18

Table 5.2 HrHPV type-specific prevalence rates in squamous cell carcinoma versus high-grade cervical intraepithelial neoplasia

	All infections *†				Single infections*			
Type	SCC N=84	CIN2/3 N=139	SCC~> CIN2/3	p	SCC N=74	CIN2/3 N=126	SCC~> CIN2/3	p
	N (%)	N (%)	OR (95% CI)		N (%)	N (%)	OR (95% CI)	
16	58 (69.9)	83 (59.7)	1.5 (0.8-3.1)	0.229	55 (74.3)	75 (59.5)	2.1 (1.0-4.5)	0.065
18	10 (12.0)	4 (2.9)	8.3 (1.9-36)	0.002	8 (10.8)	3 (2.4)	10 (2.0-52)	0.005
n16/n18	17 (20.5)	51 (36.7)	0.4 (0.2-0.8)	0.009	11 (14.9)	47 (37.3)	0.2 (0.1-0.5)	0.001
31	6 (7.2)	18 (12.9)	0.4 (0.1-1.5)	0.145	3 (4.1)	11 (8.7)	0.5 (0.1-2.1)	0.371
33	3 (3.6)	8 (5.8)	1.0 (0.2-4.6)	0.959	1 (1.4)	7 (5.6)	0.4 (0.1-3.4)	0.390
35	2 (2.4)	5 (3.6)	0.2 (0.1-3.3)	0.272	-	3 (2.4)	-	-
39	4 (4.8)	-	-	-	1 (1.4)	-	-	-
45	5 (6.0)	6 (4.3)	0.8 (0.1-6.1)	0.850	4 (5.4)	3 (2.4)	0.6 (0.1-8.1)	0.701
51	-	4 (2.9)	-	-	-	3 (2.4)	-	-
52	-	16 (11.5)	-	-	-	14 (11.1)	-	-
56	2 (2.4)	1 (0.7)	1.6 (0.1-46)	0.815	1 (1.4)	1 (0.8)	1.7 (0.1-28)	0.705
58	2 (2.4)	7 (5.0)	1.6 (0.1-11)	0.652	1 (1.4)	4 (3.2)	0.6 (0.1-9.0)	0.738
59	-	1 (0.7)	-	-	-	1 (0.8)	-	-
66	-	1 (0.7)	-	-	-	-	-	-
68	-	-	-	-	-	-	-	-

CIN2/3 indicates high-grade cervical intraepithelial neoplasia; SCC indicates squamous cell carcinoma.

*: Analyses are adjusted for age in 5-year strata.

†: Multiple and single infections combined. Analyses are adjusted for multiplicity of infection.

for the transition of CIN2/3 to SCC as an explanation of our findings, and underdetection of screen-diagnosed CIN2/3 lesions associated with HPV18 as an alternative theory. Our previous, cross-sectional, study indicated a preferential risk of HPV18 for the development of cancer from normal cytology (17). However, the estimate of the risk associated with HPV18 for cervical cancer compared to CIN2/3 obtained in this study is rather high (OR_{MH} 9.7; 95%CI: 2.4-39). To calculate approximately how robust this finding is against underdetection of HPV18-associated lesions by cytology in a cervical sceening programme (11), we estimated the prevalence difference of HPV18 between CIN2/3 and SCC using a sensitivity analysis. We found that the difference in HPV18 prevalence between CIN2/3 and SCC still remains statistically significant, even if the detection of HPV18-associated CIN2/3 lesions through screening compared to HPV18-positive SCC cases is underestimated by a factor 2. Underrepresentation of HPV18-associated lesions might be explained by the finding that HPV18 infections often occur high in the endocervical canal where lesions are less accessible to screening. Nevertheless, whether the observed association between HPV18 and SCC is mainly caused by a preferential risk of HPV18 for the development if SCC, or by underdetection of HPV18 precursor lesions, our findings have important implications for evaluations of screening programmes. Currently most, if not all, screening programmes use CIN2/3 as an intermediate endpoint for cervical cancer to evaluate screening efficacy. This approach seriously underestimates the contribution of HPV18 to the development of SCC. As a consequence, vaccination to HPV16 and HPV18 will decrease HPV18-associated carcinoma incidence more than short-term studies using CIN2/3 as an outcome measure indicate.

In conclusion, HPV18 prevalence is high in invasive SCC compared to CIN2/3 lesions. Thus, when CIN2/3 is used as intermediate endpoint for cervical cancer, the contribution of HPV18 to the development of invasive SCC is underestimated. Since the vast majority of both SCC and adenocarcinomas is attributable to HPV16 and HPV18, women with screen-detected HPV16 and HPV18 infections should be offered more intensive follow-up schemes compared to women infected with other high-risk HPV types.

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CHAPTER 6

HIGH-RISK HPV AND AN IMPROVED DETECTION OF CERVICAL CANCER

6.1 High-risk HPV may indicate cytologically false-negative smears. An analysis of "normal" smears preceding CIN2/3.

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Abstract

Cervical screening, currently performed by cervical cytology, depends for its success on the timely detection of malignant lesions. The presence of high-risk human papillomavirus (hrHPV) is associated with an increased risk of subsequent high-grade cervical intra-epithelial neoplasia (CIN2/3) and cervical cancer. In this study we determined to which extent hrHPV is present in cervical smears with a high *a priori* chance of being false negative, *ie*, in normal smears preceding CIN2/3.

Archival specimens of 187 women with CIN2/3 and preceding normal conventional smears were identified retrospectively. Of these specimens, 144 (77%) had adequate cytologic samples for further HPV DNA testing.

Of 144 CIN2/3 lesions, preceding normal smears showed hrHPV positivity in 80% of cases. Of the hrHPV positive smears 69% were upgraded cytologically at rescreening compared to 24% of hrHPV negative smears ($p<0.001$). Upgrading of smears was not associated with specific hrHPV types ($p=0.217$). In over 90% of cases, type-concordance in both smear and CIN2/3 lesion was demonstrated.

HrHPV is present in a high proportion of normal archival smears preceding CIN2/3 and false negative cytology was highly associated with the presence of hrHPV. This supports the current notion that hrHPV testing can be used as a primary cervical screening tool. If so, hrHPV positive cervical smears should be carefully examined for cytological abnormalities to reduce false negative cervical cytology.

Introduction

Invasive cervical cancer and high-grade CIN lesions, *ie*, cervical intra-epithelial neoplasia grade 2 and grade 3 (CIN2/3), are caused by high-risk types of the human papillomavirus (hrHPV) (1). Cervical cancer screening may improve with the addition of a test for hrHPV to cytology screening, since hrHPV testing has a higher sensitivity for the detection of CIN2/3 and invasive cancer than cytology testing (2). Using hrHPV testing in population-based screening, depending on the country under study, 5-7% of women of ≥30 years of age with normal cytology will be identified as being at risk for the development of cervical cancer, (3;4). Indeed, in women with hrHPV positive normal cytology, the prevalence of CIN2/3 lesions is increased, but the optimal management of this group is still a matter of debate (5-8). To resolve this issue, additional studies are required, including studies concerning the presence of hrHPV in normal smears preceding CIN2/3.

Due to the very low risk of CIN2/3 among women with normal cytology and a negative hrHPV test, prospective studies are less suitable to determine the proportion

of women with CIN2/3 after a normal cytology result and a negative hrHPV test. Instead, retrospective studies are more appropriate to investigate this issue. Indeed, in retrospective studies of invasive cervical cancer, a positive hrHPV test was often associated with a cytologically false-negative smear (9-12). However, the presence of hrHPV in smears with a high *a priori* chance of being false negative, *ie*, in normal archival smears of women with CIN2/3 lesions, has not yet been determined adequately.

Here, we present the results of a retrospective study to determine the presence of hrHPV in normal archival smears of women with CIN2/3. In addition, all archival normal smears preceding CIN2/3 lesions have been reviewed and the association of the reviewed diagnosis with hrHPV status was studied.

Materials and Methods

Study population. In order to include all women with histologic CIN2/3 lesions preceded by conventional cervical smears, we selected all women who were diagnosed with borderline or mild dsykarosis or worse (for classification: see further) between January 1st 1996 and December 31st 2000 from the archives of the department of pathology of Ziekenhuis Walcheren in the county Zeeland (Figure 1). This is a geographically-isolated region of the Netherlands. From this cohort (N=375), we retrieved the complete gynecologic history registered in the nationwide registry of histo- and cytopathology (PALGA). We then selected women with normal smears preceding the histologic diagnosis of CIN2/3 (N=187). We included both CIN2 and CIN3 lesions since the reproducibility of CIN2 and CIN3 diagnoses is relatively poor (13). All smears were taken using a Cervex brush (Rovers Medical Devices, Oss, the Netherlands) from women participating in the nationwide screening programme after 1992 (14). Hence, we evaluated smears taken 4 to 8 years preceding the histologic diagnosis. The Medical Ethics Committee of VU University Medical Center approved the study (2001/179).

Cytologic and histologic classification. Conventional smears and histologic samples, *ie*, slides and tissue blocks, were collected retrospectively and subjected to rescreening and diagnosis, respectivly. We retrieved all smears originally classified as normal that preceded the diagnosis of CIN2/3. We selected the last normal smear preceding any documented abnormality, *i.e.*, before any diagnosis of abnormal cytology or a histology diagnosis other than 'No CIN'. If more than one normal smear was available, all normal smears were revised. If the 'youngest' normal smear was upgraded at rescreening, the preceding smear was selected. In case a smear was upgraded and no normal smear preceding the upgraded smear was available, the 'oldest' available smear, classified as "normal cytology upgraded at rescreening", was selected for HPV testing.

For confirmation of the histologic diagnosis, we evaluated all histologic slides available per case, and the most abnormal slide was used for classification and the embedded tissue corresponding to this slide was selected for HPV testing.

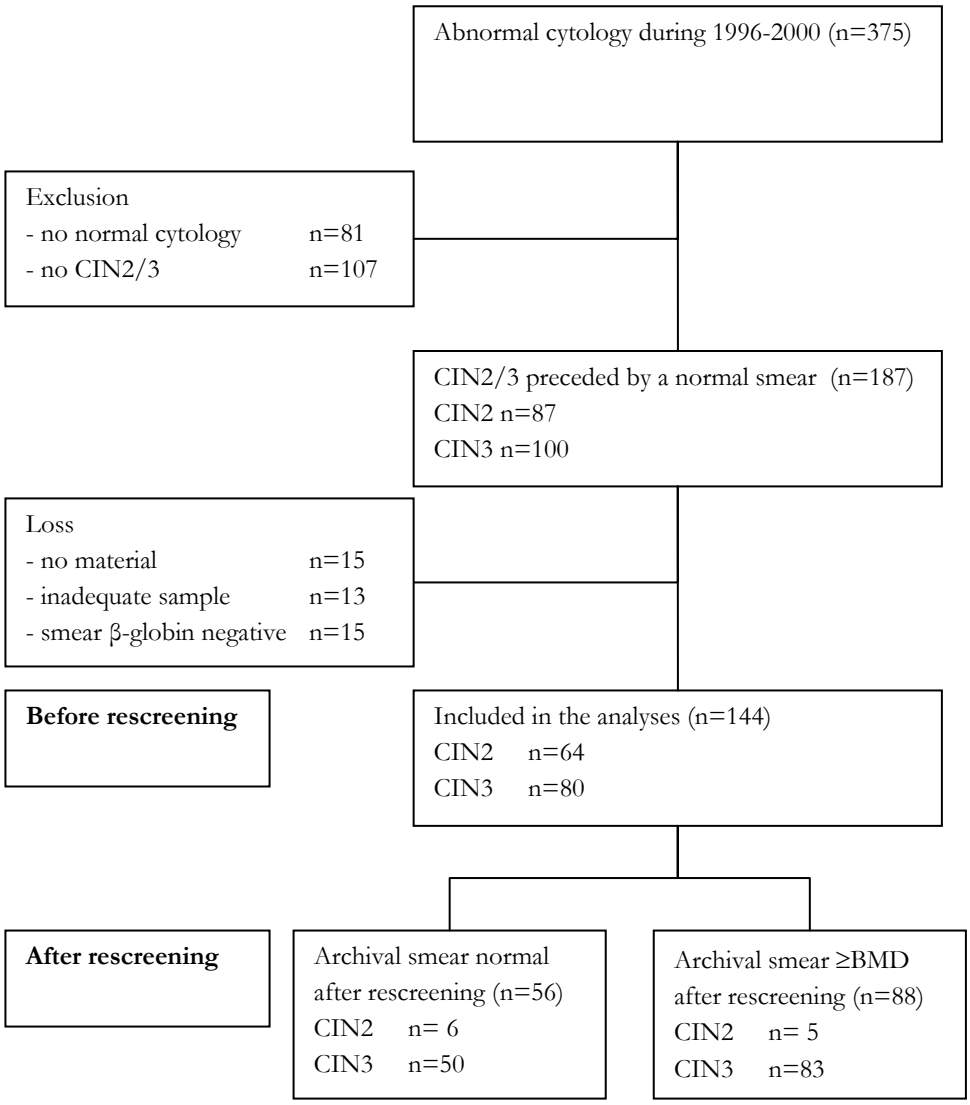

Figure 6.1. Flowchart of the study population

The reviewing pathologists were blinded to subject identity, clinical history and HPV test results. Histologic grading of dysplasia was based on standard criteria for CIN lesions (15). Cytology results were graded by CISOE-A classification and, depending on the observed squamous abnormalities, were classified as either 'normal' (corresponding to negative for intra-epithelial lesions) or 'BMD or worse' ('≥BMD; corresponding to ASC-US, ASC-H, LSIL or worse) (16;17). The mean time between the screening smear and histologic diagnosis was 3.9 years, reflecting the Dutch screening interval of 3-5 years (14).

HPV testing. Archival smears (one for each case) and representative sections of the formalin-fixed biopsies were processed for PCR analysis essentially as described before (1;18). Amplification of a 209 base pair fragment of the β-globin gene was performed to test the integrity of DNA (19). The β-globin status was not related to the storage time of histology and cytology samples ($p>0.05$).

HPV was detected by GP5+/6+-PCR-EIA using cocktail probes that detect the following HPV (sub)types: 6, 11, 16, 18, 26, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 61, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 85, 86, JC9710, and cand89. All positive samples were typed by reverse line blotting (20-22). 15 HPV types were considered high-risk, *i.e.*, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82. GP5+/6+-PCR negative samples were subjected to E7 type-specific PCR (1).

Histology samples were not available for 15 CIN2/3 cases, and of 13 more cases processed sections did not contain CIN2/3 anymore. Furthermore, smears of 15 cases tested negative for β-globin and were excluded. Thus, 144 CIN2/3 cases were included for analysis (Figure 1).

Statistical analysis. Analyses were descriptive. Results with p-values of 0.05 or less were considered statistically significant. All analyses were performed using SPSS 9.0 for Windows.

Results

From a primary selection of 375 women with abnormal cytology in the study period, we identified 144 women with CIN2/3 of whom both preceding archival normal smears and histology samples could be retrieved and analyzed for the presence of HPV (Figure 6.1).

Of these histology samples, 64 (44%) were originally diagnosed as CIN2 and 80 (56%) as CIN3. After rescreening, 133 (92%) were classified as CIN3. The archival normal smears preceding the diagnosis were revised, and 88 (61%) were upgraded. Of these smears, 28% were upgraded to BMD (n=40), and 33% (n=48) to >BMD. The median age at histologic diagnosis of the cases was 36 years (range 25 to 57 years).

Table 6.1 displays HPV results for all cases. Of the histology samples, 8 (5.6%) tested HPV negative, 7 (4.9%) were positive for low-risk types only, and 129 (90%)

tested positive for at least one hrHPV type. Of the smears, 25 tested HPV negative (17%). Four cases (2.8%) tested exclusively positive for low-risk HPV and 115 cases (80%) tested positive for at least one hrHPV type. Of cases with revised normal smears, 34% were HPV negative compared to 6.8% of cases with revised ≥BMD ($p<0.001$). When hrHPV results were used to select smears for rescreening, 115 out of 144 cases (80%) had an hrHPV positive smear originally reported as normal. Of these smears, 69% (79/115) were upgraded after rescreening. In contrast, only 24% (6/25) of the HPV negative smears were upgraded after rescreening ($p<0.001$).

Test results for smears and histology samples analyzed per case are presented in Table 6.2. Both samples tested hrHPV positive in 109 cases. In 100 of these cases, the same hrHPV type was present in the archival smear and in the CIN2/3. Nine cases tested positive for hrHPV in both samples, but different types were diagnosed in the smear and in the CIN2/3. Of smears with revised ≥BMD, 85% were hrHPV positive in both samples compared to 61% of smears with revised normal smears ($p=0.008$). A group of 20 cases (14%) tested hrHPV negative in the smear and hrHPV positive in the histology sample. Of these cases, 16 had revised normal smears and 4 revised ≥BMD smears. Five cases (3.5%) had an hrHPV negative smear and a hrHPV negative CIN2/3.

Table 6.3 presents the type-specific data of the cases that tested HPV positive in both samples. In women with normal smears, HPV16 infections occurred in 60% of cases, and HPV31 was diagnosed in 11%. In women with revised ≥BMD, HPV16 was the most frequently detected type as well (47%), and HPV31, HPV52 and HPV33 occurred less frequently in 7.7%, 7.7% and 6.4%, respectively. All other types occurred with a low frequency. There was no statistically significant difference in HPV distribution between cases with normal smears and cases with revised ≥BMD ($p=0.217$).

In four cases with revised normal smears and five cases with ≥BMD infections we could not demonstrate the same type in the smear and the CIN2/3 lesion. Multiple infections were detected in 37% of women with normal smears and 27% of women with revised ≥BMD ($p=0.121$).

We could not demonstrate a statistically significant difference in type distribution in cases with hrHPV negative smears and an hrHPV positive CIN2/3 and cases with an hrHPV positive smear and an hrHPV positive CIN2/3 ($p=0.755$).

Table 6.1 HPV test results for histology and cytology specimens

Cytologic diagnosis		Normal	Revised Normal	Revised ≥BMD
		n (%)	n (%)	n (%)
Total		144 (100)	56 (39)	88 (61)
CIN2/3				
	HPV negative	8 (5.6)	3 (5.4)	5 (5.7)
	lrHPV positive	7 (4.9)	3 (5.4)	4 (4.5)
	hrHPV positive	129 (90)	50 (90)	79 (90)
Preceding Smears				
	HPV negative	25 (17)	19 (34) *	6 (6.8) *
	lrHPV positive	4 (2.8)	1 (1.8)	3 (3.4)
	hrHPV positive	115 (80)	36 (64)	79 (90)

* p-value<0.01

Table 6.2 HPV test results for histology and cytology specimens combined

Cytologic diagnosis		Normal	Revised Normal	Revised ≥BMD
		n (%)	n (%)	n (%)
Smears and histology				
hrHPV-	hrHPV- †	5 (3.5)	3 (5.4)	2 (2.3)
hrHPV+	hrHPV+	109 (76)	34 (61)	75 (85)
same type		100 (70)	30 (54)	70 (80)
different type		9 (6.3)	4 (7.1))	5 (5.7)
hrHPV-	hrHPV+	20 (14)	16 (29)	4 (4.5)
hrHPV+	hrHPV-‡	6 (4.2)	2 (3.6)	4 (4.5)
LrHPV	lrHPV	4 (2.8)	1 (1.8)	3 (3.4)

†: includes one sample lrHPV-positive histologically (HPV82i)
‡: includes one sample lrHPV-positive histologically (HPV70)

Table 6.3 Type-specific HPV results of cases HPV positive in both cytology and histology (n=113)

Revised smears	Number (%)	HPV type in cytology	HPV type in histology
Normal* (n=35)	*1 (2.9)*	*11/16*	*11/31*
	21 (60.0)	16	16
	1 (2.9)	18	18
	4 (11.4)	31	31
	1 (2.9)	*18*	*31*
	1 (2.9)	33	33
	1 (2.9)	35	35
	1 (2.9)	*42/70*	*6*
	1 (2.9)	51	51
	1 (2.9)	52	52
	1 (2.9)	52	52
	1 (2.9)	*66*	*16*
≥BMD† (n=78)	1 (1.3)	11	11
	1 (1.3)	*16*	*45*
	1 (1.3)	*16/35*	*18/39*
	37 (47.4)	16	16
	1 (1.3)	18	18
	6 (7.7)	31	31
	5 (6.4)	33	33
	1 (1.3)	*33*	*x‡*
	1 (1.3)	*45*	*16*
	1 (1.3)	*51*	*45*
	1 (1.3)	51	51
	6 (7.7)	52	52
	3 (3.8)	58	58
	1 (1.3)	59	59
	2 (2.6)	70	70

Italics indicate discordant typing results.

*: hrHPV typing of cases with revised normal cytology involved 1 case positive for low-risk types only, and in 13 cases (37%) a multiple infection was diagnosed in either cytology or histology sample, or both.

†: hrHPV typing of cases with revised ≥BMD involved 3 cases positive for low-risk types only, and in 18 cases (27%) a multiple infection was diagnosed in either cytology or histology sample, or both.

‡: x indicates a hrHPV positive GP5+/6+ PCR-EIA with a negative typing result by RLB.

Discussion

In this retrospective study, we showed that hrHPV was present in nearly 80% of all originally normal smears preceding the diagnosis of CIN2/3. This supports the current notion that hrHPV testing may be used in primary cervical screening. Next, 69% of the hrHPV positive smears were upgraded at rescreening, compared to only 24% of hrHPV negative smears, indicating that in a routine screening setting with primary or additional hrHPV screening, hrHPV positive smears should be examined with great care to reduce the false-negativity rate of cervical cytology.

In women with cervical cancer, normal smears preceding the diagnosis are often upgraded at rescreening, indicating the need for a better test to select women at high risk for CIN2/3 and invasive cancer (9-12). Current proposals to improve cervical screening include liquid-based cytology, hrHPV testing, and rescreening of cytology slides. Although liquid-based cytology is increasingly being used in cervical screening, the gain in sensitivity expected based on the results of pilot studies has not been met in routine screening (8;23). In several countries, including Finland, Sweden and Costa Rica, hrHPV testing is presently being used in the setting of population-based cervical screening experimentally (24-26). Rescreening of cervical smears as a means to reduce the number of false negative smears is labour intensive, and the results are disappointing (27). In this retrospective study of women with CIN2/3, the proportion of upgraded smears among the hrHPV positive smears was high. Although the number of upgraded smears after rescreening, as found in our study, cannot be extrapolated to a routine screening setting, 92% (69/75) of false negative smears we detected fell into the group of hrHPV positive normal smears that constitute 5-7% of the total number of smears in population-based screening over 30 years of age. Thus, rescreening of all hrHPV positive normal smears would not only be practically feasible but might reduce false-negative cytology by 92%.

Concerning hrHPV type distribution, HPV16 occurred most often, and HPV31, HPV33 and HPV52 were also commonly associated with CIN2/3, albeit with a lower frequency. This finding confirms our earlier findings on type-specific prevalence in CIN2/3 in the Netherlands (21). In nearly 90% (100/113) of cases with both a HPV positive smear and a HPV positive CIN2/3, the same HPV type could be demonstrated. This confirms again that HPV infections precede the development of CIN2/3 (10;28;29).

We found 15 cases with an hrHPV negative smear and an hrHPV positive CIN2/3 lesion, as well as 9 type-discordant cases suggesting the occurrence of both clearance and re-infection with another HPV type. Although we have taken the utmost care to select only cases with adequate material characterized by a positive β-globin test and a smear sufficient for cytologic evaluation, failure to sample cells representative for cervical lesions or false-negative test results may be an additional explanation. Furthermore, we could not demonstrate differences in type-distribution in cases with an hrHPV positive CIN2/3 lesion and a preceding hrHPV negative smear when

compared to cases with an hrHPV positive smear. This suggests that a relatively rapid progression from normal cytology to CIN2/3 is not hrHPV type dependent (21;24;30). Therefore, we feel that close surveillance is warranted for women with a positive hrHPV test and a normal smear, even after rescreening, as these women are at risk of CIN2/3.

In conclusion, our study supports the current notion that hrHPV testing may be used in primary cervical screening. If so, the much higher proportion of false negative cervical smears among hrHPV positive samples compared to hrHPV negative normal smears should instigate great care in screening of hrHPV positive normal smears. Women with hrHPV positive normal smears account for 5-7% of the screening population over 30 years of age and may constitute 25% (36/144) of the cases with CIN2/3. We suggest that the moderately increased risk of cervical lesions among women with undoubtedly normal cytology and a positive hrHPV test, should lead to moderate careful monitoring, *e.g.*, retesting the women after 2-3 years. The screening interval for double negative women, *i.e.*, women with normal cytology and a negative hrHPV test, amounting to over 90% of the screening population of 30-60 years, may be extended to 5-8 years because of the reduced risk of CIN2/3 lesions.

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6.2 Risk of High-Grade Cervical Intra-Epithelial Neoplasia Based on Cytology and High-Risk HPV Testing at Baseline and at 6-Months

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Abstract

Adding a test for high-risk human papillomavirus (hrHPV) to cytological screening enhances the detection of high-grade cervical intraepithelial neoplasia (≥CIN2), but data are required that enable long-term evaluation of screening. We investigated the ≥CIN2 risk for women participating in population-based screening as a function of hrHPV and cytology testing results at baseline and at 6-months. We included 2,193 women aged 30-60 years participating in a population-based screening trial who received colposcopy or a repeat testing advice at baseline. The main endpoint was histologically confirmed ≥CIN2 diagnosed within 36 months.

HrHPV testing was more sensitive than cytology for ≥CIN2 (relative sensitivity 1.4, 95%CI: 1.3-1.5; absolute sensitivity 94.1% and 68.0%, respectively). The 18-month ≥CIN2 risks in women with an hrHPV-positive smear and in women with abnormal cytology were similar (relative risk 0.9, 95%CI: 0.8-1.1). Women with HPV16 and/or HPV18 had a higher ≥CIN2 risk than other hrHPV-positive women irrespective of the cytological grade. Repeat testing showed that both cytological regression and viral clearance were strongly associated with a decrease in ≥CIN2 risk. Notably, women who had a double negative repeat test at 6 months had a ≥CIN2 risk of only 0.2% (95%CI: 0.0-1.1) and hrHPV-negative women with baseline borderline or mild dyskaryosis and normal cytology at 6 months had a ≥CIN2 risk of 0% (95%CI: 0.0-0.8). Using hrHPV and/or cytology testing, risk of ≥CIN2 can be assessed more accurately by repeat testing than single visit testing. Hence, when hrHPV testing is implemented, patient management with repeat testing is a promising strategy to control the number of referrals for colposcopy.

Introduction

Invasive cervical cancer is one of the leading causes of cancer-related death in women of childbearing age worldwide (1;2). As a preventive measure, screening by cervical cytology (*i.e.*, the Pap test) has been shown to dramatically decrease the cervical cancer incidence and mortality (3). Another possibility is to screen for infections with high-risk human papillomavirus (hrHPV), the causative agent for cervical cancer, and combined cytology and hrHPV testing seems to be a promising strategy to improve cervical screening. Previous studies have shown that cytology combined with hrHPV testing improves the sensitivity to detect high-grade cervical lesions (4;5). The positive

predictive value of a single positive hrHPV test, however, remains low for women with a normal smear or mild cytological abnormalities, and referral rates for colposcopy may increase substantially with combined testing (6). Hence, implementation of hrHPV testing needs to be preceded by an evaluation of various screening strategies using hrHPV and cytological testing.

We investigated the risk of high-grade cervical intraepithelial neoplasia in women with hrHPV test results and cytology at baseline and at 6 months. We used data obtained from a population-based cervical screening trial. Since women in whom HPV16 or HPV18 is detected seem to have a substantially elevated risk of high-grade lesions compared to other hrHPV-positive women, and since 70% of all cases of cervical cancer are caused by HPV16 and HPV18, we evaluated risks for hrHPV-positive women with HPV16 and/or HPV18 and women positive for other high-risk types separately (7-9).

Materials & Methods

Study population and procedures. In this study, we included all women participating in the POBASCAM (Population-Based Screening Amsterdam) trial who had received an advice to have repeat cytology at 6 and 18 months, or who had been referred for immediate colposcopy. The POBASCAM trial is a population-based double blind randomised controlled trial to evaluate the efficacy of screening using hrHPV testing in conjunction with conventional cytology (intervention group) compared to cervical screening with classical cytology (control group). All participants gave written informed consent.

The design, methods and baseline results of the POBASCAM trial have been described previously (10). A flowchart of the randomisation, selection, and screening procedure of the POBASCAM trial is presented in Figure 6.2. Participants in the intervention group (n=21,996) were referred for colposcopy directed biopsy when the cytology result was moderate dyskaryosis or worse (>BMD) irrespective of hrHPV status. Participants returned to the next screening round after 5 years if the cytological result was normal and the hrHPV test was negative. Otherwise, participants were followed both cytologically and with hrHPV testing at 6 and 18 months. Participants with BMD at baseline were referred for colposcopy at 6 months if the 6-month result was hrHPV-positive BMD, or >BMD irrespective of hrHPV status, and participants with hrHPV positive normal cytology at intake were referred at 6 months only for >BMD. All participants in the intervention group were referred at 18 months for colposcopy if the combined test suggested the presence of a cervical lesion.

The participants in the control group (n=22,106) followed the current Dutch screening guidelines. Participants were referred for colposcopy if the result was >BMD at intake. Participants returned to the next screening round at 5 years if the result was normal. Smears were repeated at 6 or 18 months in case the result was

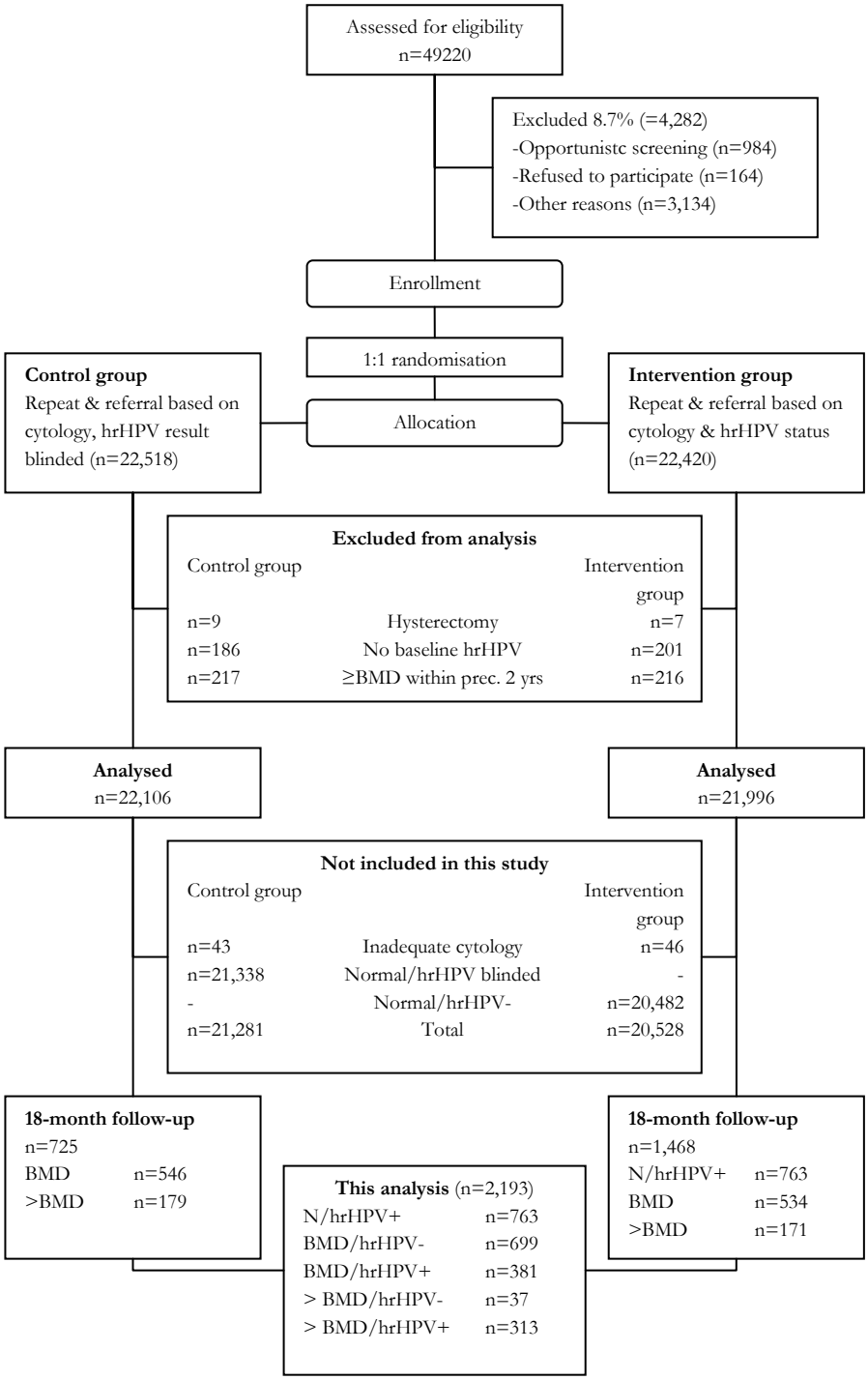

Figure 6.2. Flowchart

BMD. All participants in the control group were referred at 18 months for colposcopy if cytology was ≥BMD.

Conventional cytological smears were taken using a Cervex-Brush (Rovers Medical Devices, Oss, the Netherlands). The brush was placed in a vial containing a collection medium (*i.e.*, 5 ml PBS and 0.5% thiomersal) for hrHPV testing (10). Cervical smears were classified according to the Dutch CISOE-A classification blinded to hrHPV status of participants (11). In short, cytological results were grouped as normal, borderline or mild dyskaryosis (BMD; translating into ASC-US/ASC-H/LSIL) and moderate dyskaryosis or worse (>BMD; translating into HSIL) (12). Detection of hrHPV was performed by GP5+/6+ PCR enzyme immunoassay, using a cocktail of 14 high-risk types, *i.e.*, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV66, and HPV68 (13). HrHPV tests were performed in duplicate, and all hrHPV-positive samples were typed by reverse line blotting (14). Technicians performing hrHPV testing were blinded to the cytology results.

Colposcopically directed biopsies were taken for histological examination when suspected areas on the cervix were present according to standard procedures in the Netherlands, and abnormal results were classified histologically as CIN 1, 2, or 3, or invasive cancer according to international criteria (15;16). We included all lesions diagnosed after the referral smear and within 3 years after baseline. Histology samples were read in a community setting and were not subjected to revision (10).

Statistical analysis. All participants received cytological analyses (*i.e.*, Pap tests) and hrHPV testing at baseline. Using the screening results leading to a repeat or referral advice at baseline, we defined groups based on the combinations of cytology (normal, BMD, >BMD) and hrHPV (positive (+)/negative (-)) test result. HrHPV positive samples were further stratified on the presence of HPV16 and/or HPV18 in the baseline sample, since these two types account for approximately 70% of cervical cancer cases. Participants with follow-up were further stratified on cytology or hrHPV test result at 6 months. The outcome of interest was defined as a lesion of at least CIN2 (≥CIN2, *i.e.*, CIN2, CIN3 or invasive cancer). All analyses were repeated using lesions ≥CIN3 as outcome measure. Cumulative 18-month incidences as a measure of absolute risk of lesions ≥CIN2 were assessed using Kaplan-Meier methods and 95% CI's were calculated. 95% CI's on the original ≥CIN2 risk scale were obtained by exponentiating the upper and lower bounds of the 95% confidence intervals constructed on the log risk scale. Specific groups were compared using log-rank testing. Reported sensitivities and specificities were adjusted for non-verification occurring because women in the control group with normal cytology were sent back to routine screening regardless of the blinded hrHPV test result and because some women were lost to follow-up (17). Because the Medical Ethics Committee did not allow recalling participants with normal cytology and a negative hrHPV test for repeat

testing earlier than the regular screening interval of 5 years, we assumed women who were sent back to routine screening not to have an underlying CIN lesion. This assumption does not affect the relative sensitivity (17-19).

Results

Of 44,102 participants of the POBASCAM trial, we included 763 participants with normal cytology and a positive hrHPV test from the intervention group, 1,080 participants with a BMD result of whom 381 (35.3%) tested hrHPV positive, and 350 participants with a >BMD result of whom 313 (89.4%) tested hrHPV positive. Mean age was 38.5 years (range 29 to 60) for participants with normal cytology and a positive hrHPV test, 40.0 years (range 29 to 60) for participants with BMD and 37.3 years (range 29 to 60) for participants with >BMD. The non-response rate was 23.1% (165/713) at 6 months and 28.0% (146/522) at 18 months for participants with normal cytology. The non-response rates at 6 and 18 months for participants with BMD were 9.9% (37/374) and 28.8% (53/184), respectively.

Table 6.4 Cumulative 18-month risk of histologically diagnosed CIN2 or worse stratified by cytology and hrHPV status at baseline.

Category	Cases (n)				≥CIN2	≥CIN3
	Total	≥CIN2	≥CIN3	CxCa	Risk (95%CI)	Risk (95%CI)
N and HPV+	**763**	**58**	**29**	**1**	**12 (9.7 - 16)**	**6.1 (4.2 - 8.7)**
16+ and/or 18+	262	42	23	-	25 (19 - 33)	13 (8.9 - 19)
16- and 18-	501	16	6	1	5.3 (3.3 - 8.6)	2.2 (1.0 - 4.8)
BMD and HPV-	**699**	**15**	**8**	**2**	**2.5 (1.5 - 4.2)**	**1.8 (0.9 - 3.3)**
BMD and HPV+	**381**	**108**	**63**	**5**	**33 (28 - 38)**	**20 (16 - 25)**
16+ and/or 18+	161	59	41	5	43 (35 - 52)	31 (23 - 39)
16- and 18-	220	49	22	-	26 (20 - 33)	12 (8.3 - 18)
>BMD and HPV-	**37**	**16**	**8**	**1**	**49 (34 - 66)**	**27 (16 - 44)**
>BMD and HPV+	**313**	**246**	**192**	**9**	**79 (74 - 83)**	**62 (56 - 67)**
16+ and/or 18+	205	169	137	9	82 (77 - 87)	67 (61 - 74)
16- and 18-	108	77	55	-	71 (63 - 60)	51 (42 - 61)

+ Indicates a positive hrHPV test, and – indicates a negative hrHPV test. 16+ and/or 18+ indicates an intake hrHPV test positive for either HPV16 or HPV18, and 16- and 18- indicates an intake hrHPV test positive for types other than HPV16 or HPV18.

N indicates normal cytology, BMD indicates borderline or mild dyskaryosis, and >BMD indicates moderate dyskaryosis or worse.

CIN indicates cervical intra-epithelial neoplasia, and CxCa indicates cervical cancer (*i.e.*, squamous cell carcinoma, adenocarcinoma and adenocarcinoma *in situ*).

Table 6.5 Cumulative 18-month risk of histologically diagnosed CIN2 or worse stratified by cytology and hrHPV at baseline and cytology at 6 months.

Intake	6 months	Cases (n)				≥CIN2	≥CIN3
		Total	≥CIN2	≥CIN3	CxCa	Risk (95%CI)	Risk (95%CI)
N and HPV+	**N**	**399**	**14**	**4**	**-**	**5.5 (3.3 - 9.2)**	**1.6 (0.6 - 4.2)**
	BMD	**70**	**16**	**8**	**1**	**27 (17 - 40)**	**17 (7.0 - 25)**
	>BMD	**24**	**19**	**12**	**-**	**79 (62 - 92)**	**50 (32 - 71)**
16+ and/or 18+	N	135	12	3	-	13 (7.8 - 22)	3.3 (1.1 - 10)
	BMD	28	12	6	-	52 (34 - 73)	27 (13 - 51)
	>BMD	15	13	11	-	87 (65 - 98)	73 (50 - 92)
16- and 18-	N	264	2	1	-	1.2 (0.3 - 4.9)	0.6 (0.1 - 4.3)
	BMD	42	4	2	1	11 (4.2 - 26)	5.4 (1.4 - 20)
	>BMD	9	6	1	-	67 (38 - 92)	11 (1.6 - 57)
BMD and HPV-	**N**	**485**	**-**	**-**	**-**	**0.0 (0.0 - 0.8)**	**0.0 (0.0 - 0.8)**
	BMD	**81**	**4**	**2**	**1**	**6.4 (2.7 - 15)**	**4.0 (1.3 - 12)**
	>BMD	**7**	**6**	**4**	**1**	**86 (54 - 99)**	**71 (39 - 96)**
BMD and HPV+	**N**	**140**	**13**	**4**	**-**	**14 (8.5 - 23)**	**4.6 (1.7 - 12)**
	BMD	**109**	**41**	**20**	**1**	**38 (29 - 48)**	**18 (12 - 27)**
	>BMD	**48**	**38**	**26**	**4**	**79 (67 - 89)**	**54 (41 - 69)**
16+ and/or 18+	N	52	7	2		21 (11 - 39)	6.1 (1.6 - 22)
	BMD	43	20	15	-	46 (33 - 62)	35 (23 - 51)
	>BMD	28	23	16	1	82 (66 - 94)	57 (40 - 75)
16- and 18-	N	88	6	2	4	11 (4.8 - 22)	3.8 (1.0 - 14)
	BMD	66	21	5	-	32 (22 - 45)	7.6 (3.2 - 17)
	>BMD	20	15	10	-	75 (55 - 91)	50 (31 - 73)

+ Indicates a positive hrHPV test, and – indicates a negative hrHPV test. 16+ and/or 18+ indicates an intake hrHPV test positive for either HPV16 or HPV18, and 16- and 18- indicates an intake hrHPV test positive for types other than HPV16 or HPV18.

N indicates normal cytology, BMD indicates borderline or mild dyskaryosis, and >BMD indicates moderate dyskaryosis or worse.

CIN indicates cervical intra-epithelial neoplasia, and CxCa indicates cervical cancer (*i.e.*, squamous cell carcinoma, adenocarcinoma and adenocarcinoma *in situ*).

Participants without a cytology result at 6 months were excluded from the analyses.

In the control arm, ≥CIN2 risk for women with a baseline abnormal smear (*i.e.*, ≥BMD) was 27% (95%CI: 24 to 31). In the intervention arm, ≥CIN2 risk was 26% (95%CI: 23 to 29) for women with a baseline positive hrHPV test, 28% (95%CI: 24 to 31) for women with abnormal cytology at baseline, and 19% (95%CI: 17 to 21) for women with abnormal cytology and/or a positive hrHPV test. The relative risk of ≥CIN2 for women with a positive hrHPV result in the intervention arm compared to abnormal cytology in the control arm was 25.6/27.2 = 0.94 (95%CI: 0.8 to 1.1). The relative risk of ≥CIN2 for women with a positive hrHPV result and/or abnormal cytology in the intervention arm compared to abnormal cytology in the control arm was 0.71 (95%CI: 0.60 to 0.83). Detection rates of ≥CIN2 in women with BMD did not differ between intervention (14%, 95%CI: 11 to 18) and control group (13%, 95%CI: 11 to 17).

Because ≥CIN2 risks in women with abnormal cytology did not depend on the allocation to either intervention or control group (Figure 1), risks were pooled in further analyses. Furthermore, only results for ≥CIN2 are discussed. Results were comparable, albeit with lower absolute risks, using lesions ≥CIN3 as outcome measure.

In Table 6.4, separate ≥CIN2 risks are presented for strata defined by baseline cytology and hrHPV status. The lowest risk of 2.5% (95%CI: 1.5 to 4.2) was observed in women with BMD and a negative hrHPV test. A low ≥CIN2 risk of 5.3% (95%CI: 3.3 to 8.6) was also observed in women with normal cytology who were infected with hrHPV but not HPV16 and/or HPV18. In the subsets of hrHPV-positive women with normal cytology and HPV16 and/or HPV18, hrHPV-positive women with BMD, and women with >BMD, ≥CIN2 risks were moderate to high. Risks for HPV16-positive women only were comparable to the risk of women with HPV16 and/or 18 (data not shown).

A detailed overview of the role of repeat cytology on ≥CIN2 risks is displayed in Table 6.5, showing ≥CIN2 risks for strata defined by hrHPV and cytology at baseline, and cytology at 6-months. In women with hrHPV-positive normal cytology at baseline, cumulative risk of ≥CIN2 increased from 5.5% (95%CI: 3.3 to 9.2) to 79% (95%CI: 61 to 92) when comparing women with a second normal smear to women who had >BMD at 6-months. Women who had HPV16 and/or HPV18 at baseline with two normal smears had a risk of 13% (95%CI: 7.8 to 22), whereas women who tested hrHPV-positive for other high-risk types had a much lower risk of 1.2% (95%CI: 0.3 to 4.9). Risks in HPV16 and/or HPV18-positive women with normal cytology at baseline and BMD at 6-months were 52% (95%CI: 34 to 73), and 11% (95%CI: 4.2 to 26) for hrHPV-positive women positive for other types. In women with an hrHPV-negative BMD smear at baseline and cytological regression at 6 months (n=485), ≥CIN2 risk was 0% (95%CI: 0.0 to 0.8). In contrast, women with

hrHPV-positive BMD and cytological regression still had a substantial risk of ≥CIN2 of 14% (95%CI: 8.5 to 23). Women with >BMD at 6 months irrespective of baseline cytology had high ≥CIN2 risks.

Table 6.6 gives a detailed presentation of the role of hrHPV clearance and displays the cumulative risk of a lesion ≥CIN2 stratified for baseline results, and hrHPV test result at 6-months. In women with hrHPV-positive normal cytology at baseline, cumulative risk of ≥CIN2 was 2.4% (95%CI: 0.6 to 9.1) in women testing negative for hrHPV at 6 months and 20% (95%CI: 15 to 25) in women who tested hrHPV-positive twice. In the group of hrHPV-positive women with normal cytology at baseline and a negative hrHPV test at 6 months, all ≥CIN2 cases diagnosed tested positive for HPV16 and/or HPV18 at baseline. For the other strata with a negative hrHPV test at 6 months, ≥CIN2 risks were low but not negligible.

Finally, we evaluated the cumulative risk of a lesion ≥CIN2 based on hrHPV status and cytology at baseline and at 6 months (Table 6.7). Overall, the ≥CIN2 risk of women with an hrHPV-negative normal smear at 6 months (n=522) was 0.2% (95%CI: 0.0 to 0.8). In women with hrHPV-positive normal cytology at baseline, cumulative risk of ≥CIN2 was 1.3% (95%CI: 0.0 to 2.4) in women double negative at 6 months and 19% (95%CI: 15 to 25) in women who tested positive for cytology and/or hrHPV at 6 months. In the group of hrHPV-positive women without HPV16 and/or HPV18 at baseline with an hrHPV-negative normal test at 6 months, no cases of ≥CIN2 were diagnosed. In women with hrHPV-negative BMD at baseline, risk was 0.0% (95%CI: 0.0 to 1.1) in women who had an hrHPV-negative normal smear at 6 months and 9.8% (95%CI: 4.5 to 21) in women positive for either test at 6 months. Women with BMD cytology and a positive hrHPV test at baseline that tested hrHPV-negative with normal cytology at follow-up had a risk of 0.0% (95%CI: 0.0 to 8.4) and women who tested positive for cytology and/or hrHPV had a risk of 39% (95%CI: 31 to 47).

In order to evaluate the test characteristics of cytology and hrHPV screening, we calculated sensitivity and specificity for different thresholds of test positivity (Table 6.8). Since lesions could only have been detected in case of either ≥BMD or hrHPV positivity, the sensitivity of combined testing was assumed to be 100%. Using a threshold of ≥BMD, sensitivity for the detection of lesions ≥CIN2 was 68.0% (95%CI: 64.2 to 71.6) and specificity was 97.7% (95%CI: 97.4 to 98.1). Using hrHPV positivity as threshold, the sensitivity was 94.1% (95%CI: 91.7 to 95.9) and specificity was 96.1% (95%CI: 96.0 to 96.1). With hrHPV positivity for HPV16 and/or HPV18 as threshold, sensitivity was 62.5% (95%CI: 58.1 to 66.7) and specificity was 98.8% (98.6 to 99.0). The relative sensitivity of hrHPV testing compared to cytological testing was 0.941/0.680 = 1.38 (95%CI: 1.25 to 1.56) and the relative specificity was 0.961/0.977 = 0.98 (95%CI: 0.98 to 0.99). Using both ≥BMD and hrHPV positivity as threshold, estimates for sensitivity decreased slightly to 61.6% (95%CI: 57.7 to

65.4) for hrHPV-positive ≥BMD compared to a ≥ BMD cytology threshold, and specificity increased substantially to 99.3% (95%CI: 99.0 to 99.5). Using a positive test result of either test as threshold, in which case sensitivity was assumed to be 100%, the specificity was 94.5%.

Discussion

In this implementation study of hrHPV testing in population-based primary screening, we showed that primary hrHPV testing is more sensitive than cytology to detect ≥CIN2 lesions at the cost of slightly lower specificity. Moreover, we showed that women with HPV16 and/or HPV18 have a much higher ≥CIN2 risk than women positive for another hrHPV type. Retesting at 6 months showed that both cytological regression and hrHPV clearance are associated with decreased risks of ≥CIN2. Interestingly, women who had a double negative test at 6 months had a ≥CIN2 risk of 0.2% (95%CI: 0-1.1) and women with a hrHPV-negative BMD smear at baseline and normal cytology at 6 months had a ≥CIN2 risk of 0.0 (95%CI: 0-0.8). These data show that the risk of ≥CIN2 is better identified by 6 months with cytology and/or

Table 6.6 Cumulative 18-month risk of histologically diagnosed CIN2 or worse stratified by cytology and hrHPV at baseline and hrHPV at 6 months.

Intake	6 months	Cases (n)				≥CIN2	≥CIN3
		Total	≥CIN2	≥CIN3	CxCa	Risk (95%CI)	Risk (95%CI)
N and HPV+	**HPV-**	**165**	**2**	**-**	**-**	**2.4 (0.6 - 9.1)**	**0.0 (0.0 - 2.2)**
	HPV +	**266**	**46**	**23**	**1**	**20 (15 - 25)**	**9.8 (6.6 - 14)**
16+ and/or 18+	HPV-	48	2	-	-	7.4 (1.9 - 26)	0.0 (0.0 - 7.4)
	HPV+	112	35	20		36 (27 - 46)	20 (13 - 30)
16- and 18-	HPV-	117	-	-	-	0.0 (0.0 - 3.0)	0.0 (0.0 - 3.0)
	HPV+	154	11	3	1	7.9 (4.5 - 14)	2.4 (0.8 - 7.3)
BMD and HPV-	**HPV -**	**378**	**5**	**4**	**-**	**1.5 (0.6 - 3.6)**	**1.2 (0.4 - 3.3)**
	HPV +	**10**	**1**	**-**	**-**	**10 (1.5 - 53)**	**0.0 (0.0 - 31)**
BMD and HPV+	**HPV -**	**55**	**5**	**2**	**-**	**11 (4.7 - 26)**	**4.9 (1.2 - 19)**
	HPV +	**142**	**54**	**32**	**3**	**39 (32 - 48)**	**24 (17 - 32)**
16+ and/or 18+	HPV-	11	2	1	-	24 (6.3 - 70)	17 (2.5 - 73)
	HPV+	73	31	23	3	44 (33 - 57)	33 (23- 46)
16- and 18-	HPV-	44	3	1	-	8.2 (2.6 - 24)	2.3 (0.3 - 1.5)
	HPV+	69	23	9	-	34 (24 - 46)	14 (7.5 - 25)

+ Indicates a positive hrHPV test, and – indicates a negative hrHPV test. 16+ and/or 18+ indicates an intake hrHPV test positive for either HPV16 or HPV18, and 16- and 18- indicates an intake hrHPV test positive for types other than HPV16 or HPV18.

N indicates normal cytology, BMD indicates borderline or mild dyskaryosis, and >BMD indicates moderate dyskaryosis or worse.

CIN indicates cervical intra-epithelial neoplasia, and CxCa indicates cervical cancer (*i.e.*, squamous cell carcinoma, adenocarcinoma and adenocarcinoma *in situ*).

Participants without a HPV test result at 6 months were excluded from the analyses.

hrHPV testing than by single visit testing. Finally, our data support colposcopy referral for women with either hrHPV-positive BMD, or >BMD regardless of hrHPV status because of the magnitude of the risk of ≥CIN2.

In this study, retesting moments were 6 and 18 months as mandatory in the Dutch cervical screening programme (20). If hrHPV testing is implemented in primary screening, other retesting moments might be more cost-effective, with for example shorter intervals for HPV16 and HPV18 and longer intervals for other hrHPV types.

With lesions ≥CIN2 as the outcome of interest, screening sensitivity of classical cytology at a threshold of BMD was 68.0% and specificity was 97.7%. The sensitivity increased to 94.1% when using hrHPV testing, and specificity became slightly lower (96.1%). These data are in line with previous estimates obtained in screening studies using classical cytology and hrHPV testing by either GP5+/6+ PCR or Hybrid Capture 2 (18;21) and support the opinion that hrHPV testing should be used either

Table 6.7 Cumulative 18-month risk of histologically diagnosed CIN2 or worse stratified by cytology and hrHPV at baseline and cytology and hrHPV at 6 months.

Intake	6 months					≥CIN2	≥CIN3
						Risk (95%CI)	Risk (95%CI)
		Total	≥CIN2	≥CIN3	CxCa		
N and HPV+	**N and HPV-**	**155**	**1**	**-**	**-**	**1.3 (0.0 - 2.4)**	**0.0 (0.0 - 2.4)**
	≥BMD and/or HPV +	**274**	**47**	**23**	**1**	**19 (15 - 25)**	**9.5 (6.4 - 14)**
16+ and/or 18+	N and HPV-	47	1	-	-	3.9 (0.6 - 24.3)	0.0 (0.0 - 7.7)
	≥BMD and/or HPV +	112	36	20		37 (28 - 47)	20 (13 – 30)
16- and 18-	N and HPV-	108	-	-	-	0.0 (0.0 - 3.4)	0.0 (0.0 - 3.4)
	≥BMD and/or HPV +	162	11	3	1	7.5 (4.2 - 13)	2.3 (0.7 – 6.9)
BMD and HPV-	**N and HPV -**	**325**	**-**	**-**	**-**	**0.0 (0.0 - 1.1)**	**0.0 (0.0 - 1.1)**
	≥BMD and/or HPV +	**63**	**6**	**4**	**-**	**9.8 (4.5 - 21)**	**6.7 (2.6 - 17)**
BMD and HPV+	**N and HPV -**	**42**	**-**	**-**	**-**	**0.0 (0.0 - 8.4)**	**0.0 (0.0 - 8.4)**
	≥BMD and/or HPV +	**152**	**57**	**32**	**3**	**39 (31 - 47)**	**22 (16 – 30)**
16+ and/or 18+	N and HPV -	9	-	-	-	0.0 (0.0 - 34)	0.0 (0.0 - 34)
	≥BMD and/or HPV +	73	31	22	3	45 (34 - 57)	31 (22 – 44)
16- and 18-	N and HPV -	33	-	-	-	0.0 (0.0 - 11)	0.0 (0.0 - 11)
	≥BMD and/or HPV +	79	26	10	-	33 (24 - 45)	14 (7.4 - 24)

+ Indicates a positive hrHPV test, and – indicates a negative hrHPV test. 16+ and/or 18+ indicates an intake hrHPV test positive for either HPV16 or HPV18, and 16- and 18- indicates an intake hrHPV test positive for types other than HPV16 or HPV18.

N indicates normal cytology, BMD indicates borderline or mild dyskaryosis, and >BMD indicates moderate dyskaryosis or worse.

CIN indicates cervical intra-epithelial neoplasia, and CxCa indicates cervical cancer (*i.e.*, squamous cell carcinoma, adenocarcinoma and adenocarcinoma *in situ*).

Participants without a cytology or hrHPV test result at 6 months were excluded from the analyses.

alone or in conjunction with cytology in cervical screening. We also showed that the risk of ≥CIN2 in women with a hrHPV infection and normal cytology is higher than in women with hrHPV-negative BMD (12% *vs.* 2.5%) Besides, women with normal cytology harbouring HPV16 and/or HPV18 had a substantially higher risk of ≥CIN2 than women infected with another high-risk type (25% vs. 5.3%), although the ≥CIN2 risk of the latter group was still higher than in the group of women with an hrHPV-negative BMD smear. Similar data have been described by Khan *et al.* and Berkhof *et al.* (8;9). These results underline that hrHPV-positive women with normal cytology, and especially women with HPV16 or HPV18, should have shorter intervals for retesting than women with BMD and a hrHPV-negative test result.

We assume that among women with an abnormal smear or a positive hrHPV test at baseline the vast majority of ≥CIN2 lesions diagnosed during the study were prevalent, and the histological diagnosis was postponed due to the screening algorithm as women with BMD or normal cytology were not referred for colposcopy immediately. Thus, a difference between prevalent cases diagnosed at intake and incident cases only diagnosed during follow-up was not made. Several studies, using either histological or cytological data, have indicated that disease detected during short-term follow-up corresponds to "missed" prevalent disease (22;23).

Until now most studies evaluated the risk of ≥CIN2 for strata defined by hrHPV test results and cytology at baseline (4;8;24-27). Our study shows that for women with BMD or hrHPV-positive normal cytology a second test at 6 months, whether it be cytology or hrHPV testing, is more accurate in detecting ≥CIN2 than a single test at baseline. Tailoring the follow-up to allow for clearance of hrHPV and cytological regression of lesions will lead to a decrease in referrals for colposcopy. This is especially useful for hrHPV-positive women with normal cytology, since their baseline risk of a high-grade lesion is moderate. By retesting at 6 months, women can be distinguished with either a high or low ≥CIN2 risk. For instance, in our study HPV16 and/or HPV18-positive women with normal cytology at baseline followed by abnormal cytology at 6 months had a fourfold higher risk of ≥CIN2 than women with normal cytology at 6 months. In the group of hrHPV-positive women without HPV16 and/or HPV18, the risk of ≥CIN2 was ninefold higher when the repeat test was ≥BMD compared to normal. Results were even more pronounced when distinguishing women with an hrHPV-negative normal smear at 6 months as they had virtually no ≥CIN2 risk.

Although the negative predictive value for ≥CIN2 after a negative hrHPV test at baseline is higher than after negative cytology, the risk of a high-grade cervical lesion is not completely absent. Some participants in our study had high-grade cervical lesions but a negative hrHPV test (n=31), a phenomenon also found by others (17;23). These failures may be attributable to failure of cervical cell sampling, false-negative hrHPV test results or possibly incident disease. Additional analyses of

samples from participants with ≥CIN2 that tested negative for hrHPV using the crude sample indicate that approximately half of the samples were negative due to inadequate material for PCR analysis. Half of the remaining samples were positive by E7 PCR, suggesting that integration of hrHPV DNA caused a negative GP5+/6+ PCR result (data not shown).

In conclusion, hrHPV is a major risk factor of high-grade cervical lesions and cervical cancer. We have now shown that repeat testing for women with BMD or hrHPV-positive normal cytology using either cytology or hrHPV testing detects the risk of ≥CIN2 better than single visit testing, and that HPV16 and/or HPV18 identifies women with the highest risk of ≥CIN2. Moreover, in women with hrHPV-negative normal cytology at the second test the risk of ≥CIN2 is virtually absent. At present, we are conducting cost-effectiveness analyses to determine the optimal algorithm for the use of cytology and hrHPV testing to detect cervical cancer and its precursor lesions in cervical screening programmes

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Table 6.8 Specificity and relative sensitivity of cytology results and hrHPV test result at baseline at different thresholds for histological detection of lesions ≥CIN2 (cumulative 18-month risk).

		≥CIN2		≥CIN3	
Screening test	Threshold	Sensitivity (95%CI)	Specificity (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)
Cytology	**≥BMD**	**68.0 (64.2-71.6)**	**97.7 (97.4-98.1)**	**75.5 (71.0-79.5)**	**97.4 (97.2-97.9)**
	>BMD	**44.0 (40.1-48.0)**	**99.8 (99.6-100)**	**52.5 (47.5-57.4)**	**99.7 (99.4-99.9)**
hrHPV	**Positive**	**94.1 (91.7-95.9)**	**96.1 (96.0-96.1)**	**94.2 (91.8-95.9)**	**95.6 (95.5-95.8)**
	16+ and/or 18+	62.5 (58.1-66.7)	98.8 (98.6-99.0)	67.0 (61.8-71.8)	98.5 (98.3-98.7)
	16- and 18-	31.6 (27.7-35.8)	97.3 (97.1-97.5)	27.2 (22.7-32.2)	97.1 (96.9-97.3)
Cytology and hrHPV	**Positive and ≥BMD**	**61.6 (57.7-65.4)**	**99.3 (99.0-99.5)**	**69.3 (65.3-73.1)**	**99.0 (98.8-99.3)**
	16+ and/or 18+ and ≥BMD	39.7 (25.2-31.9)	99.7 (99.5-100)	48.4 (28.4-37.1)	99.6 (99.3-99.9)
	16- and 18- and ≥BMD	22.4 (19.2-25.9)	99.6 (99.3-99.8)	21.3 (17.5-25.6)	99.4 (99.2-99.7)
	Positive and >BMD	**40.7 (36.9-44.7)**	**99.8 (99.6-100)**	**49.6 (45.2-54.0)**	**99.7 (99.5-100)**
	16+ and/or 18+ and >BMD	28.2 (24.7-31.9)	99.9 (99.7-100)	35.7 (31.1-40.6)	99.8 (99.6-100)
	16- and 18- and >BMD	12.8 (10.4-15.8)	99.9 (99.7-100)	14.2 (11.1-18.1)	99.9 (99.6-100)
Cytology or hrHPV	**Positive or ≥BMD**	**100 (reference)**	**94.5**	**100 (reference)**	**94.0**
	16+ and/or 18+ or ≥BMD	90.8 (88.0-93.0)	96.7 (96.5-96.9)	94.1 (91.1-96.1)	96.3 (96.1-96.5)
	16- and 18- or ≥BMD	78.3 (74.6-81.7)	98.7 (98.4-98.9)	83.8 (79.5-87.3)	98.3 (98.1-98.6)
	Positive or >BMD	**97.1 (95.2-98.3)**	**96.0 (95.9-96.1)**	**96.7 (94.8-98.0)**	**95.6 (95.5-95.7)**
	16+ and/or 18+ or >BMD	77.2 (73.6-80.5)	95.4 (95.3-95.5)	81.4 (77.1-85.1)	95.1 (94.9-95.2)
	16- and 18- or >BMD	62.8 (58.7-66.7)	97.2 (97.0-97.3)	65.5 (60.5-70.2)	96.9 (96.7-97.1)

N indicates normal cytology, BMD indicates borderline or mild dyskaryosis, and >BMD indicates moderate dyskaryosis or worse.

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CHAPTER 7.

GENERAL DISCUSSION

Abstract
The aim of this thesis was to expand the epidemiologic knowledge on cervical cancer and its precursors and to evaluate the implications of HPV testing for cervical cancer screening. The studies that were performed provided data on disease occurrence and disease prevention by integration of viral risk factors in the screening test. This chapter will focus on the clinical relevance and the methodological issues of these studies. The shortcomings and merits of the individual studies presented in this thesis have been discussed in the previous chapters, but several specific methodological aspects of the studies will be discussed in this chapter. It will also provide suggestions for future research on cervical cancer screening.

Methodological considerations
Introduction. Cervical cancer epidemiology offers a unique challenge to the scientific researcher. Cervical cancer is a relatively rare disease, caused by an infection contracted at least 10 to 15 years before invasive cancer may be diagnosed. This infection with high-risk HPV is a very common condition, affecting approximately 80% of women (and presumably men as well) throughout their lifetime. The natural history of the development of invasive cervical cancer after infection with HPV has not been elucidated completely. In fact, the natural history is difficult to investigate, as cytology testing does not have a 100% correlation with the histological diagnoses, the histological diagnosis of pre-invasive lesions is subject to intra- and interoberver bias, and histological specimen taking induces regression of the lesion, thus influencing the natural history of the disease. HPV testing is also subject to error, as both sample taking and the test itself are subject to false-negative error.

Another source of epidemiological difficulty precluding the use of invasive cervical cancer occurrence as outcome measure in an epidemiological study of the course of a high-risk HPV infection is that cervical cancer is a rare condition in the Netherlands. High-grade cervical intra-epithelial neoplasia is considered a proxy for cervical cancer, but the rate of progression and regression of these lesions is not well known and high-grade cervical intra-epithelial neoplasia is by definition an asymptomatic condition. Ethically, women without cervical disease diagnosed by the usual care standards (*i.e.*, by cytology testing) cannot be subjected to invasive diagnostic procedures, and invasive diagnostic treatment cannot be withheld from women with possible cervical disease as diagnosed by cytology or HPV testing.

We have tried to meet these epidemiological challenges in the research described in this thesis, and I will now discuss the methodological considerations of the research presented in this thesis.

Study design. In epidemiological research, several study designs can be adopted in order to investigate the condition of interest.

The studies on cervical cancer incidence described in 3.1 and 3.2 were purely *ecological* in design and descriptive of nature, without taking any causative factors into account.

Several chapters (**CHAPTERS 4 AND 5**) describe *cross-sectional* studies in which the determinants, *i.e.*, HPV status and cytology interpretation, and the outcome of interest, *i.e.*, high-grade CIN and cervical cancer, were measured at the same point of time. Although these cross-sectional studies provide a relatively quick and simple way to study associations of HPV and high-grade lesions and cancer, their main drawback is that conclusions about cause and effect cannot formally be drawn. In the cross-sectional design, the temporal relation between exposure and effect is not investigated.

In 5.2, we used data on high-grade CIN lesions in a cross-sectional *case-control* design, while in reality, these data were collected in a *prospective* study (see 6.1). Patients included in this study were selected by the presence of a normal smear taken previously. Since HPV18 as outcome of interest is both related to non-detection of cervical abnormalities cytologically and to the occurrence of CIN lesions, the results of our study may have been biased towards a decreased association of HPV18 to high-grade CIN than to cervical cancer.

The design of a prospective study ensures that measurement of the determinant has taken place before the outcome of interest may occur. Therefore, this type of study may provide insight in the etiology of the disease under study. Indeed, temporality is the ultimate proof of causation. Prospective studies are described in **CHAPTER 6**. In these studies, cytology and hrHPV status were determined before the outcome of interest, high-grade CIN and cervical cancer occurred.

Potential problems with prospective studies are the duration of follow-up needed for a sufficient number of cases to occur, and loss-to-follow-up. In our prospective study, the duration of follow-up was at least three years in the study described in this thesis (presently extending to at least five years for the entire cohort), but loss-to-follow-up reached up to 30% (1). However, as the preclinical phase of cervical cancer is estimated to be approximately 12-15 years, it is obvious that cervical cancer occurrence can only be investigated using proxy outcome measures, as explained previously (2).

Exposure assessment. The validity of epidemiological research is ultimately determined by the accuracy of the measurements of determinants under study. Cervical cancer research has two main exposures under study, *i.e.*, HPV status and cervical abnormalities (cytology result or histological grading).

The presence of disease as measure of exposure can be assessed both cytologically (through Pap testing) and histologically (through biopsy sampling). The Pap test is approximately 70% sensitive and histological lesion categorisations are moderately reproducible, with lower agreement for the low-grade lesions than for the high-grade lesions (3-6). For both smear tests and biopsies there is an element of chance in sampling cells and tissue (7). Misclassification may occur due to incorrect diagnosis of

cervical specimens, especially when knowledge of the other determinants may influence the grading by the specimen. In our studies, information on hrHPV status was either not available at the original time of treatment (5.1, 5.2 and 6.1), or was blinded to cytotechnicians and pathologists performing the cytologic and histologic analysis of cervical samples (**CHAPTERS 5** and **6**). Therefore, we assumed misclassification to be non-differential.

Compared to cytology interpretation, testing for high-risk HPV seems to offer a more objective and reproducible exposure measure (8). Overall test agreement for the high-risk HPV test is good (9). Currently, different PCR and other test options can be used to diagnose the presence of high-risk HPV (10-13). Tests have to be evaluated for clinical and analytical aspects (14). A test may diagnose the presence of viral infections accurately, whereas for screening practice a test should be capable to establish the presence of infections that will lead to clinically relevant disease. While the studies we described in **CHAPTER 5** were restricted to HPV positive samples, the studies of **CHAPTER 6** showed that GP5+/6+ PCR diagnosed clinically relevant infections with a high probability of progression to high-grade CIN.

Outcome measures. In cervical cancer research, relevant issues in outcome measurement concern disease progression and the use of surrogate endpoints.

Cervical cancer is a rare condition in the Netherlands. Therefore, cervical cancer as an endpoint could only be used in our ecological studies (**CHAPTER 3**) and in the retrospective studies on cases of squamous cell carcinoma and adenocarcinoma (**CHAPTER 5**). For our prospective studies described in **CHAPTER 6**, and for most clinical research published internationally, the occurrence of high-grade CIN lesions is used as proxy for cancer incidence. High-grade CIN lesions reflect the risk of invasive cancer and are positioned in the time axis of the causal pathway between HPV infection and cervical cancer occurrence (15). As we demonstrate in 5.2, cohort studies using high-grade CIN lesions as intermediate endpoint for cervical cancer underestimate the contribution of HPV18 to squamous cell carcinoma pathogenesis. Therefore, assumptions based on analyses of proxy endpoints should be cautiously applied to cervical cancer screening in general.

Another issue in study outcome is disease progression. The study of disease progression may give more insight into the mechanisms underlying an association than a cross-sectional study would provide. In cervical cancer screening research, markers for disease progression may provide one with better risk estimates to tailor screening programmes. Disease progression studies are influenced by issues of reproducibility of outcome and exposure (8;16). We demonstrate in 6.2 that disease progression may seem to occur very rapidly for women with normal or low-grade smears especially in women who test high-risk HPV positive. We have not performed a cytologic or histologic review of all specimens included in this analysis, thereby not formally excluding false-negativity as a cause for apparent disease progression.

However, a limited analysis revealed a high correlation between the revised and the original diagnosis.

Furthermore, baseline levels of exposure may be associated with the determinant of interest, but they may also independently determine disease progression. In other words, HPV status (*e.g.*, load or type) determines the degree of abnormality diagnosed at baseline, but HPV status (*e.g.*, load or type) may also determine the probability of progression to cancer (17). For most HPV types this holds true. However, HPV18-associated lesions seem to be underrated by cytology screening, while HPV18 is associated with an increased risk of cervical cancer, especially adenocarcinomas (18-20).

Added value of a new test in cervical cancer screening. "New" factors that are found to prospectively predict the occurrence of high-grade cervical lesions and cancer independently of screening cytology may be of added value in the clinical assessment of cervical cancer risk. The existing debate whether to introduce combined testing (*i.e.*, cytology screening with high-risk HPV testing) in clinical practice, and if introduced, how, is a good example of a factor with an added value.

In cervical cancer screening research, the sensitivity of the screening modality is overestimated if all women who test double negative are assumed to be free of disease without any histological confirmation (*i.e.*, verification bias). However, performing a study adequately controlling for confirmation bias would require performing colposcopies on large numbers of disease-free women. This has been proven to be ethically unacceptable and practically infeasible (21;22). Therefore, in our studies presented in 6.2, we did not confirm the absence of disease in double-negative women. We may have overestimated sensitivity and specificity of the combined screening test although analyses were adjusted for non-verification in women with at least one high-risk HPV-positive smear at baseline.

Adding a second test will always lead to an improvement in sensitivity, as the number of false-negative tests always decreases. Specificity, on the other hand, may decrease (23). Since the specificity of a test determines whether or not the frequency of false-positives will be low enough for a screening programme to be feasible, the effects of combined testing by whatever modality (*e.g.*, general high-risk HPV testing, viral load determination or HPV typing) will have to be evaluated carefully for their cost-effectiveness. In the studies presented in this thesis, general HPV testing and HPV typing demonstrated their capacity to select high-risk women from the general population of screenees and to select the women with an extra high risk from that group respectively, while the decrease in specificity was marginal (6.2).

Implications for current practice

Addition of HPV testing to cervical cancer screening. From a clinical performance viewpoint, cervical cytology is relatively insensitive for the detection of high-grade CIN lesions and cancer, and cervical cytology screening must be repeated frequently to achieve programmatic effectiveness (24). Also, cytology is labour intensive and therefore

expensive. New screening modalities have to be developed and implemented in the screening programme to make screening both more efficient and more cost-effective. In the research presented in this thesis, we show that the addition of HPV testing to the cervical cancer screening programme allows the identification of both a very low risk group and a high-risk group.

The very low risk risk group consists of screening participants with low-grade abnormalities and a negative HPV test at intake and follow-up. Previously, these women were subjected to intensive follow-up for at least 18 months, but addition of a HPV test to screening will confirm the low-risk category these women belong to with a follow-up period of only 6 months (21;25). Indeed, once prospective research has confirmed these observations for women with double negative test results at intake, the screening interval may be extended for all women with HPV negative normal smears. Possibly, the screening interval may be extended for women with low-grade smears as well. At present, the VUSASCREEN study is investigating the occurrence of lesions in women with a double-negative test at baseline (Rijkaart *et al*. Submitted for publication).

Conversely, participants who test HPV positive with either normal cytology or low-grade abnormalities have to be considered high-risk. For these women, we have shown in 6.2 that a 6 months follow-up schedule will select those women who already have high-grade CIN lesions that have to be treated. For women with normal smears who test HPV positive, our research has suggested another avenue of intervention. It may be more efficient to subject these smears to rescreening since most false-negative interpretations will have been rendered in this group as our data in 6.1 show.

Screening interval and the age to commence screening. The addition of high-risk HPV testing in screening improves the detection of high-grade lesions at the cost of a more expensive screening programme and more false-positive tests. Thus, the remaining research questions are mainly pragmatic and concern the feasibility and cost-effectiveness of the screening regime in a given screening setting (22;26-29). Here, we discuss some of the aspects of HPV testing in relation to age and screening interval.

The addition of HPV testing to screening as an adjunct to cytology will decrease the number of false-negative smears and increase the negative predictive value of the screening test. Women with a HPV infection are at risk for cervical cancer and cervical cytology will have to be monitored either for infection resolution or for lesion progression, whereas women without a HPV infection will not develop cancer within the foreseeable future. Indeed, the negative predictive value of HPV in addition to cytology screening is such, that it has been postulated to extend screening intervals for HPV negative women who do not have cytological abnormalities (30). The regular 5-year screening interval in the Netherlands is associated with a societally accepted, very small, risk of invasive cancer after 5 years (31). This accepted level of risk can be decreased for nearly all women when the HPV test is used to distinguish between high-risk and low-risk groups. The overall risk in the HPV negative group will have

decreased to a level substantially lower than presently accepted for all women, and the screening interval may be extended to the point where the previously accepted level has been reached. Estimates are that the screening interval may be extended to 8 to 10 years for HPV negative women with normal cytology (32).

The use of HPV testing in the screening programme has not been advocated for women under the age of 30. After primary infection with HPV, levels of HPV positivity may reach up to 25 percent and cytological abnormalities are extremely common as well (22;33-35). In the studies presented in this thesis, we show that cervical cancer hardly occurs before age 25, while the incidence increases rapidly after that age until incidence reaches its first peak at age 30 (36). Modelling studies have shown that cost-effective screening in the Netherlands may begin at age 27 (37). As we show that adenocarcinoma incidence may be rising in younger women, a renewed effort should be made to improve surveillance for lesions in this young group by extension of the screening programme to include a screening round before age 30.

Another use of the HPV test is to select older women for increased screening or cessation of screening activities. Screening for cervical cancer in the Netherlands ceases at age 60. As we have shown, a substantial number of cervical cancers is still diagnosed in women over 60 years of age (36). HPV testing, especially at the last screening round, will select those women for continued screening activivties who are still at risk to develop invasive cancer. However, older women with no history of cervical disease and a normal screening history who are not at risk of acquiring HPV, are unlikely to develop CIN lesions de novo. Thus, a cytologically normal negative HPV test at age 50 may be used to advise low-risk women to stop screening (38).

HPV typing in cervical cancer screening. This study has provided some of the groundwork for the introduction of type-specific testing in the Dutch nationwide cervical cancer screening programme. We have established that type-specific testing may select women with a high risk for the presence and the development of cervical lesions from women with a lower risk. Here, we discuss some of the uses of type-specific testing in screening as highlighted in this thesis.

Types HPV16 and HPV18 are the types most often associated with invasive cervical cancer (39). HPV16 is most often associated with squamous cell carcinoma, and in adenocarcinoma HPV18 is the most frequent type. Compared to other high-risk HPV types, these two types can be defined as 'extra high-risk' types. Typing in addition to general HPV testing in screening will allow for the selection of women who are infected with any of these extra high-risk types. Whereas general HPV testing may target women at a low risk for reduced surveillance, increased surveillance of women with HPV16 and HPV18 infections will allow for a better risk stratification of women with an increased risk. Women with an intermediate risk, high-risk HPV positive for other types than HPV16 or HPV18, may undergo surveillance at a reduced intensity level compared to the extra high-risk women.

An infection with HPV18 may result in a transforming infection that is not recognized cytologically. HPV18 is preferentially associated with adenocarcinoma, and adenocarcinoma have a decreased survival compared with squamous cell carcinoma (40). We expect that HPV typing in cervical cancer screening combined with differential surveillance will result in a decrease in adenocarcinoma incidence. As mentioned in previously, adenocarcinoma incidence seems to be rising in younger women. Therefore, HPV typing is expected to have a profound public health impact in the prevention of morbidity and mortality of especially younger women.

Future research

The introduction of HPV testing in cervical cancer screening. Thus far, this thesis has focused solely on the addition of high-risk HPV testing to the existing cervical screening programme. However, cervical cancer screening may use high-risk HPV testing in several different ways.

The data of this thesis support the use of high-risk HPV testing in addition to cytology screening in cervical cancer screening programmes. Using results in a programme with higher sensitivity and a slightly lower specificity than a cytology screening programme (17;41-44). Doubts had been raised whether the increased sensitivity reflected overdiagnosis of regressive lesions, *i.e.* lesions that would have regressed before the next screening round would have occurred in regular screening (44). Data from other trials now seem to suggest that overdiagnosis is not a clinically relevant problem since the ov eral detection rate does not differ between the experimental group receiving high-risk HPV testing in addition to screening and the non-experimental group receiving regular screening (43;45). The increase in costs associated with the use of two tests instead of one may be discounted by less rigorous repeat-and-referral recommendations for women who test negative for high-risk HPV (46).

Researchers have advocated the use of high-risk HPV testing as a primary screening mode with cytological evaluation reserved for those women that test positive for high-risk HPV. Recently, the results of a large trial comparing high-risk HPV testing with cytology were published (45). While all women were tested for both cytology and high-risk HPV, results of either HPV testing or cytology screening guided the clinical management. Using this algorithm, HPV testing resulted again in an substantially increased sensitivity with a slightly decreased specificity. With cytology triage, the specificity improves to the level of conventional cytology. Therefore, the use of high-risk HPV testing as a primary screening mode with cytology screening reserved for women who test positive seems feasible and is being evaluated in large-scale screening trials (17;26).

Effects of prophylactic vaccination on cervical cancer screening. Cervical cancer screening programmes will be influenced dramatically by the introduction of prophylactic HPV

vaccinations (47-49). Extrapolating from the relatively short-term results of the vaccination trials, it is expected that HPV vaccination will confer long-term protection against new infections with types vaccinated against. Therefore, HPV vaccination will prevent the development of invasive cervical cancer associated with these types. Presently, HPV16 and HPV18 are the only high-risk types included in the vaccines. Here, we will elaborate on the expected effects of HPV vaccination on the screening programme.

If administered before an infection with HPV16 or HPV18 has occurred, the prophylactic vaccines will protect against the development of high-grade CIN and cervical cancer associated with these types and partly by the types for which cross-protection occurs after vaccination, *i.e.*, types HPV33 and HPV45. As a result, the incidence of both major histological subtypes of cervical cancer, squamous cell carcinoma and adenocarcinoma, will decrease. However, these effects will only occur after quite a long period of time has elapsed, taking the long preclinical phase of at least 10-15 years of cervical cancer into account. The cost of screening programmes will be reduced, since the number of lesions requiring invasive treatment and follow-up will decrease. The costs of the vaccination programme itself will have to be evaluated societally. At present, HPV16-associated lesions are quite effectively diagnosed in pre-invasive stages, but HPV18-associated lesions are not diagnosed well by screening cytology. Therefore, the largest (relative) reduction in incidence following vaccination will be seen in adenocarcinoma incidence.

Adenocarcinoma incidence seems to be rising in younger women especially, as shown in this thesis, and the effect of vaccination will be found most noticeably in this group. However, as vaccination is only effective before a primary infection hs occurred, vaccination will have to take place at a young age before the acquisition of HPV has taken place. Indeed, 11 to 12 years old is the age at which commencement of vaccination has been proposed (49). A screening programme using cervical cytology will have to be continued until vaccinees have reached the presently used screening ages, and cytology screening may be discontinued. Indeed, based on a vaccination age of 11 or 12 years and a screening programme commencing at age 30, about two decades will have to pass before cervical cancer screening programmes may be discontinued. Discontinuation will only be possible once a vaccine conferring protection against all high-risk HPV types has been developed.

Most high-risk types are not included in the HPV vaccine as yet. While these types do not confer a preferential risk for cancer, they are causally associated with cervical cancer and cytology screening should be continued until (nearly) all infections with high-risk HPV are prevented by vaccination (39). Tests characteristics of the screening programme, such as sensitivity and specificity, depend on the prevalence of lesions in the screened population. At present, a high false-negative rate is its most critical limitation. This limitation can be countered by the addition of HPV testing to cervical cancer screening. After the implementation of widespread vaccination, an increase in false-positives diagnosed by cytology will be a major drawback (50). The performance

parameters of cytology under conditions of low lesion prevalence, as likely after large-scale vaccination has been introduced, will have to be evaluated carefully to design new screening and triage algorithms under these conditions.

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CHAPTER 8.

SUMMARY

PREVENTION OF CERVICAL CANCER IN THE NETHERLANDS STUDIES ON CYTOLOGY AND HPV INFECTIONS

PREVENTIE VAN BAARMOEDERHALSKANKER IN NEDERLAND STUDIES BETREFFENDE CYTOLOGIE EN HPV INFECTIES

8.1 Summary

Cervical cancer prevention by cytology screening has been possible since the middle of the 20th century. In the Netherlands, organized screening was introduced nationwide in 1988. However, a new evaluation of the screening modality used is called for by the possibilities of high-risk human papillomavirus-based screening. In this thesis, cervical cancer epidemiology, cervical cancer screening and human papillomavirus infections are described. The first part of the thesis deals with the epidemiology of cervical cancer and human papillomavirus infections. In the second part, aspects of the causal relation between these entities are addressed. Integration of both approaches will result in an advice for the Dutch screening programme.

We have performed two ecological studies on cervical cancer incidence, as described in **CHAPTER 3**. Here, we confirmed that cervical cancer is a relatively rare disease in the Netherlands. Still, during the last decade, the incidence rate has decreased continuously. This decrease seems to be a decrease in the incidence of squamous cell carcinoma of the cervix only, while the incidence of adenocarcinoma of the cervix has remained stable (3.1). These findings seem to confirm that the cytological detection of squamous precursor lesions is superior than the cytological detection of precursor lesions of adenocarcinoma by cervical cancer screening. Stratified for age, the main decrease in incidence has occurred in women aged 60-74 years suggesting the influence of participation in the screening programme on cervical cancer incidence. A rising trend in incidence of adenocarcinoma may be present in women aged 15-44 years, again suggesting a decreased efficiency of screening compared of adenocarcinoma to squamous (precursor) lesions (3.2).

Thus, cervical cancer incidence has decreased in the Netherlands, and its decrease in incidence coincides with the introduction of cervical cancer screening by exfoliative cytology of the cervix (the Pap smear). In **CHAPTER 4**, we describe the cervical cytology coding classification used in the Netherlands and the effect of changes effectuated therein in order to better select women with cervical lesions from the general population screened while reducing the number of referrals for low-grade abnormalities. We have evaluated the CISOE-A scoring system for the diagnosis of cervical lesions used in the Netherlands in a cross-sectional study of one geographically defined region of the Netherlands. We found that the introduction of the CISOE-A classification in 1996 has led to a decrease in the number of women diagnosed with abnormalities, while the detection of high-grade lesions was not affected. This indicates that the cytological screening programme increased its efficacy.

Since high-risk types of the human papillomavirus cause cervical cancer, we have sought to establish whether certain high-risk types confer a preferential risk for the development of cervical cancer compared to the other high-risk types in **CHAPTER 5**. Cross-sectionally obtained data of women with normal cytology and a positive high-

risk HPV test in the POBASCAM trial was compared to data of cases with (invasive) cervical cancer obtained retrospectively (5.1). High-risk HPV types HPV16 and HPV18 occurred with the highest frequency in all carcinoma subtypes studied compared to the other high-risk types. HPV16 conferred a preferential risk for squamous cell carcinoma, and less for adenocarcinoma (in situ). HPV 18 on the other hand, conferred the highest risk for adenocarcinoma (*in situ*), and conferred a lesser risk for squamous cell carcinoma. After correction for high-prevalence types, HPV45 conferred a preferential risk for adenocarcinoma as well. Subsequently, we compared cases with (invasive) squamous cell carcinoma with cases of cervical intra-epithelial neoplasia grade 2 and 3 (CIN2/3) (5.1). In this study of exclusively squamous lesions, HPV16 was associated with both high-grade lesions and squamous cell carcinoma. HPV18 conferred a high risk of squamous cell carcinoma compared to high-grade lesions. This indicates that the contribution of HPV18 to the burden of cervical cancer may be underestimated in studies using CIN2/3 as intermediate endpoint for invasive cervical cancer.

A useful cervical cancer screening test selects participants with (a high risk of) disease from a larger population of women without disease participating in screening. Therefore, in order to implement high-risk HPV testing in the Dutch screening programme, we evaluate the addition of high-risk HPV testing to cervical cancer screening in **CHAPTER 6**. We evaluated whether a type-specific HPV test better identifies women at risk for cervical precancer than cytology (6.1). We performed a prospective study with retrospective retrieval of case material in order to evaluate this question. Women who were diagnosed with high-grade lesions in spite of adherence to the screening programme often harbored HPV infections in the normal smear taken in the screening round preceding the diagnosis of a high-grade lesion. In addition, the high-risk HPV harbouring smears previously diagnosed as normal were more often upgraded after revision than normal smears without high-risk HPV. Thus, the use of high-risk HPV testing to select smears for rescreening would facilitate early diagnosis of high-grade lesions. In 6.2, we evaluated a risk stratification in a randomized controlled trial, the POBASCAM trial, to better identify women at risk for cervical disease. Women with high-grade cervical lesions were better identified by adding a second cytology test after 6 months follow-up to the baseline cytology and HPV test than were identified by testing at intake only. Especially in the groups of women with a positive HPV test and BMD or normal cytology at intake, a subset of women with a high risk of cervical precancer could be identified by a high-grade cytology result at the repeat test. When extra high-risk types HPV16 and HPV18 were analyzed separately from the other high-risk HPV types, baseline testing for HPV16 and/or HPV18 identified a subgroup with extra high-risk for the presence of high-grade CIN lesions at short-term follow-up, while, conversely, the group with negligible risk consisted of women with a negative high-risk HPV test. These data confirm that the high-risk HPV test offers a unique possibility in the screening programme to select women at risk for precancer and thus enables a better risk

stratification than cytology only as currently employed in cervical cancer screening in the Netherlands.

In the general discussion (**CHAPTER 7**), the main findings of this thesis are considered in the context of current scientific knowledge and ongoing research in the field of cervical cancer screening. We conclude that the addition of high-risk HPV to the cervical cancer screening programme will result in an increased detection of high-grade lesions and cervical cancer in screening participants. A type-specific high-risk HPV test may further aid in the selection of women with the highest risk of cervical cancer.

8.2 Samenvatting (Summary in Dutch)

Baarmoederhalskankerspreventie is sinds het midden van de twintigste eeuw mogelijk. In Nederland is georganiseerde screening in 1988 landelijk geintroduceerd. Nu is een nieuwe evaluatie van het screeningsonderzoek noodzakelijk omdat de mogelijkheden van screening gebaseerd op hoog-risico HPV testen onderzocht moet worden. In deze thesis worden de epidemiologie van baarmoederhalskanker, baarmoederhalskanker screening en humaan papillomavirus infecties beschreven. Het eerste deel van dit promotieonderzoek betreft de epidemiologie van baarmoederhalskanker en humaan papillomavirus infecties. In het tweede gedeelte worden aspecten van de causale relatie tussen humaan papillomavirus infecties en baarmoederhalskanker beschreven. Integratie van deze twee manieren van aanpak leidt tot een voorlopig advies voor het Nederlandse screeningsprogramma.

Wij hebben twee ecologische studies naar het voorkomen van baarmoederhalskanker uitgevoerd, zoals beschreven in **HOOFDSTUK 3.** Hiermee hebben wij vastgesteld dat baarmoederhalskanker een relatief zeldzame aandoening is in Nederland. Evenzo is de incidentie over de laatste decade gestaagd gedaald. Deze daling in incidentie betrof alleen de incidentie van plaveiselcelcarcinoom van de cervix. De incidentie van het cervicale adenocarcinoom bleef stabiel (3.1). Deze bevindingen lijken te bevestigen dat de cytologische detectie van plaveiselcellige voorloperlaesies van baarmoederhalskanker beter is dan de cytologische detectie van voorloperlaesies van adenocarcinomen in het screeningsprogramma. Onderverdeeld naar leeftijd heeft de daling in incidentie met name plaatsgevonden in de groep van vrouwen in de leeftijd van 60-74 jaar, wijzend op de invloed van deelname aan het screeningsprogramma in de voorafgaande jaren. Een stijgende trend lijkt aanwezig te zijn in de groep vrouwen van 15-44 jaar, opnieuw suggestief voor een verminderde efficiëntie van cervicale screening in de opsporing van voorloperlaesies van het adenocarcinoom (3.2).

Samenvattend, de baarmoederhalskanker incidentie is gedaald in Nederland en de daling in incidentie valt samen met de introductie van baarmoederhalskankerscreening middels cervicale cytologie (het uitstrijkje). In **HOOFDSTUK 4** beschrijven wij de in Nederland in gebruik zijnde cytologie classificatie en het effect van veranderingen in deze classificatie in de detectie van vrouwen met afwijkende cytologie van de baarmoederhals. Wij hebben de KOPAC-B classificatie geëvalueerd in een cross-sectionele studie in een geografische beperkte regio van Nederland. De introductie van deze classificatie in 1996 heeft geleid tot een afname in het aantal vrouwen bij een cytologische afwijking werd vastgesteld zonder dat de histologische opsproing van laesies veranderde. Dit wijst op een verbeterde efficiëntie van het screeningsprogramma.

Aangezien hoog-risico types van het humaan papillomavirus baarmoederhalskanker veroorzaken, hebben wij in **HOOFDSTUK 5** getracht vast te stellen of er verschil is in

het vermogen van de verschillende hoog-risico types om baarmoederhalskanker te veroorzaken. Cross-sectioneel verkregen data van vrouwen met een normaal uitstrijkje en een positieve HPV test in de HPVBOB studie werden vergeleken met gegevens van cases van baarmoederhalskanker die retrospectief verkregen waren (5.1). Hoog-risico types HPV16 en HPV18 kwamen met de hoogste frequentie voor in alle histologische types van baarmoederhalskanker. HPV16 was het sterkste geassocieerd met plaveiselcelcarcinoom en in mindere mate met adenocarcinoom (in situ). HPV18 daarentegen was het sterkst geassocieerd met adenocarcinoom (in situ), en minder sterk met het plaveiselcelcarcinoom. Na correctie voor hoog-prevalente types was HPV45 ook sterk geassocieerd met adenocarcinoom. Vervolgens hebben we de type-verdeling van de hoog-risico types ook vergeleken in hoog-gradige CIN laesies (CIN2/3) versus plaveiselcelcarcinoom (5.2). In deze studie waarin alleen plaveiselcellaesies geïncludeerd werden was HPV16 met zowel CIN2/3 als plaveiselcelcarcinoom geassocieerd. HPV18 daarentegen vertoonde een sterkere associatie met plaveiselcelcarcinoom dan met CIN2/3. Dit wijst op het risico van onderschatting van de bijdrage van HPV18 in de causatie van baarmoederhalskanker indien CIN2/3 laesies als uitkomstmaat gebruikt worden in plaats van gevallen van invasieve kanker.

Een voor screening bruikbare test selecteert deelnemers met een (hoog risico op) ziekte van een groter populatie van vrouwen zonder ziekte deelnemend aan een screeningsprogramma. Daarom, teneinde testen voor hoog-risico HPV in te voeren in de Nederlandse screening, evalueren wij de additie van hoog-risico HPV testen aan het screeningsprogramma in **HOOFDSTUK 6**. We hebben geëvalueerd of de toevoeging van een type-specifieke test leidde tot een betere identificatie van vrouwen met een risico op baarmoederhalskanker en precursorlaesies dan cytologie alleen (6.1). Wij hebben een prospectieve studie verricht met retrospectieve verzameling van onderzoekspreparaten ten einde deze vraag te beantwoorden. Vrouwen bij wie een hoog-gradige laesie van de baarmoederhals werd vastgesteld ondanks deelname aan een eerdere ronde van het screeningsprogramma bleken vaak een niet gediagnostiseerde HPV infectie in het uitstrijkje van de voorgaande ronde te hebben. Bovendien werden HPV positieve uitstrijkjes vaker opgewaardeerd te worden dan uitstrijkjes zonder HPV infectie. Deze bevindingen suggereren dat HPV testen normale uitstrijkjes kunnen selecteren voor cytologische revisie ten einde gemiste afwijkingen op te sporen. In 6.2, hebben wij een risico-stratificatie voor de opsporing van vrouwen met risico op cervicale afwijkingen onderzocht binnen een gerandomiseerde gecontroleerde trial, de VUSABOB. Vrouwen met hoog-gradige laesies werden beter opgespoord door een tweede cytologisch onderzoek toe te voegen 6 maanden na de uitgangscytologie en de uitgangsHPV test dan door alleen op intake een dubbele test te verrichten. Vooral in de groep vrouwen met een positieve HPV test en normale of licht afwijkende cytologie kon een subset van vrouwen worden geïdentificeerd die verhoogd risico op het hebben of ontwikkelen van hooggradige laesies bleken te hebben zoals aangetoond door een sterk afwijkend

herhalingsuitstrijkje. Bij het separaat analyseren van de extra hoog-risico types HPV16 en HPV18 vergeleken met de andere hoog-risico types konden zowel een subgroep geïdentificeerd worden met een extra hoog-risico op de aanwezigheid van hoog-gradige laesies, de vrouwen positief voor HPV16 of HPV18, als een subgroep met een sterk verlaagd risico op hoog-gradige CIN laesies, de vrouwen met een negatieve HPV test. Deze data bevestigen dat hoog-risico HPV testen een unieke mogelijkheid voor het screeningsprogramma biedt om vrouwen met een hoog-risico te selecteren voor meer intensieve screening dan nu in het screeningsprogramma in Nederland geboden wordt.

In de algemene discussie (**HOOFDSTUK 7**) worden de belangrijkste bevindingen van dit onderzoek beschouwd in het kader van de huidige stand van zaken in de wetenschap en het lopende onderzoek op het gebied van baarmoederhalskankerscreening. We concluderen dat de toevoeging van HPV testen aan het bevolkingsonderzoek naar baarmoederhalskanker zal leiden tot een toegenomen opsporing van hoog-gradige laesies en baarmoederhalskanker in deelnemers aan screening. Een type-specifieke test kan een verdere selectie van vrouwen met hetzij een verhoogd hetzij een verlaagd risico op laesies mogelijk maken.

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Curriculum vitae

Saskia Bulk werd geboren op 29 april 1974 te Amstelveen. Na de middelbare school (Vossius Gymnasium te Amsterdam) haalde zij eerst haar propedeuse scheikunde alvorens in 1993 te beginnen aan de studie Geneeskunde aan de Universiteit van Amsterdam. Aldaar deed zij haar wetenschappelijke stage naar de diagnose van de ziekte van Creutzfeldt-Jakob door middel van het aantonen van het 14-3-3 eiwit in liquor onder leiding van professor Pim van Gool (afdeling neurologie, Academisch Medisch Centrum te Amsterdam). Tijdens de co-schappen liep zij nog een extra onderzoeksstage bij dr. Willem Hamersma naar de relatie van overgewicht op de ademarbeid (afdeling longfunctie, Onze Lieve Vrouwe Gasthuis te Amsterdam). Na het *cum laude* behalen van haar artsexamen in 2000 volgde zij de *Masters of Science* opleiding in *Genetic Epidemiology* aan het Nihes in Rotterdam, alvorens zij in 2001 het in dit proefschrift beschreven onderzoek startte.

Sinds januari 2008 werkt zij als arts-assistent op de afdeling Medische Genetica van het Universitair Medisch Centrum Utrecht. Sinds 1 september is zij in opleiding tot klinisch geneticus (opleider professor Dick Lindhout).

Saskia Bulk woont in Bussum met haar man Marko, zoon Baruch en hond Luca.

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