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Innovative targets for diagnostics and therapy in cervical neoplasia

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Nathalie Reesink-Peters

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door

Nathalie Reesink-Peters

geboren op 24 februari 1972
te Sleen

Misschien is niets geheel waar,
en zelfs dàt niet

(Multatuli)

Aan Gerard

CONTENTS

General introduction

ABSTRACT

In chapter 1 an introduction to the thesis is given, by discussing the epidemiologic features of cervical cancer; the classification and treatment of preneoplastic CIN lesions, the development and functioning of cervical cancer screening programs, and the staging and treatment of cervical cancer. Furthermore the outline of this thesis is presented by describing the background and aims of the different chapters.

GENERAL INTRODUCTION

Cervical cancer is an important cause of death in women worldwide. An estimated number of 370,000 – 470,000 new cases occur per year, and 230,000 women die of cervical cancer per year.[1-3] It is the second to third most common cancer in women with large incidence differences between developed countries with cervical cancer screening programs and less developed countries. The cumulative lifetime risk for a woman to develop cervical cancer varies from 0.4% in Israel to 5.3% in Colombia.[4] Infection with high-risk human papillomavirus (HPV) has been identified as the main causative factor in cervical carcinogenesis.[5] The prevalence of high-risk HPV infection is much more common than the prevalence of cervical cancer, indicating that although HPV infection is a necessary cause, it is not a sufficient cause for full cervical carcinogenesis. Additional factors, both viral and host-cell-related factors are required.

THE EPITHELIAL SURFACE OF THE UTERINE CERVIX

The uterine cervix is covered with non-keratinizing squamous epithelium mainly at the ecto-cervix and glandular columnar epithelium at the endo-cervix. The border between the two different epithelial cell types is called the squamo-columnar junction (SCJ). Metaplastic transformation in the SCJ starts from puberty onwards and columnar epithelium is replaced by squamous epithelium, shifting the original SCJ towards the ecto-cervix, forming a neo-SCJ. The area between the original and neo-SCJ is called the transformation zone. It is assumed that the transformation zone is most susceptible to oncogenic influences due to the local high cell-turnover.[6]

CERVICAL INTRAEPITHELIAL NEOPLASIA: CLASSIFICATION AND SCREENING

Richart suggested in the late 1960's that cervical cancer develops from non-invasive cervical cancer precursor lesions, called cervical intraepithelial neoplasia (CIN).[7] CIN lesions have been subdivided according to the severity of dysplasia. CIN I describes mild dysplasia, restricted to the basal one-third of the epithelium, CIN II describes moderate dysplasia, restricted to the basal two-third of the epithelium and CIN III describes severe dysplasia, dysplasia in the full thickness of the squamous cervical epithelium.[8] The risk of developing cervical cancer has been estimated to rise with increasing severity of the CIN lesion. Approximately 1% of patients with CIN I and 5-45% of the CIN II/III lesions will progress to cervical cancer when the CIN lesion is left untreated. It is estimated that the progression from CIN to cervical cancer generally takes 10-15 years.[9,10]

Papanicolaou and Traut were among the first to demonstrate that morphologic review of exfoliated cells could be used to detect carcinoma in situ and invasive cancer of the cervix.[11] Based on the assumption that treatment of precancerous lesions would prevent the development of cervical cancer and that early detection of cervical cancer would positively influence survival, many countries started to organize nation-wide screening programs. Nowadays cytomorphological examination of cervical smears is the most widely applied screening-method for cervical cancer and its precursors. The observed

decline in the number of cervical cancer related deaths in the last decades has generally been attributed to the implementation of such screening programs.[12,13] Cervical smears are classified according to a modified Papanicolaou system (CISOE-A) or the Bethesda classification system. In table 1 an overview is given of the different nomenclatures used to describe cervical cytological and histological abnormalities of the squamous epithelium.

Table 1: Cytolomorphological and histolmorphological nomenclature

Dysplasia	CIN	Bethesda	Papanicolaou
Normal	Normal	Within normal limits	Pap 1
Benign atypia	Inflammatory atypia	Benign cellular changes	Pap 1
Atypical cells	Squamous atypia	ASCUS	Pap 2
Mild dysplasia*	CIN I	Low-grade SIL	Pap 3A1
Moderate dysplasia	CIN II	High-grade SIL	Pap 3A2
Severe dysplasia	CIN III	High-grade SIL	Pap 3B
Carcinoma in situ	CIN III	High-grade SIL	Pap 4
(microinvasive) cancer	(microinvasive) cancer	(microinvasive) cancer	Pap 5

CIN   Cervical intraepithelial neoplasia

SIL   Squamous intraepithelial lesion

* The term 'dysplasia' is used for a histological diagnosis. In cytological reviews the term dysplasia is replaced by the term 'dyskaryosis'.

TREATMENT OF CERVICAL INTRAEPITHELIAL NEOPLASIA

Although the exact indications for referral differ between countries, in general women with abnormal cytology reports will be referred to a gynecologist for colposcopic examination. CIN lesions and cervical cancer can be visualized after acetic whitening and diagnozed by histological examination of colposcopy-directed biopsies of suspect areas of the uterine cervix. Because no markers exist that identify those CIN lesions that will progress, clinicians have felt compelled to treat at least all CIN II/III lesions. A large part of all women treated for CIN would never actually have developed cervical cancer.[10,12] Treatment options for CIN are: Large loop excision of the transformation zone (LLETZ), laser evaporation, cryocoagulation, cone biopsy or hysterectomy depending on the preference and expertise of the attending gynecologist. In general, LLETZ is preferred because it can be performed in an outpatient setting, the tissue removed can be histo-morphologically reviewed and a LLETZ seems to have no effect on menstruation or fertility.[14] With LLETZ a cure rate of more than 90% can be achieved.[14]

STAGING AND TREATMENT OF CERVICAL CANCER

The main histologic types of cervical cancer are squamous cell carcinoma and adenocarcinoma. Over 80% of all cervical cancers are of squamous cell type. Clinical staging of cervical cancer occurs by the FIGO criteria for which, during a pelvic examination under general anaesthesia, tumour-size and involvement of the vagina and parametrium are estimated.[15] Traditionally, clinical stage is the most prominent

prognostic parameter and therefore determines the choice of treatment modality to a great extent. Stage IA, microinvasive cancer, can be treated by LLETZ or exconisation to maintain fertility or by simple hysterectomy. Stages IB1, IB2 and IIA are considered early stages for which radical hysterectomy (combined with adjuvant radiotherapy when indicated) and primary radiotherapy are equally effective treatments.[16,17] Most gynecologic oncologists prefer radical hysterectomy with pelvic lymphadenectomy for their relative young and healthy low-risk early stage patients because of the shorter treatment course, opportunity for ovarian preservation, and presumed better post-treatment vaginal function.[18] It is generally accepted to treat patients with lymph node metastases, positive resection margins or parametrial involvement with adjuvant radiotherapy.[18,19] Patients with stage IIB-IV disease are usually treated with primary radiotherapy in combination with chemotherapy because of the increase in survival (15%) compared to the treatment with radiotherapy alone.[20-23] The five year relative survival rates for cervical cancer patients vary from approximately 80% for patients with localized disease to 55% for those with lymph node metastases.[24] Survival rates for patients with more advanced disease at diagnosis are considerably worse.[25]

OUTLINE OF THIS THESIS

Important drawbacks of morphology based cervical cancer screening are high numbers of false positives and false negatives.[26-28] Therefore objective methods based on molecular changes in cervical cancer might improve the current screening approach. In **Chapter 2** data on cervical cancer epidemiology, etiology and the current cervical cancer screening approach are summarized. Available data on new molecular based approaches for screening are reviewed in more detail and the potential of these approaches to replace or augment current Pap-smear screening is discussed.

Apart from the high false positive and false negative rates another drawback of cervical cancer screening is the referral of patients with abnormal cervical cytology reports who would never had developed cervical cancer.[10,12] These unnecessary referrals cause unfavorable effects such as high anxiety levels, overtreatment and reduced cost-effectiveness of cervical cancer screening programs.[29-31] **Chapter 3** describes whether HPV, hTERT, hTR DNA detection or telomerase activity assessment in cervical scrapings can identify those patients that harboured CIN II or CIN III within a group of patients with borderline, mild or moderate dyskaryosis.

Although the overall mortality of cervical cancer has been markedly reduced by the widespread availability of effective cytologic screening programs, survival by stage did not change significantly untill radiotherapy with concurrent chemotherapy was introduced for primary and adjuvant therapy.[25,32] Even if we are not able to influence survival we can improve patient care by reducing treatment related morbidity. In **Chapter 4** it was therefore studied whether the known squamous cell cervical cancer serum tumor marker, serum SCC-ag, could be used to identify a group of patients that might preferably be treated with primary radiotherapy in stead of primary radical hysterectomy in order to reduce morbidity associated with double modality treatment.

The development of diagnostic tools starts with knowledge of cervical carcinogenesis. After Zur Hausen in 1973 suggested that viral oncogenesis was involved in the development of cervical cancer our understanding of HPV mediated carcinogenesis has grown.[33][35] Parallel with this increasing understanding thousands of studies have provided insight in the epidemiology of HPV. Although HPV infection is an initiating event for cervical carcinogenesis, other factors need to be involved, because the prevalence of high-risk HPV infection is much more common than the prevalence of cervical cancer. Knowledge of these factors is limited and we therefore explored the role of possible infectious (co)factors in cervical carcinogenesis and reported the results of three epidemiologic studies. In **Chapter 5** the prevalence of, risk factors for, and the impact on histologic changes of bacterial vaginosis was studied in patients with cytological abnormalities of the uterine cervix. **Chapter 6** describes an explorative study on the possible association between the presence of *Chlamydia trachomatis* antibodies and the severity of neoplastic lesions in patients with cervical dyskaryosis. **Chapter 7** describes the last and most important infectious factor studied, HPV. In a previous study using the GP5/6 primers based HPV PCR we observed that the prevalence of HPV rises with increasing histological severity of neoplasia, more cigarettes smoked per day and higher lifetime number of sexual partners in patients with cervical dyskaryosis.[36] Some years after that initial epidemiologic study the highly sensitive SPF10 primers and Inno-LiPA (line probe assay) HPV prototype research assay became available for the detection and typing of HPV. Using the SPF10-LiPA system, the stored cervical scrapings of the earlier described study population were re-analyzed.

Knowledge of the role of infectious cofactors only reflects a small part of our increasing understanding of carcinogenesis in general. Partly due to the development of new technologies, scientific achievements have been made to unravel the molecular pathogenesis of cervical cancer. In **Chapter 8 and 9** translational research on two topics that have been subject of major interest in the last decade is described. It has become increasingly clear that not only mutations occur during cancer development, but epigenetic mutations such as silencing of tumor suppressor- or other cancer-associated genes by methylation of CpG islands, are common features of cancer tissues as well. Hypermethylation of tumor suppressor genes also occurs frequently in cervical cancer. Although these aberrant methylation patterns represent excellent targets for novel diagnostic approaches, it was unknown whether a cervical scraping could reflect the hypermethylation status of the underlying epithelium. It was therefore unclear whether quantitative hypermethylation specific PCR (QMSP) on cervical scrapings could be used as a future screening method augmenting the current approach. In **Chapter 8** the use of QMSP on cervical scrapings was explored by analyzing cervical scrapings and paired fresh frozen cervical tissue samples obtained from cervical cancer patients and controls by QMSP for the genes APC, DAPK, MGMT and GSTP1.
Programmed cell death, or apoptosis, is a genetically controlled mechanism that plays an important role in the regulation of cellular homeostasis.[37] Many triggers can lead to apoptosis induction, including binding of apoptosis-inducing 'death ligands' such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) to their cognate cell-

surface death receptors, Fas and DR4/DR5, respectively.[38] The Fas death receptor-mediated apoptosis pathway has been most extensively investigated. Since cross-linking of Fas by anti-Fas antibodies resulted in apoptosis in certain hematopoietic malignancies[3941] the Fas-mediated apoptosis pathway attracted a lot of attention as a possible target for application for cancer treatment.[3943] Expression of Fas and TRAIL receptors have been reported in many normal (epithelial) cells and neoplastic cells.[42,44-47] Increasing imbalance between proliferation and apoptosis is thought to be important in cervical carcinogenesis. Downregulation of Fas expression is observed in CIN II/III lesions.[48] In cervical cancer DR4 and DR5 downregulation did not occur.[49] No data were available on the expression of TRAIL and its death receptors in different grades of CIN lesions. The aim of the study, described in **Chapter 9,** was to examine the role of FasL and TRAIL and their death receptors in the increasing imbalance between proliferation and apoptosis.

Finally the results of our studies are summarized and some perspectives for future studies are discussed.

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CHAPTER 2

New options for cervical cancer screening: a review

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ABSTRACT

Although current morphology based cervical cancer screening has reduced the incidence of cervical cancer, the Pap-smear is associated with high false positive and false negative rates. These negative aspects have spurred the search for new technologies to improve cervical cancer screening. Preferably, these new technologies should be based on molecular changes associated with cervical carcinogenesis. In this review we will summarize data on cervical cancer epidemiology and etiology and the current cervical cancer screening approach in short. Available data on new molecular based approaches to screening such as quantitative cytochemistry, detection of loss of heterozygosity and tumor suppressor gene promotor hypermethylation will be reviewed in more detail and we discuss the potential of these approaches to replace or augment current Pap-smear screening.

INTRODUCTION

World-wide cervical cancer is an important cause of death. Infection with human papillomavirus (HPV) plays an important role in cervical carcinogenesis. Current cervical cancer screening occurs by morphological assessment of cervical smears, but because of high false positive and false negative rates efforts have been made to improve cervical cancer screening by the use of HPV DNA testing or by using other new molecular markers. In this review we will summarize data on cervical cancer epidemiology and etiology and the current cervical cancer screening approach in short. Available data on molecular diagnostic targets such as microsatellite alterations, telomerase activity and tumor suppressor gene promotor hypermethylation for cervical cancer detection will be reviewed in more detail.

EPIDEMIOLOGY AND ETIOLOGY OF CERVICAL CANCER

The cumulative lifetime risk for a woman to develop cervical cancer varies from 0.4% in Israel to 5.3% in Colombia. Cervical cancer represents the third most frequent gynecological malignancy among women worldwide, with the highest incidence rates in less developed countries.[1] It is estimated that 12,200 new cervical cancer cases will be diagnosed in 2003 in the US and that an estimated 4,100 deaths from cervical cancer will occur (accounting for 1.9% of all cancers in women and 1.5% of all cancer related deaths in women).[2] In sharp contrast to these relatively low incidence numbers of cervical cancer are the high number of surgical treatments each year for premalignant cervical lesions in countries with nation-wide screening programs.[3-5] Cervical cancer develops from these premalignant lesions, also called cervical intraepithelial neoplasia (CIN). The mildest form, CIN I, regresses in most cases, while 20-45% of the CIN II/III lesions will progress to cervical cancer when left untreated. It is estimated that the progression from CIN to cervical cancer generally takes 10-15 years.[6]

Sufficient evidence for a causal role of HPV in cervical carcinogenesis has been provided by both epidemiological as well as experimental studies in different parts of the world. It has been proposed that HPV infection is the first identified necessary cause of cervical cancer, implying that cervical cancer, with very rare exceptions, can not develop without HPV infection.[7] Over 100 HPV types have been identified of which more than 35 types can be found in the genital tract and of which 18 are associated with cervical carcinogenesis.[8] For cervical oncogenesis expression of the viral proteins E6 and E7 is pivotal because E6 and E7 facilitate increased degradation of respectively p53 or pRB proteins, leading to inactivation of two important cellular regulatory proteins. HPV 16 accounts for 50-70% of the cervical cancer cases in most countries. The second most frequent HPV type is HPV 18 (10-12%) followed by HPV 31 and 45.[7,8] Despite of all the evidence for the important role of HPV in cervical carcinogenesis it is clear that additional factors, both viral and host-cell related have to be involved because the majority of patients infected with HPV will not develop invasive cervical cancer.[9]

CURRENT CERVICAL CANCER SCREENING

Cytomorphological examination of cervical smears is the most widely applied screening-method for cervical cancer and its precursors. In 1941 it became clear that cytomorphological assessment of cervical smears could be used to detect cervical cancer and its precursors and many countries started to organize screening programs.[10] Cervical smears are classified according to a modified Papanicolaou system (Pap/CISOE-A) or the Bethesda classification system. An overview of the different nomenclature used in cervical cytomorphology and histomorphology is given in Table 1.

Table 1: Cytolomorphological and histolmorphological nomenclature

Dysplasia	CIN	Bethesda	Papanicolaou
Normal	Normal	Within normal limits	Pap 1
Benign atypia	Inflammatory atypia	Benign cellular changes	Pap 1
Atypical cells	Squamous atypia	ASCUS	Pap 2
Mild dysplasia	CIN I	Low-grade SIL	Pap 3A1
Moderate dysplasia	CIN II	High-grade SIL	Pap 3A2
Severe dysplasia	CIN III	High-grade SIL	Pap 3B
Carcinoma in situ	CIN III	High-grade SIL	Pap 4
(microinvasive) cancer	(microinvasive) cancer	(microinvasive) cancer	Pap 5

ASCUS	atypical suamous cells of undetermined significance
CIN	cervical intraepithelial neoplasia
SIL	squamous intraepithelial lesion

Although the introduction of nation-wide screening programs have lead to decreasing incidences of cervical cancer it has been questioned whether the disadvantages counter-balance the relatively low reduction in cervical cancer deaths.[11] Disadvantages include the high numbers of false-positive and false-negative cervical smears, leading to an overshoot of diagnostic procedures or even a delay in the diagnosis of cervical cancer.[4,5,12] Up to 14 % of all cervical smears are cytomorphologically abnormal without the presence of a (pre)malignant cervical lesion.[13] These false-positive results can cause unnecessary anxiety and invasive procedures. False-negative cytology may be found in about 50% of cases when previous negative smears are reviewed from the small proportion of screened women who develop invasive cancer.[14,15] High-grade CIN or microinvasive cervical cancer has cure rates close to 100% with appropriate treatment. False-negative screening results will leave CIN or cancer undetected. Even when symptoms start to occur, the falsely given assurance by a false negative smear may lead to a further delay in the diagnosis and treatment of the cancer which will negatively influence curation chances.

Women can be emabarrassed to undergo a vaginal examination, which leads to low attandance rates. Low attandance to the cervical cancer screening programs is another major drawback of cervical cancer screening. It has been investigated wether self-sampling can overcome this problem and wether it can be a reliable alternative for cervical cancer screening on physician-collected samples, because half of cervical

cancer cases arise in women who are not adequately screened.[16,17] Preference for self-sampling has been reported.[18,19] However, although feasible, patient-collection seems an inferior alternative to physician-collected cervical cytology.[18 20] Possibly, for newly developed options for cervical cancer screening self-collected cervico-vaginal samples may be less inferior alternatives for physician-collected samples than for cytology-based-methods. The possible use of self-collected samples would improve the current screening approach.

TECHNICAL IMPROVEMENTS FOR MORPHOLOGICAL SCREENING

AUTOMATED PAP-SMEAR ANALYSIS

For automated slide microscopy digital photographs are taken of ordinary Papanicolaou-stained cervical smears. The data thus obtained are documented and images can be analyzed by computer systems such as done by the neural-network-based approach and by the AutoPap system.[21 23] These automated methods have the potential to improve the accuracy of cervical cytologic examinations as well as to make a positive impact on the productivity of cytology laboratory personnel.[23] For the detection of high-grade cervical neoplasia (CIN II/III or HSIL and cervical cancer) in a primary screening setting, both systems show slightly improved specificity and equivalent sensitivity to the Pap test.[21 23] Confortini *et al.* therefore stated that comparison of the AutoPap system and conventional reading should focus mainly on cost analysis.[22] The current expense of the automated technologies in comparison to conventional screening limit their widespread implementation.[24] At present it is not clear whether the potential benefits of computerized screening relative to conventional Pap test are sufficient to justify a possible increase in costs.[25 26] However, in the future widespread implementation of automated Pap-smear may become cost-effective since costs of manpower will further increase, whereas automated technologies in general become less expensive in time.

LIQUID BASED CYTOLOGY

For liquid based cytology, also called thin-layer test or ThinPrep Pap test, cervical cells are collected by scraping the cervix with a sampling device after which the sampling device is transferred directly to a vial of liquid preservative. Collected cells are mechanically dispersed into the liquid medium before a representative aliquot is transferred to the slide. Possible advantages of this procedure compared to conventional Pap-smear testing are: prevention of inadequate air drying of cells after sampling; debris and blood are removed before cell transfer to slides and cellular separation is enhanced; several slides can be tested from one sample and the residual fluid can be used for a variety of molecular analyses.

Two studies compared conventional Pap-smear classification with classifications obtained by liquid based cytology and showed 85-87% and 97% correspondence within one diagnostic category, respectively.[27 28] When 8,636 Costa Rica women were tested, specificity for histological CIN II or worse was 99% for both conventional and liquid based cytology. Sensitivity of smears showing at least moderate dyskaryosis for histological CIN II or worse was 64% for conventional cytology and 69% for liquid based cytology.[29] In a multicentre screening study liquid based cytology showed a 39% reduction in unsatisfactory slides and a 44% reduction in limited reports. A randomized trial in 1,999 women showed that the quality of conventional Pap-smear sampled after removal of mucus and cellular debris with a cellulose swab was better than of liquid based cytology.[30] The correlation between cytologic and histological diagnoses was also

better for 'conventional' Pap-smears than for liquid based cytology. However, most studies report that liquid based cytology provides more satisfactory results and has slightly higher sensitivity for high-grade cervical neoplasia compared to conventional Pap-smears.[27,2831-37] The use of liquid based cytology is associated with increasing costs of cervical cancer screening because the average cost of a thin-layer test is $2.4 higher than that of the conventional smear test.[38] The higher price of liquid based cytology is only justifiable if this screening technique outperforms the conventional method.[38] Current evidence appears to be insufficient to recommend for or against the routine use of liquid-based cytology especially because it is unclear whether its use will reduce the incidence of and mortality from invasive cervical cancer despite the reported increase in sensitivity.[25,26] The American Cancer Society reports in its guideline for the early detection of cervical neoplasia and cancer that cervical screening using liquid-based cytology may be an alternative to conventional screening.[39] In our opinion efforts should be made to develop a less expensive way to perform liquid-based cytology by comparing the commercially available cell preservatives with alternative preservatives, for example ethanol-carbowax (7% polyethylene-glycol, 50% ethanol). When the average costs of a thin-layer test would be comparable to the average costs of a conventional Pap-smear test the integration of liquid based cytology in existing screening programs would be justifiable. We can then benefit from the better sensitivity and improved number of satisfactory results. At the same time the residual fluid can be used to further develop and evaluate new screening concepts and technologies based on the presence of molecular markers present in cervical cancer.

Improvement of specificity of cervical cancer screening would lead to the reduction of costs associated with false positive results. Improvement of sensitivity could result in a higher detection percentage of premalignant or early stage malignant cervical lesions. Early detected cervical cancer can be treated more effectively than advanced stages of disease. In the following parts of this review we describe whether molecular markers may be able to improve cervical cancer screening.

STAINING OF MOLECULAR MARKERS SUPERIMPOSED OVER THE CONVENTIONAL PAP-SMEAR

QUANTITATIVE CYTOCHEMISTRY
IMMUNOCYTOCHEMICAL STAINING OF MARKERS
OF CELL PROLIFERATION

The first markers to be discussed are the ones that can be used superimposed over the conventional Pap-smear. These markers exploit the advantage of both Pap-smear analysis and the molecular identification of dysplastic cells. Dysplastic cells can be recognized by their increased persistence in cell cycle compared with normal epithelial cells that exit the cell cycle during maturation and differentiation.[40] Ki-67 antigen is a proliferation marker, which is expressed during late-G1, S and G2M phases of the cell cycle, but not during G0.[41,42] Immunohistochemical staining of Ki-67 antigen by the MIB1 antibody is strongly related to the severity of cervical neoplasia.[43-45] The same phenomenon was observed for immunohistochemical staining of proliferating cell nuclear antigen (PCNA) which is a protein essential for the synthesis of DNA during cell proliferation.[46] PCNA immunocytochemical staining has not been investigated on Pap-smears. Ki-67 staining on Pap-smears can be used to distinguish high-grade CIN lesions from atrophic cervical epithelium.[47-50] Furthermore, Ki-67 staining on cervical cytology was also proposed as a triage tool for patients with minor abnormalities on Pap-smear cytology.[51] However, neither Ki-67 nor PCNA staining have been evaluated for a possible use in primary cervical cancer screening. Both Ki-67 and PCNA staining have been compared with more recently identified markers of cell proliferation.[46] Cdc6 and Mcm5 are proteins assembled into a prereplicative complex that is essential for the initiation of DNA replication. Higher percentages of dysplastic cells in high and low-grade squamous intraepithelial lesions were immunohistochemically stained by both Mcm5 and Cdc6 compared to Ki-67 and PCNA staining.[40] In the same study sensitivity of Mcm5 and Cdc6 immunocytochemical staining on 58 cervical smears taken from patients attending a colposcopy outpatient clinic was higher on direct comparison with conventional Pap-smear analysis. However, these markers of DNA replication may also detect immature phases of squamous metaplasia which can lead to false positive results.[40] The data on Mcm5 and Cdc6 immunocytochemistry need to be further evaluated in larger studies comprising pre-clinical screening populations.

MARKERS FOR CHROMOSOMAL AND DNA ANEUPLOIDY

Other markers of dysplasia to be detected in the same smears as used for morphological Pap-smear analysis are markers to analyze chromosomal and DNA aneuploidy. In cervical smears and tissue samples, aneuploidy is found to be related with the severity of neoplasia using Feulgen-stained image analysis.[52,53] Monsonego and coworkers found aneuploidy to be present in 78% of smears taken from patients with high-grade CIN and in 21% of smears taken from CIN I patients.

Both aneuploidy measurements and immunocytochemistry of Mcm5 and Cdc6 proteins should preferable be used superimposed over conventional Pap-smear analysis, which will lead to the development of a labor intensive and therefore expensive screening approach. New PCR-based detection techniques may be used without concurrent morphological screening and may therefore provide a more clear-cut approach.

DETECTION OF MOLECULAR MARKERS BY PCR-BASED TECHNIQUES

HUMAN PAPILLOMAVIRUS AND CERVICAL CANCER SCREENING

HPV plays an important causative role in cervical carcinogenesis. It is estimated that for women the lifetime risk of contracting a genital HPV infection is 80%, leading to genital warts in 5%, abnormal cervical scrapes in 35%, CIN in 25%, and invasive cervical cancer in less than 1%.[54-57] HPV can be detected in almost all CIN III lesions and cervical cancers and can be detected in cervical smears.[58] It is therefore suggested that the detection of high-risk HPV in cervical smears may well improve cervical cancer screening because of high sensitivity.[59,60] Over 100 studies were undertaken to proof this suggestion right. In general, the sensitivity of high-risk HPV testing by general primer PCR (GP5+/6+; MY 09/11; CP1/2G; SPF10) or Hybrid Capture 2 to detect CIN III and cervical cancer is more than 90%. However, women without cervical dysplasia can also be HPV positive. Therefore, due to low specificity, HPV testing alone will not sufficiently improve cervical cancer screening.[8,13] Still, HPV testing may be used in specified patient groups. The testing of HPV might improve cervical cancer screening in women with smears showing borderline abnormalities (borderline dysplasia or atypical squamous/glandular cells of undetermined significance (ASCUS/AGUS)), because it could prevent a number of referrals. The women with ASCUS who are HPV negative do not need referral because women with ASCUS who are HPV negative very rarely will have CIN III or cervical cancer. In women with ASCUS, HPV testing will identify more patients with high-grade neoplasia compared to repeat cytology. However, a specificity of 59-64% in this group of patients will still lead to more referrals than necessary.[61] HPV testing may also be used together with cytology to define a patient group in which screening intervals can be prolonged. HPV negative women with normal cytology have an extremely low risk to develop cervical cancer in 10 or more years.[7] Another group of patients that may benefit from HPV testing is the group of women aged 35 years or older. In these women HPV testing has a higher specificity because HPV infections are less frequent in this group and when present, infections more often represent HPV persistence.[62] In women with cytological abnormalities HPV persistence is strongly related to the development of CIN III and cervical cancer.[63] Resuming, HPV testing can be used as a diagnostic tool for screening purposes, but it has a relatively low specificity due to frequent HPV infections without dysplasia. HPV DNA testing can best be restricted to a subset of females in which specificity of HPV testing is higher. Currently the definitive evidence of efficacy from long-term follow-up studies and from randomized trials should best be awaited before considering implementation of HPV DNA testing in existing screening programs. Trials are underway that should soon clarify the role of HPV testing for cervical cancer screening.[25,26,64]

The psychological consequence of a positive high-risk HPV test without the presence of CIN III or cancer can be considerable. Regardless of how it is implemented, the incorporation of HPV DNA testing into primary screening will inevitably result in informing millions of women with normal Pap-smears world-wide that they are at

increased risk for cervical cancer. Then, it will be necessary to assure HPV positive women that they do not have to feel unduly alarmed or stigmatized while convincing them of the need for proper follow-up in order to identify those who will actually develop cervical cancer.[65] In light of these considerations, the use of other markers of molecular changes occurring in cervical carcinogenesis should also be investigated. Most of the markers and assays in the following parts of this review have only recently been identified or developed. For most of these new techniques it will be difficult to already estimate possible cost-effectiveness which we will therefore not discuss.

LOSS OF HETEROZYGOSITY, MICROSATELLITE ALTERATIONS

Since HPV infection not always leads to cervical cancer, other genetic alterations must also play a role in tumor-development. Oncogenes and tumor suppressor genes are genes involved in cell growth and regulation. Oncogenes may foster malignant processes if activated, whereas tumor suppressor genes usually will be inactivated during malignant transformation, necessary because of their natural repressive function on malignant processes. Possible mechanisms to down-regulate a tumor suppressor gene are point mutations resulting in aberrant gene products, methylation of the promotor sequences or deletions of (parts of the) tumor suppressor genes. To inactivate tumor suppressor genes located on autosomal chromosomes both alleles need to be inactivated, often by combinations of different mechanisms. For instance, the loss of one of the alleles can be detected quite easily by the analysis of loss of heterozygosity (LOH). To determine LOH, DNA from tumor tissue is compared with normal DNA of the same individual after amplification using PCR based microsatellite analysis.

Microsatellites are short tandem repeats on chromosomes that can be used as markers for a region in which a tumor suppressor gene is suspected. Most microsatellite PCR markers are highly polymorphic and can therefore distinguish both alleles. In case of a deletion, the intensity of one of the alleles in the tumor sample will decrease, referred to as allelic imbalance and in this particular case LOH. In cervical neoplasia. using tissue samples, frequent LOH has been found on several chromosome arms, including 3p, 4p, 4 q, 5p, 6p, 9p, 11p, 11q and 17p.[66] The frequency of LOH in premalignant lesions increases with severity.[67] Using Pap-smear DNA and 9 markers, microsatellite alterations (LOH or microsatellite instability) were present in 85% of 13 smears taken from patients with cervical cancer[68]. Due to the necessary comparison between tumor and normal DNA, LOH analysis requires large quantities of cancer-cell DNA. In most cases 50% of the total DNA in the sample has to be tumor-derived to reliably detect LOH which makes cervical scrapings unsuitable for cervical cancer detection by LOH analysis.[69]

The short tandem repeats of microsatelites might be prone to alteration as the result of loss of mismatch repair function. Microsatellite instability (MSI) can be detected when several changes in the tandem repeat sequence and length of microsatellites are present. MSI is easier to detect than LOH. Approximately one microsatellite instable cell among 200 normal cells can be detected.[70] Despite the fact that improved techniques

have reduced the time required, MSI analysis is still difficult to carry out on a large number of clinical samples for a reliable detection of cancer.[71] Furthermore, MSI is only present in a subset of cervical cancers. Using five markers, MSI was only detected in 29% of 93 squamous cell cervical cancer samples.[72] Although LOH and MSI analyses are not suitable as high-through-put techniques yet, these analyses are useful to identify future markers of cervical cancer with diagnostic and prognostic value.

TELOMERASE ACTIVITY

Telomerase is a nuclear enzyme that is able to synthesize the short stretches of repeat nucleotides that are lost from telomeric ends of chromosomes with each round of replication. Studies in cancer cell lines, as well as in human tumors showed that, in contrast to normal somatic cells, the vast majority of malignant cells (>90%) are characterized by increased telomerase activity.[73,74] Therefore, determination of telomerase activity was suggested for early cancer detection. Telomerase activity is associated with severity of cervical neoplasia.[75-78] The percentage of telomerase activity in cervical cancer tissue varies from 86% to 97%.[74,75,79,80] However, in cervical scrapings positivity for telomerase activity was found in only 31% of cervical cancer patients.[75] For CIN lesions percentages of telomerase activity are reported between 0 and 68%.[74,75,80-82] Ngan *et al.* reported that telomerase activity assessment was unable to improve the detection of high-grade CIN in a study population comprising 86 women with normal cytology and 114 women with abnormal cervical smears.[83] Testing for both telomerase activity and the telomerase components hTR and hTERT in cervical scrapings led to the conclusion that detection of telomerase activity and components are not suitable for the detection of CIN II or more severe lesions in women with cytological borderline, mild or moderate dyskaryosis. Furthermore, the combined sensitivity and specificity of these tests were too poor to suggest a role in primary screening.[82]

DNA METHYLATION

Some 30 years ago it became clear that, as a 'fifth base', methylcytosine is formed postreplicatively in DNA by addition of a methyl group to a cytosine already incorporated into previously synthesized DNA.[84] Methylation forms a modification of DNA and is referred to as an epigenetic change since it does not alter the primary DNA sequence. The function of DNA methylation may be a contribution to overall genetic stability and maintenance of chromosomal integrity and to facilitate organization of the genome into active and inactive regions with respect to gene transcription.[85,86]

Genes with promoter region CpG islands are virtually always unmethylated in normal tissues. Exceptions are inactivated genes on the female X-chromosome and the inactivated allele for selected imprinted genes on autosomal chromosomes. For the past 15 to 20 years, abnormal patterns of DNA methylation have been recognized as molecular changes in neoplasia. Areas of hypermethylation involve CpG islands in 5' regulatory regions of genes and these represent 'epimutations' in which genes are silenced. Hypermethylation of tumor suppressor genes contributes to an immortalized

phenotype by silencing expression of genes responsible for control of normal cell differentiation and/or inhibition of cell growth.

It has been shown that promotor hypermethylation of at least one of the genes *p16, DAPK, MGMT, APC, HIC-1,* and *E-cadherin* was present in 79% of 53 cervical cancer tissues and in none of 24 normal cervical tissues.[87] Furthermore, aberrant methylation of at least one of the genes *p16, RARß, FHIT, GSTP1, MGMT and hMLH1* was detected in 14 of 19 cervical cancer tissue samples; in 12 of 17 high-grade CIN samples; in 11 of 37 women with no dysplasia or with CIN I and in none of 22 negative controls.[88] All these experiments were carried out using conventional methylation specific PCR (MSP) which takes advantage of the sequence differences resulting from bisulfite modification by designing PCR primers in CpG islands to distinguish methylated from unmethylated DNA. An advancement of conventional MSP is real-time quantitative methylation specific PCR (QMSP) which permits reliable quantification of methylated DNA after adjusting for DNA input. QMSP is more sensitive than conventional PCR and can therefore detect aberrant methylation patterns in human samples with substantial (1:10.000) contamination of normal DNA,[89] which is important for the use of cervical scrapings. Moreover, QMSP is amenable to high-throughput techniques allowing the analysis of close to 400 samples in less than 2 hours without requirement for gel-electrophoresis, which can facilitate the implementation of QMSP in nation-wide screening programs. It has been demonstrated by QMSP analysis that cervical scrapings can reflect the hypermethylation status of the underlying cervical epithelium well and 32 of 48 (67%) cervical cancer patients could be identified by analyzing cervical scrapings for *APC, DAPK, GSTP1* and *MGMT* hypermethylation.[90] However, a lot of developmental work has yet to be performed. First, it will be necessary to identify other genes more specifically methylated in (cervical) cancer to compose a sensitive and specific cervical cancer hypermethylation panel. The candidate tumor suppressor gene 'tumor suppressor in lung cancer 1' *(TSLC1)* and the TRAIL decoy receptor *DcR1* may be interesting candidates. *TSLC1* hypermethylation was present in 59% of 49 cancer tissues, 35% of 20 high-grade CIN lesions and in none of 11 low-grade CIN lesions.[91] Hypermethylation of DcR1 was present in 100% of 50 cervical cancer tissue samples of unknown tumor stage and in 2 of 112 normal tissues of different origin.[92] As soon as a cervical cancer sensitive and specific panel has been identified, the use of QMSP on cervical scrapings has to be established in a large preclinical population.

CONCLUSION AND FUTURE PERSPECTIVES

Current morphology based cervical cancer screening is associated with significant false positive and false negative results. In this review we show that to date no other diagnostic tools are available with proven cost-effectiveness to replace or augment current Pap-smear screening. HPV DNA detection stands closest to implementation in nation-wide screening programs of all markers reviewed. However, low specificity for (progressive) high-grade CIN and cervical cancer and negative psychologic effects of knowledge of HPV positivity are important drawbacks. Even when the trials that are underway would show cost-effectiveness of implementing HPV DNA testing in cervical cancer screening new markers and technologies could theoretically still lead to major improvements. Implementation of liquid based cytology would by itself slightly improve Pap-smear screening. Perhaps more importantly it would aid the development of new technologies which can be tested in residual fluid and the results thus obtained can be compared with the paired cytologic classification.

In our opinion, of the molecular markers and molecular-changes-based technologies, two techniques especially shoul be further evaluated. Immunocytochemical staining of cervical smears by Cdc6 and Mcm5 will be the fastest option. Apart from further evaluation, QMSP will also need further development. In comparsion to morphology based Pap-smear analysis and immunocytochemical staining QMSP has three major theoretical advantages: 1) clear-cut results after the definition of cervical cancer cut-off levels; 2) QMSP is amenable to high-throughput analysis, resulting in cost-effectiveness and 3) promise of high sensitivity combined with high specificity.

Primary prevention of cervical cancer by effective prophylactic HPV vaccines may be possible in the near future which will further challenge cervical cancer screening.[54] If implemented, HPV vaccination will lower the prevalence of cervical cancer and its precursor lesions. Positive screening results will thereby be more likely to represent false positives. Whenever HPV vaccination will be implemented nation-wide it will be only acceptable to screen for residual cervical cancer if we have highly specific screening tools at our disposal. Morphology based Pap-smear analysis does not meet those criteria. It therefore seems to be of eminent importance that the search for and development of new cervical cancer screening approaches are continued at present, now that cervical cancer prevalence is still high.

New screening options

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Detection of telomerase, its components and human papillomavirus in cervical scrapings as a tool for triage in women with cervical dyskaryosis

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ABSTRACT

AIM

To examine whether detection of either telomerase and its components, or high risk human papillomavirus (HPV) are of value in predicting the presence of cervical intraepithelial neoplasia grade II/III in women referred because of cervical cytology reports showing at most moderate dyskaryosis.

METHODS

Cervical smears of 50 women referred with cytological borderline, mild or moderate dyskaryosis were analyzed. Telomerase activity was assessed by a commercially available TRAP-assay and its components human telomerase RNA (hTR) and human telomerase reverse transcriptase (hTERT) by reverse transcriptase-PCR. HPV was detected by GP5+/6+ PCR enzyme immunosassay. Histological findings on colposcopy guided biopsies or excised cervical tissue were regarded as pathological final diagnosis. Sensitivity and specificity for detecting CIN II/III were calculated.

RESULTS

Twenty-eight women (46%) were diagnosed with CIN II/III. In the women diagnosed with CIN II/III telomerase activity was detected in none, hTR in 88%, hTERT in 23% and high risk HPV in 79%. As a diagnostic test none of the described analyses combined a sensitivity of at least 90% with a specificity $\geq$ 90%. Despite the small numbers, calculation of the 95% confidence intervals excluded a combined sensitivity and specificity of at least 90% for any of the evaluated parameters.

CONCLUSIONS

Neither detection of telomerase or its components, nor detection of high risk HPV seem suitable for the triage of women with borderline, mild and moderate cytological dyskaryosis.

INTRODUCTION

Cervical cancer is an important cause of death in women world wide, which develops from cervical intraepithelial neoplasia (CIN).[1] A CIN-lesion can either regress, persist or progress towards (micro)invasive carcinoma. Most of the low grade CIN lesions (CIN I) will regress, while in the long term 12% to 40% of high grade CIN lesions (CIN II/III) will progress to squamous cell carcinoma.[2,3] Because no markers exist that identify those lesions that will progress, clinicians have felt compelled to treat at least all CIN II/III lesions.

Cytomorphologic examination of cervical smears is the most widely applied screening-method for cervical cancer and its precursors. Disadvantages are high numbers of false-negative and false-positive cervical smears. Cervical cytology alone is a good predictor for the presence of CIN II/III when it shows severe dyskaryosis or carcinoma in situ. In women with these severely abnormal smears CIN II/III or cancer was found in 89-93% of women.[4,5] In contrast, 51-58% of women with cytological mild or moderate dyskaryosis is diagnosed with CIN II/III.[5,6] All of the women with cytological mild or moderate dyskaryosis are subjected to colposcopic evaluation, implying an overshoot of diagnostic procedures.[7,8] Therefore parameters in cervical scrapings are needed which more accurately predict the presence of CIN II/III or cancer in women with borderline, mild or moderate dyskaryotic smears.

Although it has been suggested that high risk HPV testing may well improve cervical cancer screening,[9,10] its role in the triage of women with cytological borderline, mild or moderate dysplasia is less clear.

Another possible parameter that is reported on is telomerase activity assessment by PCR-based telomere repeat amplification protocol (TRAP) assay.[11,12] Telomerase is an enzyme that replenishes short stretches of repeat nucleotides lost from telomeric ends of chromosomes with each round of replication. Studies in tumour cell lines, as well as in human tumor specimens have shown that, in contrast to normal somatic cells, the vast majority of malignant cells (>90%) are characterized by increased telomerase activity.[11,13] Therefore determination of telomerase activity has been suggested for early cancer detection. In a previous study of our group it was shown that although telomerase activity was associated with severity of cervical neoplasia, it was only detected in 27% of scrapings from women with CIN II/III and cervical cancer.[14] In contrast, Reddy et al. detected telomerase activity in 96.5% of cervical cancer samples and in 68.7% of premalignant cervical scrapings.[11] In a previous study of our group and in the study of Reddy *et al.* telomerase activity was detected by a non-commercially available TRAP-assay.

Low sensitivity has been found for telomerase activity assessment in urine and screening for bladder cancer. However, when detection of the human telomerase RNA component (hTR), was performed, sensitivity for detecting bladder cancer increased.[15]

In another study hTR detected by in situ hybridization in frozen cervix samples was related to grade of CIN.[16] We therefore speculate that detection of hTR in cervical scrapings might be a more sensitive alternative than telomerase activity assessment in screening for CIN II/III. Another option could be the detection of human telomerase reverse transcriptase mRNA (hTERT) in cervical scrapings. hTERT is the catalytic subunit of telomerase which is thought to be the rate-limiting component in the formation of functional telomerase.[17 20]

Aim of the present study was to examine whether the detection of telomerase activity hTR, hTERT and HPV in cervical scrapings has clinical value in the triage of women referred because of cytological borderline, mild or moderate dyskaryosis.

PATIENTS AND METHODS

PATIENTS

Patients were recruited from the outpatient clinic of the Department of Gynecology, University Hospital Groningen. Eligible for participation in the study were all patients referred by their general practitioner in the period May 1999 - August 2000 because of a cervical cytology report showing two times a borderline or mild dyskaryosis or with a single moderately dyskaryotic smear. In the Netherlands cervical smears are classified according to a modified Papanicolaou system in which no ASCUS diagnosis exists.[21] Instead a cervical smear can be classified as borderline dyskaryotic, but the two terms do not necessarily describe identical abnormalities. Exclusion criteria were previous colposcopic examination because of an abnormal cervical cytology report and pregnancy at the time of the diagnostic or therapeutic procedure.

All patients were asked to participate in the study during their initial visit to the outpatient clinic. All specimens used for the study were collected during the initial visit, before diagnostic procedures. All patients underwent colposcopically directed biopsies. CIN was diagnosed and graded according to international criteria.[22] Only if CIN II or III was diagnosed, the transformation zone was excised 4 to 6 weeks later by diathermic loop excision. Cervical neoplasia was classified according to the most severe lesion found on histological examination of biopsy and loop excision specimen and categorized in low grade lesions for no dysplasia (CIN 0) and CIN I and high grade lesions for CIN II and III. This categorization corresponds well with the classification of low grade and high grade squamous intraepithelial lesions, which is also widely used. The study-protocol was approved by the medical ethical committee of the University Hospital Groningen. All patients gave informed consent.

CERVICAL SCRAPINGS

The cervix of all eligible women was scraped with the blunt or pointed end of an Ayre's spatula and with an endocervical brush. The scraped cells were suspended in 5 mL ice-cold phosphate buffered saline (PBS: 6.4 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 M NaCl; 2.7 mM KCl (pH 7.2)) and kept on ice until further processing. Of this cell suspension 4 mL was centrifuged and washed with wash buffer (10 mM HEPES-KOH (pH=7.5); 1.5 mM $MgCl_2$; 10 mM KCl; 1 mM dithiothreitol) (as described previously),[14] after which half of the pellet was snap frozen in liquid nitrogen and then stored at −80 °C until further use for telomerase activity assessment with TRAP assay. The other half of the pellet was suspended in 500 μL guanidine isothiocyanate (GT) buffer, quickly frozen in liquid nitrogen and stored at −80 ºC until further use for rt-PCR.

TRAP ASSAY

The TRAP assay was performed with TRAPeze XL Telomerase Detection Kit (Intergen Company, Purchase NY, USA) in accordance with the manufacturers' directions for use. Lysis of the pellet was performed as described previously.[16] All samples were tested in duplicate and average results were categorized as positive when peaks represented

≥ 10 GLC4 (a human small-cell lung cancer cell line) cell equivalents (quantification comparable to the normalized fluorescence of 10 GLC4 cell equivalents is 10 U/µg protein).

HPV ANALYSIS

DNA was prepared from the pellets of scrapings, obtained after extraction of proteins with TRAP lysis buffer using the GT-diatom procedure and dissolved in 100 µL of 10 mM tris/HCI, 1mM EDTA (pH 7.0). This procedure did not affect the HPV-PCR results of cervical carcinoma cell lines with known HPV-status: Hela S3; Caski; SiHa and C33a (results not further shown). The GP5+/6+ PCR enzyme immunoassay for high risk HPV detection was performed as described previously.[23]

REVERSE TRANSCRIPTASE PCR FOR HTR AND HTERT

Isolation of RNA and rt-PCR was performed as described previously by Wisman *et al.*[16] PCR was performed separately for hTERT mRNA (35 cycles), hTR (35 cycles) and a housekeeping mRNA, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH)(30 cycles). GAPDH, hTR and hTERT levels in 1 µg total RNA of GLC4 cells were set at 100%. Expression levels in the cervical samples were relatively expressed to the expression levels in GLC4, after which the expression levels were normalized to the house-keeping gene GAPDH. Expression levels of hTR and hTERT were categorized as follows: no expression = negative; expression <10% of 1 µg total GLC4 RNA = very low; expression between 10% and 75% GLC4 = low; expression between 75% and 200% GLC4 = moderate and expression between 200% and 1000% GLC4 = high.

STATISTICS

To test for differences in expression levels of hTR and hTERT in women with no dysplasia/CIN I and CIN II/III the Mann-Whitney-U test was used. Independent associations of HPV and age with the two diagnostic categories were estimated using a multiple logistic regression model. A difference associated with a p-value ≤ 0.05 was considered to be significant. Diagnostic-test characteristics were calculated by using the proportion of women with CIN 0/I and CIN II/III. The 95% confidence intervals were calculated using the CIA (©Gardner & British Medical Journal. London, GB) software. All other analyses were performed using the statistical analysis program SPSS (©SPSS Inc. Chicago, IL).

RESULTS

In the period May 1999 - August 2000, 50 consecutive patients participated in the study. The median age was 37 years (Inter Quartile range (IQ-range) 31-45). Of the 50 women 22 (44%) were diagnosed with CIN 0/I (no dysplasia n=11, CIN I n=11) and 28 (56%) with CIN II/III (CIN II: n=17, CIN III: n=11). The median age of women with CIN 0/I was 43.5 (IQ-range 34.8 - 48.8) and of women with CIN II/III 35.5 (IQ-range 30-42.5) (p=0.01). The association between age and the diagnostic categories remained borderline significant (p=0.065) when adjusted for the presence of HPV. Table 1 shows the final histological diagnosis in relation to the cytology results of the cervical smear at referral.

Table 1: Histological diagnosis related to cytology results of cervical smears at referral

		Histology	
Cytological dysplasia	N	CIN 0/I	CIN II/III
Borderline	4	4	
Mild	24	12	12
Moderate	22	6	16
Total	50	22	28

In Table 2 the proportion of women with CIN 0/I or CIN II/III positive for telomerase components or high risk HPV is shown. All scrapings could be analyzed for the presence of telomerase activity and HPV, but the quality of three cervical scrapings was too poor to analyse with rt-PCR for hTR and hTERT.

Table 2: Telomerase components and HPV in cervical scrapings of women referred for a borderline, mild or moderate dyskaryotic smear.

	Histology	
	CIN 0/I N=22	CIN II/III, N=28
Telomerase activity[1]	1 (4.5%)	0
hTR expression	22 (100%)	22[2] (88%)
hTERT mRNA expression	4 (18%)	7[2] (28%)
High risk HPV	11 (50%)	22 (79%)

[1] >10U/µg protein

[2] Quality of 3 samples was too poor to analyze

Telomerase activity was only detected once, in a cervical scraping from a woman with CIN 0/I. Because hTR was detected in 44 of 47 scrapings we analyzed whether the frequency of low and high hTR expression levels differed between the two diagnostic categories. In women with CIN 0/I, hTR expression was negative in none, weak in 13 (59%), moderate in 6 (27%) and high in 3 (14%). In women with CIN II/III, hTR expression was negative in 3 (12%), weak in 8 (32%), moderate in 11 (44%) and high in 3 (12%) (p=0.74). For hTERT most samples had very low or low expression, without

differences between CIN 0/I and CIN II/III. One sample was found to have moderate expression and one high expression. Both samples were taken from patients with CIN II/III.

Of the 6 women diagnosed with CIN II/III who were high risk HPV negative four were diagnosed with CIN II and two with CIN III. The 33 HPV positive women were younger (median age 35, IQ-range 30 - 43.5) than the 17 HPV negative women (median age 44, IQ-range 33.5-49.5) (p=0.017). The presence of HPV was independently associated with the severity of the histological diagnosis (p=0.023), after adjusting for age.
It was analyzed whether detection of one of the telomerase components or HPV analysis could be used as a diagnostic test by calculating sensitivity, specificity, positive predictive value and negative predictive value for the detection of CIN II/III (Table 3). For none of the analyzed tests a combination of high (90% or higher) sensitivity and high specificity was observed. The 95% confidence intervals excluded a combined sensitivity and specificity of at least 90% for any of the evaluated parameters (Table 3).

Table 3: Test characteristics of telomerase components and HPV when used for the detection of CIN II/III in women referred because of cervical cytology reports showing at most moderate dyskaryosis

| | Test characteristics in % (95% Confidence Interval) | | | |
	Sensitivity	Specificity	PPV[1]	NPV[2]
Telomerase activity [3]	0 (0-12)	95 (77-100)	0	43 (29-58)
hTR expression	88 (69-98)	0 (0-15)	50 (35-65)	0 (0-71)
hTERT mRNA expression	28 (12-49)	78 (60-95)	58 (31-89)	49 (33-67)
High risk HPV	79 (59-92)	50 (28-72)	67 (48-82)	65 (38-86)

1 Positive predictive value

2 Negative predictive value

3 >10U/µg protein

DISCUSSION

In recent literature it is debated whether low grade cervical lesions should be diagnosed and treated.[24] Since CIN I is estimated to progress to cervical cancer in approximately 1% of all cases,[2] immediate treatment of these lesions seems rather drastic. A major drawback of identifying women with CIN 0/I during screening is that women may become worried that they are at risk for cervical cancer development, when in fact the far majority will never develop this disease.[25] In most cervical-screening programs patients are referred for colposcopy when cytological atypia or borderline dyskaryosis is found more than once, or directly when cytological abnormalities are more severe. Using this policy a significant number of women with CIN 0/I are referred and disturbed with time consuming and invasive diagnostic procedures. A diagnostic tool is therefore needed, that can sensitively detect CIN II/III in women with cytological borderline, mild or moderate dyskaryosis, and that also has high specificity so few women are bothered with invasive procedures incorrectly. Such a diagnostic test should best be performed on cervical scrapings, as was done for all of the presented parameters in this article.

In our study CIN II/III was diagnosed in 46% of all patients. Others found 4.7-26% in comparable groups of women.[4,8,26] The high percentage found in our study probably represents the fact that more than half of the patients were referred because of moderate dyskaryosis and only four women were referred because of a borderline dyskaryotic smear. Furthermore, in the Netherlands women are not referred with a single borderline or mildly dyskaryotic smear but only when such an abnormality is found twice, leading to a higher percentage of women diagnosed with CIN II/III. Lanham *et al.* found percentages of histological proven CIN II/III in women referred because of moderate dyskaryotic smears that correspond well with the percentage in which CIN II/III was diagnosed in our population.[4]

At present TRAP assays are commercially available which makes the clinical use of telomerase activity assessment accessible. However, with a commercially available TRAP assay we found only one scraping with telomerase activity, indicating a much too low sensitivity for the detection of CIN II/III to be clinically applied. In a previous study of our group a different (home made[14]) TRAP assay was used, which detected telomerase activity more frequently. Even the sensitivity of the more sensitive home made TRAP assay was too low, because telomerase activity was found in only 7 cervical scrapings of 48 (15%) women with cytological borderline, mild or moderate dyskaryosis with histological proven CIN II/III. In a review of recent literature Nowak[27] showed that studies using cytology specimens of more than 10 women report telomerase activity detection rates of 0-11% in normal cases; 12-31% in low grade lesions; 6-66% in high grade lesions and 31-100% in cervical cancer cases. In the same review detection percentages in frozen tissue samples were reported to be between 0 and 92% for normal tissue; 17-96% for high grade lesions and 82-100% for cervical cancer tissue. These widely varying numbers demonstrate that telomerase activity assessment has a too high variability and a too low sensitivity and specificity for the detection of CIN II/III or cervical cancer to be clinically applied.

We found low specificities in predicting CIN II/III for both hTR (100% positive in CIN 0/I) and hTERT (18% positive in CIN 0/I) analyses in cervical scrapings. Low specificity of hTR assessment for the detection of CIN II/III was also found by others.[4] Snijders et al.[28] found hTERT mRNA expression in 40% of CIN 0/I tissue samples, also demonstrating low specificity. Lanham et al.[4] used cytology specimens for the detection of hTERT and found positivity in only 6 % of CIN 0/I cases and in none of the 40 women with CIN II/III.

It is known that the prevalence of HPV declines with age.[29] Therefore, our finding that HPV negative women were older than HPV positive women, was not surprising. However, we did not expect the women with CIN 0/I to be older than the women with CIN II/III. This difference in age might be explained by the lower percentage of HPV positive women in the CIN 0/I group. The association between age and diagnosis was only borderline significant when adjusted for the presence of HPV. Another explanation for the difference in age between the two diagnostic categories might be that reactive changes in cervical smears, mistaken for cervical dyskaryosis, are more frequent in older women, leading to a high percentage of CIN 0/I in older women.

HPV studies in women with cervical smears showing more than mild dyskaryosis, as presented here, are rare. In a Dutch none intervention study[30] high risk HPV was found in 182/297 (61%) women referred for mild or moderate dyskaryosis. This corresponds well to the 66% of positives found in our study population. HPV testing as a screening tool for women at risk for CIN II/III has been studied extensively in women with cervical cytology showing Atypical Squamous Cells of Undetermined Significance (this corresponds best with borderline dyskaryosis) or mild dyskaryosis. The ALTS trial (Randomized Atypical Squamous Cells of Undetermined Significance/Low-Grade Squamous Intraepithelial Lesion Triage Study) showed that HPV DNA testing represents a promising approach for colposcopy triage of atypical squamous cells of undetermined significance, but not for low-grade squamous intraepithelial lesions.[10] High risk HPV testing in women with cervical cytology showing low-grade squamous intraepithelial lesions had good sensitivity but low specificty for the detection of CIN III. One may think that specificity of high HPV viral load is higher than the specificity of HPV presence alone, because viral load is associated with the severity of the histological diagnosis.[29] However, Sun et al. reported that specificity remains low because high HPV-viral load is also found in 12.5% of women without dysplasia and in 25% of women with low grade lesions.[31] We therefore think that HPV detection is not useful.

We realize that the number of patients in our study is low, leading to broad 95% CI's for the calculated test characteristics (Table 3). However, for all of the presented tests the calculated 95% CI's exclude the possibility for a test to combine 90% sensitivity with 90% specificity. This shows that the chance that one of the tests will prove to be of clinical value in a larger study is rather low. We therefore conclude that other diagnostic tests are needed for the triage of women with cytological borderline, mild or moderate dyskaryosis because high risk HPV detection by PCR and detection of telomerase activity and components are not suitable for this purpose.

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Preoperative serum squamous cell carcinoma antigen (SCC-ag) levels in clinical decision making for patients with early stage cervical cancer.

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ABSTRACT

PURPOSE

In order to prevent increased morbidity associated with double modality treatment early stage cervical cancer patients should only be offered radical surgery when there is a low likelihood for adjuvant radiotherapy. We analyzed whether serum Squamous Cell Carcinoma-antigen (SCC-ag) analysis allows better preoperative identification of a group of patients with a low likelihood for adjuvant radiotherapy than currently used clinical parameters.

PATIENTS AND METHODS

In a historical cohort study, FIGO stage, tumor size and preoperative serum SCC-ag levels, as determined by enzyme immunoassay, were related to the frequency of postoperative indications for adjuvant radiotherapy in 337 surgically treated FIGO stage IB/IIA squamous cell cervical cancer patients.

RESULTS

In patients with normal preoperative SCC-ag 16% of stage IB1 and 29% of stage IB2/IIA had postoperative indications for adjuvant radiotherapy, in contrast to 57% of stage IB1 and 74% of stage IB2/IIA patients with elevated (>1.9 ng/mL) serum SCC-ag (p<0.001). Serum SCC-ag was the only independent predictor for a postoperative indication for radiotherapy (odds ratio 7.1, p<0.001). Furthermore, in stage IB1 patients that did not have indications for adjuvant radiotherapy 15% of patients with elevated preoperative serum SCC-ag levels recurred within 2 years in contrast to 1.8% of patients with normal serum SCC-ag levels (p=0.02).

CONCLUSION

In early stage cervical cancer patients determination of serum SCC-ag levels allows more refined preoperative estimation of the likelihood for adjuvant radiotherapy than current clinical parameters and simultaneously identifies patients at high-risk for recurrence when treated with surgery only. Preoperative serum SCC-ag analysis deserves implementation in clinical decision making.

INTRODUCTION

Cervical cancer is an important cancer related cause of death in women worldwide.[1] Clinical staging of cervical cancer occurs by the criteria of the International Federation of Gynecology and Obstetrics (FIGO) for which, during a pelvic examination under general anesthesia, tumor-size and involvement of the vagina and parametrium are estimated.[2] Traditionally, clinical stage is the most prominent prognostic parameter and therefore determines the choice of treatment modality to a great extent.

The NIH consensus statement of 1997 on cervical cancer reported that radical hysterectomy (combined with adjuvant radiotherapy when indicated) and primary radiotherapy are equally effective treatments for early stage (FIGO IB/IIA) cervical cancer.[3] This consensus opinion was later confirmed by a randomized trial.[4] In daily practice however, most gynecologic oncologists prefer radical hysterectomy with pelvic lymphadenectomy for their relative young and healthy early stage patients because of the shorter treatment course, opportunity for ovarian preservation, and presumed better post-treatment vaginal function.[5]

'Classic' indications for adjuvant radiotherapy such as lymph node metastases; positive resection margins and parametrial involvement, are generally accepted in early stage cervical cancer after radical hysterectomy.[5,6] Using these indications, adjuvant radiotherapy is given to approximately 30% of stage IB/IIA patients.[7] Using both 'classic' and so-called 'intermediate' indications, approximately 38% stage IB1 (tumor < 4 cm) and 72% stage IB2 (tumor > 4 cm) patients will be treated with adjuvant radiotherapy.[2]

When patients receive adjuvant radiotherapy after surgery, treatment related morbidity and costs will increase and benefits of primary surgery alone will disappear.[4,8] Two-modality treatment (radical surgery followed by adjuvant radiotherapy) should be avoided as much as possible[3,5,8] and in some institutions radical surgery is therefore already restricted to stage IB patients with tumors equal to or less than 3 cm, thus reducing the frequency of adjuvant radiotherapy to approximately 12% of the operated patients.[9] In an editorial on treatment choices in early stage cervical cancer, Morris suggested that criteria for selecting patients for radical hysterectomy should be defined such that postoperative adjuvant radiotherapy will be given to a maximum of 10-20% of the patients.[8]

Squamous cell cancer antigen (SCC-ag) is a known serum tumor marker for squamous cell cervical cancer. Most studies reported that elevated preoperative serum SCC-ag is associated with pelvic lymph node metastases and independently from lymph node metastases with worse survival.[10-16] All these (often too small) studies used different single cut-offs for preoperative serum SCC-ag analysis and analyzed predictive values for lymph node metastases only, which is just one of the three 'classic' indications for radiotherapy. Due to these limitations, until now not enough nuances were available to

allow wide spread implementation of pretreatment serum SCC-ag analysis in clinical decision making.

To obtain a better informed choice between primary surgery and primary (chemo)radiotherapy in early stage squamous cell cervical cancer patients, we analyzed in the present study the predictive value of a continuum of preoperative serum SCC-ag levels for either one or a combination of postoperative indications for adjuvant radiotherapy and compared the performance of serum SCC-ag analysis to currently world-wide used clinical parameters, i.e. FIGO stage and tumor-size.

PATIENTS AND METHODS

PATIENTS

We retrospectively analyzed the data of patients primarily treated with radical hysterectomy, because of invasive ($\geq$ 3 mm) squamous cell cervical cancer in the University Hospital of Groningen or in the Academic Medical Centre (Amsterdam) in the period 1991 – 2002. Tumors were classified according to the criteria of Kurman et al.[17] The data of patients who were treated with cold knife exconisation or loop excision before serum sampling were excluded from analysis because a study by Maruo et al. showed that 80% of patients with elevated serum SCC-ag seroconverted within 24 hours after surgical excision of cervical tumors.[18] Follow-up data were completed for all patients of both hospitals till November 2002.

STAGING, TREATMENT AND FOLLOW-UP

Pelvic examination under general anesthesia was performed for staging in accordance with the FIGO criteria.[2] During this procedure lesion size (largest diameter) and tumor spread beyond the cervix were estimated routinely. Tumor size was expressed in cm. Patients with FIGO stages IB and IIA were eligible for surgical treatment which consisted of a class III radical hysterectomy with pelvic lymphadenectomy in both institutions.[19] Exceptions were made for patients with poor general conditions for whom surgery determined an unacceptable risk. When primary radical surgery was abandoned during operation due to obvious extracervical tumor mass, patients were treated with primary radiotherapy. Radiotherapy was combined with chemotherapy (3 cycles of carboplatin (300 mg/m^2, day 1) and 5-FU (600 mg/m^2 , day 2 -5) and from 1-1-2002 with Cisplatin 40 mg/m^2/week for 6 weeks) in the University Hospital of Groningen.

In both hospitals patients received adjuvant radiotherapy when considered to be at high risk for locoregional recurrence. 'Classic' high-risk indications were: one or more pelvic lymph node metastases; close (<2 mm) or positive resection margins and parametrial involvement. Patients considered to be at intermediate risk (with either deep stromal infiltration (> 2/3 of the cervical stroma) and/or massive capillary/lymphatic space involvement (at least 3 different tumor localisations in capillary or lymphatic space to be seen outside of the main tumor mass in at least two slides using a 10x20 enlargement) only received adjuvant radiotherapy in the University Hospital of Groningen. Adjuvant radiotherapy consisted of external radiotherapy (6-10 MV) to the pelvis, using the box-technique, 5 fractions a week of 1.8 Gy to a total dose of 45 Gy.

FOLLOW-UP

Standard follow-up surveillance consisted of a clinical history, physical examination and blood analysis starting 6 weeks after treatment, then every 2 months for the first year, every 3 months in the second year, then every 6 months until the sixth year and yearly thereafter until 10 years after treatment.

SCC-AG ANALYSIS

During the study period it was clinical practice to use serum SCC-ag analysis for the confirmation of complete remission after surgery and for the early detection of recurrences. To serve as a reference, a serum sample was routinely taken at the patients initial visit. SCC-ag was measured by the IMx SCC-ag microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL, USA). Treatment decisions were taken independently of the preoperative SCC-ag value. The standard cut-off value used for serum SCC-ag was >1.9 ng/mL, which is the 99th percentile in a population of 250 healthy women tested at the University Hospital of Groningen.

STATISTICAL ANALYSIS

Differences in the distributions of age, stage and other patient-characteristics were analyzed with the χ^2-test. The ability to preoperatively identify those patients with a 'classic' indication for adjuvant radiotherapy by serum SCC-ag analysis was compared with the performance of tumor-size and FIGO stage by calculating receiver operating characteristic (ROC) curves. Multivariate analysis was performed using the COX-regression model entering serum SCC-ag, tumor-size and stage into the model in a one step analysis. For the ROC curves and regression analysis one cut-off level was chosen for the three variables used: for SCC-ag 1.9 ng/mL; for tumor-size > 4 cm and stage IB versus IIA. For evaluation of serum SCC-ag analysis as a diagnostic tool to preoperatively identify those patients who will receive adjuvant radiotherapy for 'classic' indications, positive and negative predictive values were calculated for the SCC-ag cut-off values 1.0; 2.0; till 10.0 separately. The association between increasing levels of SCC-ag and the chance of receiving adjuvant radiotherapy was evaluated with the χ^2-test for trend. As most locoregional recurrences of early stage cervical cancer occur within 2 years after primary treatment,[20,21] analysis of two year recurrence percentages was performed, using the χ^2-test or Fisher-exact test when appropriate. Differences associated with a p-value ≤ 0.05 were considered significant. All these analyses were performed using SPSS software(version 11, © SPSS Inc. Chicago, IL, USA). 95% confidence intervals for proportions of patients with indications for adjuvant radiotherapy were calculated using the CIA software (© Gardner & British Medical Journal. London, GB).

RESULTS

DATA SELECTION
From 1991 - 2002 a total number of 537 stage IB/IIA squamous cell cervical cancer patients was primarily treated with a radical hysterectomy in either of both hospitals. The data of 200 women were not included in the final analysis because of the following reasons; in 184 patients serum samples were drawn after exconisation or loop excision; in five patients the data of clinical staging were incomplete and 11 patients were not treated according to protocol (6 patients because tumor-spill occurred during surgery; 2 patients could not be radically operated due to severe prolaps and 3 patients were treated with a trachelectomy and laparoscopic lymphadenectomy). The data of the 337 remaining patients were analyzed.

STUDY GROUP CHARACTERISTICS
Median age at time of diagnosis was 41 years (Interquartile-range (IQR) 34 - 50). FIGO stage IB cervical cancer was diagnosed in 276 (82%) patients (198 (72%)stage IB1; 61 (18%) stage IB2) and FIGO stage IIA in 61 (18%) patients. Adjuvant radiotherapy was given for 'classic' indications in 110 (33%) patients (presence of pelvic lymph node metastases in 80 (73 %), parametrial involvement in 16 (15%), tumor positive resection margins in eight (7.3%) and a combination of these three in six (5.5%) patients).

In 18 patients macroscopic extracervical disease (confirmed by frozen section) was found during surgery after which the radical hysterectomy was abandoned and the patients were treated with primary (chemo)radiation. For the evaluation of the value of serum SCC-ag to predict 'classic' indications for adjuvant radiotherapy, these 18 patients were categorized as having 'classic' indications for adjuvant radiotherapy, as these patients finally received both surgery and radiotherapy (combined with chemotherapy in 4 patients). These 18 patients were not included for the evaluation of recurrence percentages in patients treated with radical hysterectomy and adjuvant radiotherapy.

Only nine patients received adjuvant radiotherapy because of 'intermediate'-risk. Therefore it was decided not to analyze the predictive value of serum SCC-ag levels for 'intermediate'-risk separately. For the evaluation of the value of serum SCC-ag to predict 'classic' indications for adjuvant radiotherapy these nine patients were classified as having no 'classic' indication for adjuvant radiotherapy. The data of these nine patients were not used in the analyses of recurrence percentages in patients only treated with radical hysterectomy.

The overall frequency of indications for adjuvant radiotherapy, including the 27 patients who were reclassified, is shown in Table 1. In stage IB2 and IIA patients a comparable frequency of indications for adjuvant radiotherapy and of elevated serum SCC-ag was observed and therefore both groups were combined for further analysis. There was no difference in the distribution of age, stage, frequency of adjuvant radiotherapy for 'classic' indications, or frequency of recurrence between the two hospitals.

Table 1: Adjuvant radiotherapy and elevated preoperative SCC-ag levels according to FIGO stage

FIGO stage		Adjuvant RT*	SCC > 1.9 ng/mL
Stage	Number of patients	Number of patients (%)	Number of patients (%)
IB1	198	51 (26)	47 (24)
I82	78	43 (55)	48 (62)
IIA	61	34 (56)	33 (54)
Total	330	128 (38)	128 (38)
* 'Classic' indication for adjuvant radiotherapy			

FIGO STAGE, TUMOR SIZE AND SERUM SCC-AG IN RELATION TO INDICATIONS FOR ADJUVANT RADIOTHERAPY

Preoperative serum SCC-ag levels were elevated (>1.9 ng/mL) in 128 (38%) patients (Table 1). Table 2 represents the frequency of elevated serum SCC-ag levels for patients without any indication for adjuvant radiotherapy and for the different indications for radiotherapy separately. The frequency of elevated serum SCC-ag was highest in patients with more than one 'classic' indication for adjuvant radiotherapy. .

Table 2: Elevated serum SCC-ag according to different indications for adjuvant radiotherapy

Indication for radiotherapy		SCC >1.9 ng/mL
Indication	Number of patients	Number of patients (%)
No adjuvant radiotherapy	209	41 (20)
Positive resection margins only	8	3 (38)
Known intermediate risk only	9	4 (44)
Positive lymph nodes only	80	53 (66)
Parametrial invasion only	16	11 (69)
Abortion of surgery	18	14 (78)
Combination of 'classic' indications	6	6 (100)

Figure 1, comprising the ROC curves, represents the identification of patients with a 'classic' indication for adjuvant radiotherapy by the criteria FIGO stage, tumor-size and serum SCC-ag, respectively. The better performance of serum SCC-ag compared to tumor-size and stage is expressed by its ROC curve, which is closest to the upper left corner of the graph.

Figure 1: ROC-curve for tumor-size, FIGO stage and serum SCC-ag in predicting a 'classic' indication for adjuvant radiotherapy in 330 stage IB/IIA squamous cell cervical cancer patients
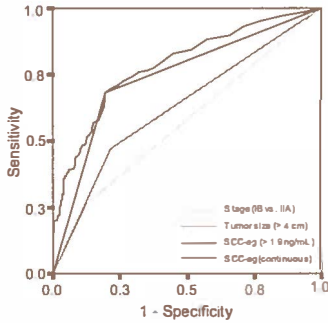
Elevated serum SCC-ag was the only independent predictor for indications for adjuvant radiotherapy with an odds-ratio of 7.1 in multivariate analysis including tumor-size and FIGO stage.

Table 3: Odds ratios for a 'classic' indication for adjuvant radiotherapy, estimated using multiple logistic regression analysis with tumor-size, FIGO stage and serum SCC-ag as independent variables

	Number of patients	Odds ratio (95% CI)*	p
FIGO stage			
IB	276		
		1.8 (0.96 – 3.5)	0.07
IIA	61		
Tumor-size			
≤ 4 cm	233		
		1.7 (0.98 - 3.0)	0.06
> 4 cm	104		
SCC-ag			
≤ 1.9 ng/mL	209		
		7.1 (4.2 - 12)	<0.001
> 1.9 ng/mL	128		
* 95% Confidence interval			

Figure 2 shows the frequency of adjuvant radiotherapy because of 'classic' indications for increasing levels of preoperative serum SCC-ag. The higher the level of preoperative serum SCC-ag the higher the frequency of adjuvant radiotherapy (p<0.001).

Figure 2: Serum SCC-ag for individualized risk assessment
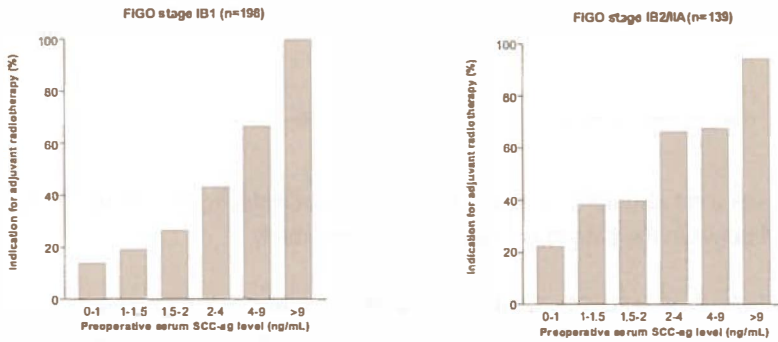
Standard deviations can not be given because the observed frequency of indication for adjuvant radiotherapy in one category is not independent from the frequencies observed in the other categories.

Using our classic cut-off value (1.9 ng/mL), serum SCC-ag analysis allowed the identification of a group of patients at high-risk for adjuvant radiotherapy in both stage IB1 and stage IB2/IIA patients. In 27 of 47 (57%) stage IB1 patients with elevated serum SCC-ag 'classic' indications for adjuvant ra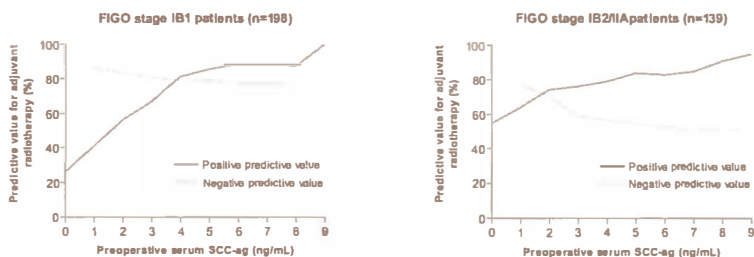diotherapy were present in contrast to 24 of 151 (16%) stage IB1 patients with normal preoperative serum SCC-ag (Figure 4, p<0.001). In 60 of 81 (74%) stage IB2/IIA patients with elevated serum SCC-ag 'classic' indications for adjuvant radiotherapy were present in contrast to 17 of 58 (29%) stage IB2/IIA patients with normal preoperative serum SCC-ag (p<0.001).

The predictive value of serum SCC-ag for 'classic' indications for adjuvant radiotherapy is shown in figure 3 for a continuum of different cut-off values. This figure allows evaluation of the implications of choosing a single cut-off value at different levels for serum SCC-ag for all early stage cervical cancer patients. When a cut-off value of 2.0 ng/mL is chosen, the likelihood of having an indication for adjuvant radiotherapy in the patients treated with surgery is approximately 16% in stage IB1 and 22% in stage IB2/IIA patients. Choosing a higher cut-off, for example 4.0 ng/mL, will especially lead to higher positive predictive values. When preoperative serum SCC-ag is higher than 4.0 ng/mL the likelihood of having and indication for adjuvant radiotherapy will be approximately 80% for both stage IB1 and stage IB2/IIA patients.

All analyses were also performed for both hospitals individually. No differences were observed between the data of the two hospitals separately.

THE ASSOCIATION OF ELEVATED SERUM SCC-AG AND RECURRENCE OF DISEASE

The median time in follow-up in our population of 337 early stage cervical cancer patients was 3.9 years (IQR 1.4 – 6.2). Twenty-one patients were lost to follow-up for a variety of reasons after a median time in follow-up of 3.3 years (IQR 0.9 – 5.4). Overall 54 recurrences were observed and the median time to recurrence was 1.0 year (IQR 0.6 – 1.8, range 0.3 – 10.3 years). Of these 54 recurrences 22 occurred in patients with normal and 32 in patients with elevated preoperative serum SCC-ag. At the time of recurrence nine (41%) patients with normal and 15 (47%) patients with preoperatively elevated SCC-ag levels had distant metastases.

In the group of 198 patients with stage IB1 disease 144 were not treated with adjuvant radiotherapy. In patients with normal preoperative serum SCC-ag two of 124 (1.6%) had a recurrence within two years, while in the remaining 20 patients with elevated serum SCC-ag three (15%) patients had recurrent disease (p=0.02, figure 4). In 49 stage IB1 patients that received adjuvant radiotherapy for 'classic' indications no differences in frequency of recurrences were observed between patients with normal (4 recurrences in 23 patients) and elevated preoperative serum SCC-ag (4 recurrences in 26 patients).

198 stage IB1 patients
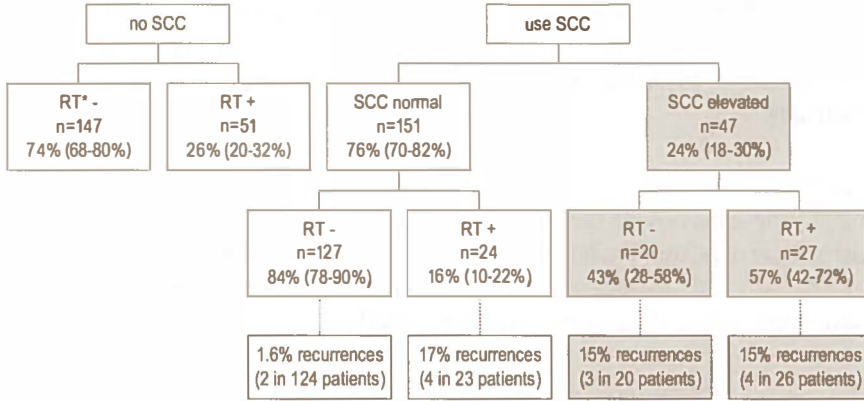
Based on the data of our cohort of IB1 patients two flow charts were constructed of actual treatment that patients received. On the left side the frequency of adjuvant radiotherapy when classic preoperative staging is performed and on the right side the hypothetical situation when IB1 patients would have been divided in two groups based on serum SCC-ag values. The grey shaded blocks indicate which patient group would, in the future, receive primary radiotherapy upfront when a preoperative serum SCC-ag level of 1.9 ng/mL or higher would be used as a cut-off for primary therapy. The data of 5 patients were not used in the calculation of recurrence percentages because they either received adjuvant radiotherapy for 'intermediate' indications or surgery was aborted because of extracervical tumor mass (* Adjuvant Radiotherapy (RT)).

In stage IB2/IIA patients serum SCC-ag levels did not identify patients at higher-risk for recurrence, neither in patients only treated with surgery nor in patients treated with surgery and adjuvant radiotherapy.

DISCUSSION

Our study shows for the first time that in early stage squamous cell cervical cancer patients prediction of 'classic' indications for adjuvant radiotherapy after radical surgery is improved by preoperative serum SCC-ag analysis. Using the exact serum SCC-ag value allows an individualized and refined pre-treatment risk assessment of risk for ending-up with combined modality treatment, which is associated with increased morbidity.

Patient characteristics of our study population are comparable to the characteristics of other early stage cervical cancer populations previously described.[2,4,22] The relative high percentage of adjuvant radiotherapy in our study can be explained by the exclusion of 184 patients with previous loop excision or exconisation, as these patients particularly have small tumors at low risk for adjuvant radiotherapy.

Discussing treatment choices in early stage cervical cancer, Morris suggested that only patients with a preoperative likelihood below 10-20% for adjuvant radiotherapy after surgery should be eligible for radical hysterectomy.[8] As a consequence patients with a higher likelihood should be treated with primary radiotherapy in order to avoid the increased morbidity of two-modality treatment in a significant proportion of patients. Our study shows that when knowledge of FIGO stage and SCC-ag levels are combined the group of patients with FIGO stage IB1 and preoperative serum SCC-ag levels between 0 and 1.5 ng/mL (figure 2) indeed fulfills this criterion. When in stage IB1 patients the 'classic' cut-off for SCC-ag of 1.9 ng/mL is used, approximately 16% of the patients will ultimately have an indication for adjuvant radiotherapy (figure 4) which also lays within the range, as suggested by Morris. Our study also indicates that in stage IB2/IIA only patients with serum SCC-ag levels between 0 and 1.0 ng/mL have a frequency of adjuvant radiotherapy close to 10-20%. However, these data should be used with caution since only a small group of stage IB2/IIA patients (n=31) had a preoperative serum SCC-ag value of 1.0 ng/mL or lower. Although thoroughly discussed, the choice to use a pretreatment likelihood of 10-20% for adjuvant radiotherapy as a criterion for eligibility for radical surgery is an arbitrary one. One great advantage of our present study is, that figure 2 and 3 allow implementation of serum SCC-ag analysis in existing clinical guidelines in which, based on figure 2 and 3 different SCC-ag levels corresponding to different likelihoods for two modality treatment can be chosen as cut-offs in choice between primary surgery and (chemo)radiation.

An obvious drawback of implementation of preoperative serum SCC-ag as a cut-off in choice between primary surgery and primary radiotherapy is the treatment with primary radiotherapy of patients who based on 'classic' indications would not have received adjuvant radiotherapy after surgery (e.g. 43% of the stage IB1 patients with preoperative serum SCC-ag > 1.9 ng/mL, figure 4) and one could argue that treatment by primary radiotherapy may cause unnecessary higher long-term morbidity in these patients. However, our data suggest that especially this group of patients is at higher

risk for recurrence when treated with radical surgery only. In 15% of stage IB1 patients with elevated serum SCC-ag and no 'classic' indication for adjuvant radiotherapy recurrent disease was observed in contrast to 1.6% recurrent disease in otherwise comparable stage IB1 patients with normal serum SCC-ag. Preoperative serum SCC-ag analysis apparently also allows identification of patients at greater risk for recurrence after radical surgery who would not have been identified otherwise. Especially this patient group may benefit from choosing (chemo)radiotherapy as primary treatment as prognosis after a recurrence is poor, with most patients dying as a result of uncontrolled disease.[20] A randomized trial will of course be the only way to really prove such a benefit. Radiotherapy as primary treatment can be combined with chemotherapy because recent trials (primarily in more advanced stages of disease) show a survival benefit for this combined modality treatment in cervical cancer.[23]

Until now previous studies on preoperative serum SCC-ag levels have not led to a wide implementation of preoperative serum SCC-ag analysis into clinical practice, due to small study populations, the lack of including parametrial involvement and positive resection margins into a predictive model, and/or inclusion of more advanced staged patients into the predictive model. Despite the restrictions mentioned, these studies also showed strong associations between elevated preoperative serum SCC-ag levels and the presence of lymph node metastases, thus underlining the strength of the data reported in our present study.[11,13,15,16,24]

In both our institutions imaging techniques such as magnetic resonance imaging or computer tomography are not routinely used in staging patients with cervical cancer although it has been reported that these imaging techniques can be used for either staging of cervical cancer or for preoperative identification of lymph node metastases.[25] However, it has also been shown that interobserver correlations are rather low with Kappa values between 0.28 and 0.62 and that the quality of images largely depend on the equipment and different techniques used.[26,27] In contrast, serum SCC-ag analysis can easily be performed everywhere with a relatively cheap, commercially available enzyme immunoassay, which is not subject to interobserver variation. Serum SCC-ag analysis can be reliably used by every oncologist and therefore our results should be used to identify future squamous cell cervical cancer patients at different levels of risk for adjuvant radiotherapy after surgery (figure 2).

Above each cut-off level one may consider to choose radiotherapy as primary treatment in stead of surgery. Radiotherapy as primary treatment can be combined with chemotherapy because recent trials (primarily in more advanced stages of disease) show a survival benefit for this combined modality treatment in cervical cancer.[27] Another application of preoperative serum SCC-ag analysis is stratification of early stage cervical cancer patient in different risk groups when designing future clinical trials. In patients with early stage squamous cell cervical cancer clinical trials should not only stratify for tumor size and FIGO stage, but also for patients with elevated serum SCC-ag levels, because of the clearly different risk profile. Studies on more intensive

treatment regimens (such as combining chemotherapy and radiotherapy) could be restricted to early stage cervical cancer patients at high risk for recurrence, preventing additive morbidity in patients that are adequately treated with surgery.

In conclusion, our current study shows that serum SCC-ag analysis allows more refined and individualized preoperative risk assessment for adjuvant radiotherapy than currently used clinical parameters. Preoperative serum SCC-ag analysis deserves implementation in existing treatment guidelines for early stage cervical cancer.

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Bacterial vaginosis is not important in the etiology of cervical neoplasia: a survey on women with dyskaryotic smears

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ABSTRACT

BACKGROUND AND OBJECTIVES

It has been suggested that bacterial vaginosis may play a role in the etiology of cervical neoplasia. The authors analyzed the prevalence, risk factors, and impact on histologic changes of bacterial vaginosis in women with cytological abnormalities of the uterine cervix.

METHODS

Two-hundred-eighty women with dyskaryotic smears were surveyed. Using a questionnaire, data were obtained on smoking habits and sexual history. Bacterial vaginosis was the diagnosis if the vaginal discharge produced a fishy odor upon alkalinization and if clue cells were seen in the wet smear.

Cervical scrapes were analyzed for the presence of human papillomavirus DNA, and cervical tissue, specimens were analyzed for the presence and severity of (intraepithelial) neoplasia and the proliferation rate (mitotic index) of the lesion. *Chlamydia Trachomatis* was identified by culture of an endocervical swab.

RESULTS

Bacterial vaginosis was found in 56 (20%) out of the 280 women. The presence of bacterial vaginosis was significantly associated with the number of cigarettes smoked per day, age at first sexual intercourse, the lifetime number of sexual partners, and current *Chlamydia Trachomatis* infection. The number of cigarettes currently smoked per day and the lifetime number of sexual partners were independent significant risk factors for the presence of bacterial vaginosis. There was no relation between the presence of bacterial vaginosis and the human papillomavirus infection. Bacterial vaginosis did not influence the severity of the (intraepithelial) neoplasia or the mitotic index.

CONCLUSION

In women with dyskaryotic cervical smears, the prevalence of bacterial vaginosis did not seem to be increased, and bacterial vaginosis did not influence the histologic changes. Therefore, bacterial vaginosis is unlikely to be important in the etiology of cervical neoplasia, despite the similarity between its epidemiologic features and those of cervical human papillomavirus infection and cervical neoplasia.

INTRODUCTION

Cervical intraepithelial neoplasia (CIN) is a morphologically defined lesion associated with the development of cervical cancer. In the conventional morphogenetic model, CIN is separated into three grades according to the degree of cellular atypia and disturbance of the epithelial architecture.[1]

The proliferation rate of the lesion increases with increasing CIN grade.[2,3] Risk factors for cervical neoplasia are smoking and lifetime number of sexual partners.[4] Human papillomavirus (HPV) is strongly associated with CIN, and the frequency depends on the CIN grade. In women with cytologic abnormalities, cervical HPV was found in 27% of those without neoplasia, 37% with CIN I, 43% with CIN II, and 68% with CIN III.[5] Epidemiologically, the risk factors for cervical HPV infection are similar to those for cervical neoplasia.[6] Bacterial vaginosis is defined as replacement of the lactobacilli of the vagina by characteristic groups of bacteria accompanied by changed properties of the vaginal fluid. It is a polymicrobial condition, and the micro-organisms most often found are *Gardnerella vaginalis, Prevotella species,* anaerobic gram-positive cocci, *Mycoplasma hominis* and *Mobiluneus species* 7. The clinical features of bacterial vaginosis are, 1) a thin, homogenenous vaginal discharge; 2) a vaginal pH of more than 4.5; 3) vaginal epithelial cells heavily coated with bacilli (clue cells); and 4) release of fishy odor from the vaginal discharge on alkalinization with 10% potassium hydroxide (KOH).[8]

It has been reported that bacterial vaginosis occurs significantly more often in patients with the colposcopic or cytologic suspicion of pre-cancerous changes or early cervical carcinoma than in women with normal cervical findings.[9] Pavic hypothesized that the local production of nitrosamines in patients with bacterial vaginosis may act synergistically with other agents in the development of cervical neoplasia.[10] In the present study, we explored the possible importance of bacterial vaginosis in the etiology of cervical neoplasia. We analyzed whether the occurrence of bacterial vaginosis is determined by the same features as the occurrence of cervical HPV infection in women with documented cervical cytologic abnormalities. More specifically, we analyzed 1) the prevalence of bacterial vaginosis, 2) the risk factors for bacterial vaginosis; 3) the occurrence of bacterial vaginosis in relation to HPV; and 4) the relation between bacterial vaginosis and the severity of histomorphologic changes.

MATERIALS AND METHODS

PATIENTS

Patients were recruited from the outpatient clinic of the Department of Gynecology, University Hospital Groningen. They were referred either by their general practitioner because of an abnormal cervical cytology report, or the cervical cytologic abnormality was discovered during gynecologic examination. Patients were eligible to participate in the study if they had two mildly or moderately dyskaryotic cervical smears, or one severely dyskaryotic cervical smear. These cytologic criteria for eligibility correspond with the grounds for colposcopy as agreed on by cytopathologists and gynecologists in The Netherlands. In the case of mild or moderate dyskaryosis, the interval between the two abnormal smears was a maximum of 1 year. Patients were not eligible if they had previously undergone a colposcopic examination because of an abnormal cytology report or if their cervical smear was taken during pregnancy.

For the 5-year period, September 1, 1988 to September 1, 1993, 343 consecutive patients were eligible for participation in the study. Sixty-three patients were not included for the following reasons: two patients did not want to be involved in the study; two had insufficient command of Dutch or English; at the enrolment visit, 24 patients had their menstrual period or bloody vaginal discharge; and 16 had an insufficient volume of vaginal discharge for examination; four patients were pregnant at the time of colposcopy; and 15 were not treated in accordance with the study protocol. Thus, 280 newly diagnosed patients were included in the study. The age of the patients ranged from 20 to 66 years with a mean value of 34.7 years (standard deviation, 7.7 years).

QUESTIONNAIRE

Using a structured questionnaire, we asked the women to state the mean number of cigarettes they were smoking per day, their age at first sexual intercourse, their lifetime number of sexual partners, and whether they had ever had a sexually transmitted disease or genital warts. All of the women were told beforehand that the questionnaire contained some intimate questions and that they were not obliged to answer.

MICROBIOLOGICAL ANALYSES

Some vaginal discharge was taken from the anterior or lateral vaginal vault or the proximal side walls of the vagina. Care was taken to avoid contact with the cervical mucus. Examination for fishy odour followed after the addition of 10% KOH. A small drop of discharge was mixed with 0.9% NaCl and examined microscopically for the presence of clue cells. Bacterial vaginosis was diagnosed if the vaginal discharge produced a fishy odor after the addition of 10% KOH and clue cells were seen under the microscope.

Then, an endocervical swab was taken for *Chlamydia Trachomatis* analysis. Within 4 hours of collection, this specimen was inoculated on cycloheximide-treated McCoy cell

monolayers on cover slips, as described by Ripa and Mårdh.[11] Detection of
C. trachomatis inclusions was done after 48 hours using fluorescein-conjugated
monoclonal antibodies (MikroTrak Culture Confirmation; Syva Corp., Palo Alto, CA).
Finally, the cervix was scraped with the blunt and pointed end of a wooden cervical
spatula and with an endocervical brush. The scraped cells were suspended in 5 ml of
phosphate buffered saline, pH 7.2, supplemented with merthiolate 1: 10,000
volume/volume. The cell suspension was sent to the laboratory and processed the
following morning. The samples were analyzed for the presence of HPV using a general
primer-mediated polymerase chain reaction, as described by Snijders *et al.*[12] The
laboratory staff were unaware of the histologic diagnoses.

MORPHOLOGICAL EXAMINATION

Four weeks after the cervix had been scraped, we took colposcopically directed
biopsies. If CIN was diagnosed from the biopsies, we subsequently excised the whole
transformation zone by loop electrosection or cold knife conization. Diathermic loop
excision was used if the squamocolumnar junction could be visualized entirely and did
not extend up into the canal more than 5 mm from the anatomical os externum. The
details of the technique have been described in a previous report.[13] Cervical neoplasia
was diagnosed and graded according to the criteria of the World Health Organization.
In the individual patient, the most severe grade found by histologic examination was
her diagnosis.

For counting mitoses, new sections 4 µm thick were cut from all of the tissue blocks to
achieve uniform quality of the study material. These sections were used to identify the
area with the highest mitotic index. This area could be localized in one of the biopsies
or in the electrosectioned slice or in the cold knife cone. The mitotic index was assessed
by using a grid to count the number of mitotic figures per 1,000 nuclei from basal to
proximal through the epithelial layer. Mitotic figures were defined as figures without a
nuclear membrane, indicating that the cell had passed the prophase, and with clear
hairy extensions of nuclear material. Pyknotic nuclei or nuclei with basophilic cytoplasm
were not counted.[14] Sixty-one of the 280 women were not included in the morphologic
part of the study for the following reasons: no neoplasia was found in 37 patients;
invasive carcinoma was found in 11 patients; the tissue specimen was morphologically
unsatisfactory in five patients; and the specimens were not available for evaluation in
eight patients. Therefore, this part of the study was performed on 219 patients.

The microscopic examinations were performed with a Leitz dialux 20 EB microscope
with a x40 npl fluotar Leitz objective (numeric aperture, 0.70) and a x10 wide-field
periplan ocular piece.

STATISTICAL ANALYSIS

To test for a significant difference between two groups of patients regarding qualitative
or quantitative variables, we used the chi-squared test or the MannWhitney U test,
respectively. If there were low numbers for a qualitative variable (frequency, <5% in a

cell of the contingency table), Fishers's exact test was performed. Spearman's rank correlation coefficient was used to express the relations between continuous variables. These statistical procedures were performed with the SYSTAT software package (SYSTAT, Inc. Evanston, IL, USA, 1990). The 95% confidence interval of Spearman' s rank correlation coefficient was obtained with the Confidence Interval Analysis software package (BMJ, London, UK,1991). The logistic regression analysis and the 2xk-table analysis were performed using the EGRET software package version 0.26.6 (Statistics and Epidemiology Research Corporation, Seattle, USA, 1992). In the logistic regression analysis, the variables were treated as continuous or as unordered categorical variables. Contrasting likelihood values revealed that the descriptive power of the univariate logistic models did not change if the continuous variables were converted into unordered categorical variables. No two-factor interactions were found. P values of less than or equal to 0.05 were considered significant.

RESULTS

Fifty-six (20%) of the 280 women had bacterial vaginosis. Table 1 shows the distribution of selected factors according to the presence or absence of bacterial vaginosis. An association was found between the presence of bacterial vaginosis and the number of cigarettes smoked per day, age at first sexual intercourse, lifetime number of sexual partners, and current *C. trachomatis* infection. No association was found between bacterial vaginosis and current age, past history of genital warts, past history of venereal disease, or current HPV infection.

Table 1: Distribution of selected factors among patients with abnormal cervical cytology reports, according to the presence or absence of bacterial vaginosis

	Bacterial Vaginosis		
Factor	Absent (n = 224)	Present (n = 56)	P Value
Age (yr)			
Median	35	35	0.86*
Interquartile range	29-39	28.5-42	
Number of cigarettes			
smoked per day (total population)			
Median	10	17.5	0.009*
Interquartile range	0-20	0-25	
Age at first sexual			
intercourse (yr)†			
Median	17.5	17	0.03*
Interquartile range	16-19	15-18	
Lifetime number of partners‡			
Median	4	6	0.003*
Interquartile range	2-10	4-15	
Past history of venereal disease n(%)	36 (16.1)	10 (17.9)	0.90§
Past history of genital warts n(%)	19 (8.5)	6 (10.7)	0.79§
Human papillomavirus present n(%)	157 (70.1)	42 (75)	0.58§
***Chlamydia Trachomatis* present n(%)**	3 (1.3)	4 (7.1)	0.03*

*Mann-Whitney U test.

†Data missing for three cases.

‡Data missing for seven cases.

§Chi-squared test for two groups with Yates' correction.

‖Fisher's exact test.

We used 2xk-table analysis and logistic regression analysis to appraise the relations between bacterial vaginosis and smoking behavior, age at first sexual intercourse, and lifetime number of sexual partners. *Chlamydia Trachomatis* infection was not included in this part of the study because of the low numbers. Table 2 shows the results of the analysis. The proportion of women with bacterial vaginosis increased significantly as the number of cigarettes smoked a day increased (chi-squared = 5.86, degrees of

freedom = 1, P = 0.02; test for trend). Compared with women who did not smoke, the point estimate of the odds ratio increased to 1.44 in women who smoked more than 20 cigarettes a day. Each cigarette produced a 3% increase in risk (odds ratio, 1.03 with 1.01 and 1.06 as the limits of the 95% confidence interval) for bacterial vaginosis when the number of cigarettes a day was analyzed as a continuous variable.

The proportion of women with bacterial vaginosis increased significantly as the lifetime number of sexual partners increased (chi-squared = 6.91, degrees of freedom = 1, P = 0.009; test for trend). Compared with women who have had one to two partners in their lifetime, the point estimate of the odds ratio increased to 3.24 in women who have had more than 10 sexual partners. Each additional partner conferred a 2% increase in the risk for bacterial vaginosis when the lifetime number of sexual partners was analyzed as a continuous variable.

Age at first sexual intercourse was inversely associated with the occurrence of bacterial vaginosis (chi squared = 5.72, degrees of freedom = 1, P = 0.02; test for trend). Compared with women who had their first intercourse before 16 years old, the point estimate of the odds ratio decreased to 0.29 for women who first had sexual intercourse after 20 years of age.

The number of cigarettes smoked per day, the lifetime number of sexual partners, and the age at first sexual intercourse were interrelated variables. Spearman's rank correlation coefficient between the number of cigarettes smoked per day and the age at first sexual intercourse was -0.29 (95% confidence limits, -0.40 to -0.18), and between the number of cigarettes smoked per day and the lifetime number of sexual partners was 0.18 (95% confidence limits, 0.07 to 0.30). Spearman's rank correlation coefficient between the age at first sexual intercourse and the lifetime number of sexual partners was -0.37 with 95% confidence interval limits -0.46 to -0.26. Because of these interrelations, we performed a multivariate logistic regression analysis. We showed that the relationship between bacterial vaginosis and the number of cigarettes smoked per day and lifetime number of partners was largely unaltered (Table 2). No relation was found between bacterial vaginosis and the age at first sexual intercourse when the analysis was adjusted for smoking behavior and number of partners. We concluded that smoking behavior and lifetime number of partners are independent risk factors for bacterial vaginosis.

Table 2: Odds Ratios for bacterial vaginosis, by number of cigarettes smoked per day, lifetime number of sexual partners, and age at first sexual intercourse in patients with reported cervical cytological abnormalities

Variable	Number (%) with Bacterial Vaginosis		Odds Ratio (95% Confidence Interval) for Bacterial Vaginosis	
			Crude	Adjusted*
No. of cigarettes smoked per day				
0	16/104	(15.4)	1 (reference)	1 (reference)
1-10	6/47	(12.8)	0.81 (0.29-2.21)	0.92 (0.33-2.57)
11-20	18/77	(23.4)	1.68 (0.79-3.55)	1.44 (0.64-3.23)
≥ 21	16/52	(30.8)	2.44 (1.11-5.41)	2.39 (1.03-5.53)
Trend per cigarette			1.03 (1.01-1.06)	1.03 (1.00-1.06)
			(P=0.01)	(P=0.03)
Lifetime number of sexual partners [†]				
1-2	8/72	(11.1)	1 (reference)	1 (reference)
3-4	12/70	(17.1)	1.66 (0.63-4.33)	1.39 (0.52-3.72)
5-10	18/79	(22.8)	2.36 (0.96-5.83)	1.90 (0.75-4.81)
≥11	15/52	(28.8)	3.24 (1.26-8.38)	2.46 (0.90-6.72)
Trend per partner			1.02 (1.00-1.04)	1.02 (1.00-1.04)
			(P=0.03)	(P=0.06)
Age at first sexual intercourse [‡]				
15	14/50	(28.0)	1 (reference)	1 (reference)
16-17	22/97	(22.7)	0.75 (0.35-1.64)	1.10 (0.47-2.55)
18-19	14/81	(17.3)	0.54 (0.23-1.25)	0.89 (0.35-2.25)
>20	5/49	(10.2)	0.29 (0.10-0.89)	0.52 (0.16-1.70)
Trend per year	0.87		(0.77-0.99)	0.94 (0.83-1.07)
			(P=0.03)	(P=0.35)

*The estimates were adjusted for both other factors through logistic regression by treating these other factors as continuous variables.

[†]Data missing for seven cases.

[‡]Data missing for three cases.

Table 3 shows the histologic diagnosis according to the presence or absence of bacterial vaginosis. We dichotomized the spectrum of histologic diagnoses between CIN I and CIN II. No difference was found regarding the presence of bacterial vaginosis between minor lesions (no neoplasia or CIN I) and major lesions (CIN II or a more severe lesion; 15 out of 68 versus 41 out of 212, P = 0.75, chi-squared test).

Analysis of the mitotic index was performed only on patients with CIN. The median values (range of values) of the mitotic index in the patients with CIN I, CIN II, and CIN III were 3 (0-8), 4 (1-12), and 7 (0-51), respectively. In the group of patients with bacterial vaginosis and in the group without bacterial vaginosis, the median values of the mitotic index were 6 (0-28) and 5 (0-51), respectively. No relation was found between the presence of bacterial vaginosis and the mitotic index (P = 0.27, Mann-Whitney U test).

Table 3: Presence of bacterial vaginosis in patients with abnormal cervical cytology reports in relation to the final histologic diagnosis

Histologic Diagnosis	Number (%) of Patients With Bacterial Vaginosis	
No neoplasia	11/37	(29.7)
CIN I	4/31	(12.9)
CIN II	5/43	(11.6)
CIN III	30/158	(19.0)
Invasion	6/11	(54.6)
CIN = cervical intraepithelial neoplasia.		

DISCUSSION

It may seem peculiar to study the epidemiologic features and possible morphologic sequelae of bacterial vaginosis using a scientific design with one sample of women with cytologic abnormalities of the uterine cervix. However, cytologic abnormalities result from a spectrum of changes with diverse etiologies and clinical outcomes.[15] Therefore, the composition of any sample of patients with cytologic abnormalities is heterogeneous. An investigation on bacterial vaginosis and cervical neoplasia is not possible in patients with invasive carcinoma because the vaginal environment is disturbed by blood and tumor necrosis. A regular case-control study with CIN patients is difficult to perform because the prevalence of bacterial vaginosis in both groups, patients and control subjects, will not differ very much. Thus, very large groups of patients and control subjects would be needed.

We defined bacterial vaginosis as vaginal discharge that released a fishy odor after being mixed with a drop of 10% KOH and that showed the presence of clue cells in the wet smear during microscopic examination. These two criteria are the most relevant for diagnosing bacterial vaginosis and permit a correct diagnosis of bacterial vaginosis to be made in almost all cases.[16-18] An elevated pH value of the vaginal fluid is the least specific indicator for the diagnosis of bacterial vaginosis[17] and is a physiologic phenomenon in post-menopausal years. Assessing whether the discharge has a thin, homogeneous appearance is a subjective process and is less reliable for clinical purposes.[16]

In our series, bacterial vaginosis was found in 56 (20%) of the 280 women. This prevalence seems comparable with the prevalence normally found in samples of patients attending gynecology outpatient clinics. The prevalence of bacterial vaginosis, as reported by other investigators, depends on the characteristics of the patient population. In one study, bacterial vaginosis was diagnosed in six (12%) of 52 virginal post-menarchal girls.[19] In populations of outpatients at gynecology clinics, bacterial vaginosis was found in 19 (15%) of 126 patients who came for a routine annual or contraceptive examination and did not have symptoms of vaginitis (i.e., vaginal discharge, vaginal odor, and/or vulvovaginal irritation or itching),[8] in 32 (29%) of 111 patients who presented with symptoms of vaginitis,[8] and in 142 (20.9%) of 680 fertile nonpregnant women who visited the clinic for various reasons.[18] At clinics for sexually transmitted diseases, one group of investigators diagnosed bacterial vaginosis in 210 (33%) of 640 women they examined,[20] while other researchers diagnosed 364 (36%) cases in their group of 455 patients.[16] The reported prevalences indicate that the occurrence of bacterial vaginosis is partly determined by sexual behavior.

In the univariate analyses, we found that bacterial vaginosis was associated with age at first intercourse, lifetime number of sexual partners and current *C. Trachomatis* infection. These associations corroborate the relation between the presence of clue cells reported, Papanicolaou smears and lifetime number of sexual partners and age at first

intercourse.[20] In women who attended a sexually transmitted disease clinic, the presence of bacterial vaginosis was associated with the number of sexual partners during the 30 days before examination.[21] The associations discussed also indicate that sexual behavior influences the risk of contracting bacterial vaginosis.

We found that bacterial vaginosis is significantly associated with being a smoker. Other investigators have reported an association between the presence of clue cells in Papanicolaou smears and smoking.[20] A question that arises is whether smoking is an independent risk factor for bacterial vaginosis or simply a covariable of sexual behavior. An association between smoking and the lifetime number of male sexual partners has been demonstrated in a study on women attending a university health services clinic[22] and in a study on women attending a sexually transmitted disease clinic.[23] An association between smoking and a comparatively young age at first intercourse has been demonstrated in studies of healthy young women attending medical contraception clinics[24,25] and in a study of junior high school students.[26] In the present study, we too demonstrated a relation between smoking and sexual behavior. The findings indicate that sexual behavior could be a confounder in studies on the relation between smoking and the presence of bacterial vaginosis. In the multivariate analysis, we demonstrated that the number of cigarettes smoked per day and the lifetime number of sexual partners are independent risk factors for bacterial vaginosis. Other investigators found that both of these factors also have an independent effect on the occurrence of the cervical HPV infection.[6] Hence, there was a similarity between the epidemiologic characteristics of bacterial vaginosis and the cervical HPV infection.

We did not find a relation between the presence of bacterial vaginosis and current age. Other investigators who have reported on patients attending an outpatient gynecology clinic also were unable to find such a relation.[8,18]

There was no relation between bacterial vaginosis and cervical HPV infection; nor was there a relationship between bacterial vaginosis and the severity of the histomorphologic changes. In the latter analysis, we combined "no neoplasia" and "CIN I" into one category of "minor lesions." All of the patients had cytologic abnormalities, and discriminating between benign reactive changes and CIN I is a subjective process.[27]

The number of mitoses in the lesions classified as CIN was counted to obtain a mitotic index and we confirmed that the mitotic index increased with increasing CIN grade.[2,3] The mitotic index is estimated quantitatively and its use provides increased statistical power to detect a relation between bacterial vaginosis and morphologic changes. The presence of bacterial vaginosis did not have a demonstrable effect on the mitotic index, which supports the conclusion that bacterial vaginosis does not influence the severity of the morphologic changes. This contrasts with the reports on HPV that stated the prevalence of HPV is strongly associated with the CIN grade.[5,28-30]

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No association of anti-*Chlamydia trachomatis* antibodies and severity of cervical neoplasia

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ABSTRACT

OBJECTIVE

To explore whether the presence of *Chlamydia trachomatis* antibodies is associated with the severity of neoplastic lesions in women with cervical dyskaryosis.

METHODS

In a cross-sectional study in two groups of women referred for an abnormal Pap smear (group A: 296, group B: 331 women) blood samples were analyzed for antichlamydial antibodies by Enzyme Immunoassay. Cervical neoplasia was graded histologically.

RESULTS

In group A no association was found between increasing grade of CIN and the presence of antichlamydial antibodies. The proportion (93%) of women with antichlamydial antibodies was higher in 14 women with (micro)invasive carcinoma than in women with CIN (35%). As the high prevalence of antichlamydial antibodies in women with cervical carcinoma is not consistent with prevalences reported in recent literature, we analyzed a second group of women in which indeed the high prevalence was not confirmed

CONCLUSION

Our results suggest that the presence of circulating antichlamydial antibodies is not associated with the severity of neoplastic lesions and it seems unlikely that *C. trachomatis* plays a role in the progression of cervical neoplasia.

INTRODUCTION

Human papillomavirus (HPV) plays an important role in the development of cervical intraepithelial neoplasia (CIN) and cervical carcinoma. However, compared to the high rates of HPV infections in women without cervical neoplasia, the occurrence of CIN and cervical cancer is rare.[1] The search for risk-factors for cervical neoplasia, other than HPV, is therefore still going on. A candidate risk-factor is *Chlamydia trachomatis*. Case-control studies have reported that serum antibodies against *C. trachomatis* are relatively more frequent in women with CIN or cervical carcinoma compared to controls.[2-7]

In the present study we explored the hypothesis that the presence of *C. trachomatis* antibodies is associated with the severity of neoplastic lesions using a cross-sectional study design. An (unexpected) high prevalence of antichlamydial antibodies in women with (micro) invasive carcinoma ((M)IC) was found. This high prevalence of antichlamydial antibodies in women with (M)IC and a higher prevalence in women with (M)IC than in women with CIN III was not reported previously.[2-7] Therefore a second study population was selected in an attempt to confirm our results.

PATIENTS AND METHODS

PATIENTS

Two groups of women referred for an abnormal Pap smear to the gynecological-outpatient clinic of the University Hospital Groningen, the Netherlands, were recruited for this study. Group A comprised 296 women referred between September 1988 and September 1993[8] and group B comprised 331 women referred between November 1995 and June 1999. For both groups separately, the study was approved by the ethical review board of the hospital.

QUESTIONNAIRE

Using a structured questionnaire, women were asked about their smoking habits and their lifetime number of sexual partners.

DETECTION OF SERUM ANTIBODIES AGAINST CHLAMYDIA TRACHOMATIS

Blood samples were taken at the enrolment visit of all women. Periodate treated Enzyme Immuno Assays (EIA) were carried out as described previously.[9,10] Treatment with sodium periodate results in enhanced specificity of the assay compared to the native EIA.[9,10] For both groups of patients, the same reference serum was used ensuring comparability of the results.

MORPHOLOGICAL EXAMINATION

Colposcopically directed biopsies were taken and graded according to the criteria of the World Health Organisation.[11] If CIN was diagnosed, except for CIN I in group B, the whole transformation zone was subsequently excised by loop electrosection (LETZ) or cold knife conization. Cervical neoplasia was classified according to the most severe histological lesion found.

RESULTS

In group A 114 (39%) of 296 women tested positive for serum antibodies against *C. trachomatis*. The prevalence of antichlamydial antibodies did not increase significantly with increasing severity of CIN. However, the prevalence of *C. trachomatis*-antibodies was significantly higher in the group of women with (M)IC than in women with CIN (table 1).

Table 1: Antibodies against *C. trachomatis* and the grade of neoplasia

	No (%) of positives for antibodies against *C. trachomatis*, and the 95% CI				
	Grade of neoplasia				
	None	CIN I	CIN II	CIN III	(M)IC
Group A	16/40 (40)	8/34 (24)	14/43(33)	63/165 (38)	13/14 (93)*
	25-57	10-41	19-49	31-46	66-100
Group 8	8/19 (42)	26/50 (52)	41/82 (50)	74/153 (48)	15/27(55)
	20-67	37-66	39-61	40-56	35-75

* X²-test, p < 0.001
X²-test for trend was not significant for both groups

Patient characteristics in group B met the characteristics of group A apart from the proportion of women diagnosed with no dysplasia or CIN II, for which the 95% confidence intervals did not overlap (table 2).

Table 2: Patient-characteristics for group A and 8

	Group A		Group B	
Age (median, interquartile range)	35	29-39	35	31-42
Life time number of sexual partners				
(median, interquartile range)	4	2-10	4	2-8
Sexarche ≤ 17 years (%, 95%CI)	52	46-57	49	43-54
Smoker (%, 95%CI)	65	59-70	69	64-75
No dysplasia (%, 95%CI)	14	9.6-17	5.7	3.5-8.8
CIN I (%, 95%CI)	12	7.9-15	15	11-19
CIN II (%, 95%CI)	15	11-19	25	20-29
CIN III (%, 95%CI)	56	50-61	46	41-52
(M)IC (%, 95%CI)	4.7	2.6-7.8	8.2	5.5-11.7

Serum antibodies against *C. trachomatis* were found in 164 (50%) of 331 women in group B. No increasing trend was observed for the proportion of women positive for antichlamydial antibodies with increasing severity of CIN (table 1). The prevalence of *C. trachomatis*-antibodies in women with (M)IC was not significantly higher than in women with CIN.

DISCUSSION

Differences between group A and B might occur because of systematic differences between the two groups or due to chance. Effort was made to reduce systematic differences: periodate treated EIA was performed for both groups separately, but the same reference serum was used. Criteria for eligibility for group A and group B corresponded. We therefore have no other explanation than that the difference in the proportion of women with no dysplasia and CIN II is due to chance. The reported differences appeared to have no implication for our results.

Overall prevalences of antichlamydial antibodies were comparable for group A and B. However, 93% of the women with (M)IC in group A had antichlamydial antibodies compared to 54% in group B. Prevalences reported by others are comparable to the prevalence found in group B.[2 7] The number of women with (M)IC in group A is low. The 95%CI of the prevalence is very wide in this group and overlaps the 95%CI of the proportion observed in the same category of group B (table 2). Chance has a great effect on small study populations.[12] Considering what is discussed above we conclude that the high prevalence in the (M)IC group of A is due to chance.

The role of C. trachomatis in the etiology of cervical neoplasia is hard to interpret. Many studies reported antichlamydial antibodies to be more frequent in women with cervical neoplasia than in controls.[2 7] This might indicate that C. trachomatis plays a causal role in cervical carcinogenesis. Our results suggest that C. trachomatis does not favor the progression from CIN to invasive disease. However, it should be kept in mind that these serological data can not exclude the possible involvement of local factors induced by (chronic) C. trachomatis infections.

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Using a new HPV detection system in epidemiological research: change of views on cervical dyskaryosis?

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ABSTRACT

OBJECTIVE

In the past we reported the prevalence of human papillomavirus (HPV) to be higher with increasing histological severity of neoplasia, more cigarettes smoked per day and higher lifetime number of sexual partners in women with cervical dyskaryosis. Recently the highly sensitive SPF10 primers and Inno-LiPA HPV prototype research assay became available for the detection and typing of HPV. Using this system, we challenged the previously reported findings.

STUDY DESIGN

The study group comprised 304 women referred because of abnormal Pap smears in whom a histological diagnosis was made. Data on the lifetime number of sex-partners and smoking behaviour were obtained by questionnaire. HPV analysis was performed on cervical scrapes obtained at the enrolment visit.

RESULTS

Oncogenic HPV was found in 288 (95%) women. A total of 86 (30%) out of these 288 women disclosed multiple types. HPV 16 occurred significantly less often in multiple infections than was expected on the basis of chance alone.
The grade of neoplasia was significantly associated with the presence of oncogenic HPV and this association depended on the presence of HPV type 16. No association was found between grade of neoplasia and the presence of multiple HPV types. Neither the lifetime number of sexual partners nor smoking were associated with oncogenic HPV, the five most frequent HPV types separately or the presence of multiple types.

CONCLUSION

We conclude that the association between the detection of HPV and the epidemiological risk factors, as found with the GP5/6 PCR in the past, could not be confirmed when using SPF10 PCR primers and LiPA HPV genotyping. We suggest that the number of sex-partners and smoking may be determinants of high HPV viral load, rather than determinants of the presence of HPV per se.

INTRODUCTION

Human papillomavirus (HPV) has been the subject of a lot of research since 1976 when zur Hausen published on a possible role for HPV in the etiology of cervical cancer.[1] HPV is now considered to be the most important factor in the multi-step process leading to cervical cancer. HPV is a small (7,9kB) double stranded epitheliotropic virus of which more than 90 types are known.[2] These types can be divided into a mucosal and cutaneous group. Part of the mucosal HPV types are considered to be high-risk or oncogenic because they are found in cervical carcinomas. Among these are HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 69.[1] With PCR based methods HPV is found in nearly all cervical carcinomas.[3,4]

In the past we reported the prevalence of HPV in women with cervical dyskaryosis to be higher with increasing histological severity of neoplasia, more cigarettes smoked per day and higher lifetime number of sexual partners.[5-7] In these studies we used the GP5/6 PCR for detection of HPV. Other investigators, using the MY 09/11 PCR for HPV detection, have reported similar associations in various populations.[8-15]

Recently a new and highly sensitive broad-spectrum HPV-PCR, the SPF10 PCR, was developed.[16,17] With the Line Probe Assay (LiPA) typing method 25 different HPV types can be identified simultaneously.[17] In this report, we challenge our previously reported findings by re-analyzing the data with the test results from the SPF10/LiPA system. Because 25 HPV types can be identified in one test, we considered the mere presence of oncogenic HPV, the five most prevalent HPV types separately and the presence of multiple oncogenic types.

PATIENTS AND METHODS

PATIENTS

Between September 1988 and September 1993 a total of 343 consecutive patients with cervical dyskaryosis were seen in the outpatients colposcopy clinic of the Department of Gynecology, University Hospital Groningen, as described previously.[7] Briefly, patients had had either two cervical smears showing mild or moderate dyskaryosis, or one smear with severe dyskaryosis. These cytological criteria for eligibility correspond with the grounds for colposcopy as agreed on by the cytopathologists and gynecologists in the Netherlands at that time. A total of 39 patients were not included in the present study: two of them did not want to be involved in the study; 15 patients were not treated in accordance with the study protocol, 2 patients had insufficient command of Dutch or English, 4 patients were pregnant at the time of colposcopy and the cervical scrapes of 16 patients were not available for re-testing on HPV with the SPF10/LiPA system. The data of the remaining 304 patients are presented here. The mean age was 35.0 years (SD 8.0; range 19-67 years). The study was approved by the ethical committee of the hospital.

QUESTIONNAIRE

Using a structured questionnaire, women were asked about their smoking habits and to state the lifetime number of sexual partners. All the women were told beforehand that the questionnaire comprised some intimate questions they were not obliged to answer.

MORPHOLOGICAL EXAMINATION

Approximately four weeks after the cervix had been scraped for virological analysis, colposcopically directed biopsies were taken. Cervical neoplasia was diagnosed and graded according to the criteria of the World Health Organisation.[18] If CIN (either CIN I, CIN II or CIN III) was diagnosed, the whole transformation zone was subsequently excised by loop electrosection (LETZ) or cold knife conization. LETZ was performed if the squamocolomnar junction could be visualized entirely and did not extend up into the cervical canal more than 5 mm from the anatomical external os. The tissue was examined histologically. The cervical neoplasia was classified according to the most severe lesion found on histological examination.

CERVICAL SCRAPES AND HPV DETECTION

At the enrolment visit, the cervix was scraped with an Ayre's spatula and with an endocervical brush. The scraped cells were suspended in 5 ml phosphate-buffered saline, pH 7.2, supplemented by merthiolate 1:10 000 v/v. Part of the cell suspension was stored at -80 °C until analysis with the SPF10/LiPA system.[17] After amplification, PCR products were analyzed by 3% agarose gel electrophoresis and the HPV DEIA, a microtiter-plate based assay for general detection of SPF amplimers. Typing of the SPF amplimers was performed with reverse hybridization in the Line Probe Assay (the Inno-LiPA HPV prototype research assay). By LiPA low risk HPV types 6, 11, 34, 40, 42, 43, 44, 53, 54, 59, 70 and 74 can be identified, as well as the high risk types 16, 18, 31,

33, 35, 39, 45, 51, 52, 56, 58, 66 and 68.[16] The high risk types as a group are denoted as oncogenic HPV. SPF amplimers not identified by LiPA are designated as type X. Isolated DNA was controlled by the ß-globin primers PC03 and PC04.[19]

STATISTICAL ANALYSIS

In the analysis, we considered all oncogenic HPV types as a group, the five most prevalent HPV types separately, and the presence of multiple oncogenic HPV types. The five most prevalent HPV types could be analyzed separately because combinations between these types did not occur more frequently than expected by chance alone (as determined with the χ^2-test; see Results section). The severity of the cervical lesion was divided into five diagnostic categories. The lifetime number of sexual partners and the number of cigarettes smoked a day were divided into four categories according to the calculated quartiles. In the analysis of the association between HPV and these variables, the χ^2-test for trend was used. P-values of 0.05 or less were considered to be statistically significant.

To compare the prevalence of specific HPV types, as determined with SPF10/LiPA, between the GP5/6 positive and negative group, we used the χ^2-test. To correct for multiple testing according to Bonferroni, P-values of 0.005 or less were considered to be statistically significant. The SPSS software package was used for the statistical analysis.

RESULTS

SPF10/LiPA RESULTS

A total of 299 women (98%) tested positive for one or more HPV types. Oncogenic HPV was detected in 288 women (95%). The five most prevalent HPV types were HPV 16 (50%), HPV 18 (16%), HPV 31 (21%), HPV 33 (12 %) and HPV 56 (9.9%) (Table 1). In 86 (30%) of the 288 women infections with more than one oncogenic HPV types were identified: 68 women disclosed 2 types, 15 disclosed 3 types, 1 disclosed 4 types and 2 women disclosed 5 oncogenic HPV types.

Table 1: HPV types detected with SPF/LiPA in 304 women with cervical dyskaryosis*

HPV	N	(%)	HPV	N	(%)	HPV	N	(%)
Negative	5	(1.6)	35	13	(4.3)	53	2	(0.7)
			39	2	(0.7)	54	1	(0.3)
6	9	(3.0)	40	2	(0.7)	56	30	(9.9)
11	16	(5.3)	43	1	(0.3)	58	14	(4.6)
16	152	(50)	44	3	(1.0)	66	3	(1.0)
18	49	(16)	45	8	(2.6)	68	1	(0.3)
31	65	(21)	51	7	(2.3)	Type X	7	(2.2)
33	35	(12)	52	19	(6.3)			

* A total of 86 women harbored multiple types

HPV 16 was present in 50% and HPV 18 in 16% of the women in the study population. If these two HPV types were to occur independently, HPV 16 would be found together with HPV 18 in 8% of the women. Instead, their conjoint presence was actually found in only 4% (p<0.001; χ^2 test) (Table 2). Likewise, the combinations of HPV 16 with HPV 31 and HPV 16 with HPV 33 were also seen significantly less frequently than expected by chance alone (p<0.001 and p=0.002, respectively; χ^2 test) (Table 2). Similar differences were not observed for the combinations of the HPV types 18, 31, 33 and 56.

Table 2: Combinations between HPV 16 and the other four most prevalent HPV types

HPV types	no (%) of combinations			
	expected		observed	
16 and 18	24	(7.9)	12	(3.9)*
16 and 31	33	(11)	16	(5.3)*
16 and 33	18	(5.9)	9	(3.0)$
16 and 56	15	(4.9)	11	(3.6)*

*	<0.001, χ^2-test
$	p=0.002, χ^2-test

HPV AND GRADE OF CERVICAL NEOPLASIA

The overall occurrence of oncogenic HPV was significantly related to the histological severity of the neoplastic lesion (Table 3, p < 0.001, χ^2 for trend).

When the five most prevalent HPV types were analyzed separately, HPV 16 was the only HPV type for which a significant association with the grade of the neoplastic lesion could be observed (Table 3, p < 0.001, χ^2 for trend). When all oncogenic HPV types other than HPV 16 were analyzed together, no association with the grade of neoplasia could be observed. No association was found between the presence of multiple oncogenic HPV types and the grade of the neoplastic lesion.

Table 3: HPV and the grade of cervical neoplasia

	grade of neoplasia				
	no dyspl. N=40	CIN I N=35	CIN II N=44	CIN III N=172	(M)IC N=13
HPV	n (%)	n (%)	n (%)	n (%)	n (%)
Oncogenic HPV	34 (85)	31 (89)	41 (93)	170 (99)	12 (92)*
HPV 16	10 (25)	10 (29)	15 (34)	108 (63)	9 (69)*
HPV 18	6 (15)	11 (31)	10 (23)	20 (12)	2 (15)
HPV 31	10 (25)	11 (31)	12 (27)	30 (17)	2 (15)
HPV 33	3 (7.5)	6 (17)	3 (6.8)	22 (13)	1 (7.7)
HPV 56	7 (18)	4 (11)	8 (18)	10 (5.8)	1 (7.7)
Number of oncogenic types					
1	25 (63)	17 (49)	27 (61)	124 (72)	9 (69)
≥ 2	9 (23)	14 (40)	14 (32)	46 (27)	3 (21)
* p < 0.001, χ^2 for trend					

HPV AND EPIDEMIOLOGICAL RISK FACTORS

The lifetime number of sexual partners was categorized according to the calculated quartiles into 1, 2-3, 4-9 and ≥10. No association was found between the lifetime number of sexual partners and oncogenic HPV, the five most prevalent HPV types and the presence of multiple oncogenic types (Table 4).

Table 4: HPV and the lifetime number of sexual partners

	Lifetime number of sexual partners			
	1	2-3	4-9	≥ 10
	N=48	N=73	N=78	N=94
HPV	n (%)	n (%)	n(%)	n(%)
Oncogenic HPV	44 (92)	65 (89)	76 (97)	91 (98)
HPV 16	20 (42)	33 (45)	42 (54)	51 (54)
HPV 18	4 (8.3)	18 (25)	8 (10)	16 (17)
HPV 31	9 (19)	7 (9.6)	19 (24)	26 (28)
HPV 33	5 (10)	5 (6.8)	9 (12)	15 (16)
HPV 56	6 (13)	5 (6.8)	7 (9.0)	9 (11)
Number of oncogenic types				
1	35 (73)	51 (70)	51 (65)	59 (63)
≥ 2	9 (19)	14 (19)	25 (32)	33 (35)

The number of cigarettes smoked per day was categorized into 0, 1-10, 11-20 and ≥21. Again, no association was found between smoking and oncogenic HPV, the HPV types 16, 18, 31, 33 and 56 separately, and the presence of multiple oncogenic HPV types (Table 5).

Table 5: HPV and lifetime number of cigarettes smoked per day

	number of cigarettes per day			
	0	1-10	11-20	≥21
	N=109	N=50	N=83	N=62
HPV	n(%)	n(%)	n(%)	n(%)
Oncogenic HPV	100 (92)	49 (98)	78 (94)	61 (98)
HPV 16	47(43)	29(58)	45(54)	31(50)
HPV 18	19(17)	8(16)	12(15)	10(16)
HPV 31	21(19)	9(18)	22(27)	13(21)
HPV 33	15(14)	3(6.0)	9(11)	8(13)
HPV 56	11(10)	3(6.0)	7(8.4)	9(15)
Number of oncogenic types				
1	71(65)	41(82)	49(59)	41(66)
≥ 2	29(27)	8(16)	29(35)	20(32)

COMPARISON OF TWO HPV PCR SYSTEMS

In the 304 patients of the present study, a total of 187 (73%) women had been tested positive with the GP5/6 PCR. It follows that the SPF10/LiPA detects significantly more women with cervical HPV than the GP5/6 (98% vs. 73%, p<0.001, McNemar). For all but four types (HPV 43, 54, 62 and 68) additional positives were found with the SPF10/LiPA (data not shown). Therefore, the higher sensitivity of the SPF10/LiPA applies to almost all of the tested HPV types. However, the higher sensitivity did not apply equally to all types. HPV 16 was found significantly less often in GP5/6 negative women than in GP5/6 positive women (18% vs. 62%, p<0.001 McNemar), whereas the opposite was true for HPV 31 (33% vs. 17%, p=0.003 McNemar).

DISCUSSION

We could not confirm previously reported associations between HPV and lifetime number of sexual partners and smoking when a new and highly sensitive HPV PCR system, the SPF10/LiPA, was used for the detection and typing of HPV. However, the association between the presence of oncogenic HPV (any type) and the grade of neoplasia remained and turned out to be determined by the influence of HPV type 16 alone.

The SPF10/LiPA was performed five years later than the GP5/6. The results do not suggest that the quality of cell suspensions diminished due to storage, because the HPV detection rate was higher when the SPF10/LiPA was used. High HPV detection-rates using the SPF10/LiPA were described previously.[16,17] The use of the new test disclosed a substantial number (30%) of multiple infections, which enabled us to analyse co-occurrences of specific HPV types. Although HPV 16 was the most prevalent HPV type in the presented population, it was detected significantly less often in multiple infections than expected. We can only speculate about the origin of this phenomenon. A possible explanation is competition in the PCR which can happen when the SPF primers are more sensitive for the detection of HPV 16 than for other types. Annealing of primers could than occur only to HPV 16 DNA although DNA of other HPV types is present in the same sample. Another reason for competition in the PCR-reaction could be that viral loads of HPV 16, when present, are higher than that of other HPV types. Then the excess of HPV 16 DNA can lead to a false negative PCR result for other HPV types present, which can result in observing HPV16 infections as single type infections. Alternatively, HPV 16 might be more oncogenic than the other types. HPV 16, present as a single type infection, might lead to cervical neoplasia more easily than single type infections of other HPV types. These other types might need more factors present, for example other HPV types, to lead to the same grade of neoplasia. Our data do not support the theory that the other types have additive effects in oncogenesis. The presence of multiple HPV types was not associated with the histological severity of the neoplastic lesion, although an increasing percentage of multiple infections with increasing grade of neoplasia was reported previously. A much more speculative explanation is that HPV 16 induces mechanisms which inhibit infections with other HPV types.

HPV 16 was the most common HPV type in this study population and therefore determined the association found for the oncogenic HPV as a group to a great extent. The fact that HPV 16 is the only HPV type significantly associated with the grade of neoplasia has been reported by another group of investigators.[20] In most epidemiological HPV research all oncogenic HPV types are taken together, for example in determining risk factors for a HPV infection.[10,14,15,21,22] It will be interesting to analyze in other populations whether the strength of risk factors for HPV infections is a type-dependent phenomenon, although our data revealed no indication for that.

The GP5/6 is no longer used in epidemiological HPV research. In more recent studies the GP5+/6+ and MY 09/11 PCR's are the most widely applied. These latter two PCR systems have comparable sensitivities.[23] As with the GP5/6 PCR, associations between HPV and smoking[11,12] and number of sexual partners[8-10,12-15] have been reported when the MY 09/11 PCR was used for the detection of HPV. We could not reproduce our findings when the SPF10/LiPA system was used for detection and typing of HPV.

The discrepancies between the results with the GP5/6 and SPF10/LiPA cannot be explained by a difference in the spectrum of HPV types detected by the two systems. Only for HPV types 16 and 31 the spectra differed. For these two types no association was found with the lifetime number of sexual partners or smoking behaviour. Women with one partner, or anyhow a comparatively low number of partners, might have a lower viral load of HPV. Whether this is indeed true and what the mechanism of action is, needs further investigation. Sexually transmitted agents might play a role in this respect. Promiscuity has been reported as a risk factor for the development of cervical neoplasia, independent of HPV.[13] Furthermore a number of studies reported an association between cervical neoplasia and the presence of antibodies against *Chlamydia trachomatis,*[24-27] and underlying possible pathogenetic mechanisms have been discussed.[28] We suggest that the presence of *Chlamydia trachomatis* might contribute to persistence of an HPV infection and/or an increase of the HPV viral load. Likewise, smoking might increase viral load by its impact on the immune system. In this respect, it is worthwhile mentioning that the number of Langerhans cells is decreased in smokers as compared to non-smokers.[29,30] The possible impact of risk factors, like number of partners and smoking, on viral load of HPV was recently also discussed by another group of investigators.[31]

The use of a new detection method in this epidemiological study has put associations between HPV on the one hand and lifetime number of sexual partners and smoking on the other into a different perspective. We suggest that the number of sexual partners and smoking are determinants of a high HPV viral load, rather than determinants for the presence of HPV per se.

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Detecting cervical cancer by quantitative promotor hypermethylation assay on cervical scrapings: A feasibility study

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ABSTRACT

PURPOSE

Current morphology based cervical cancer screening is associated with significant false positive and false negative results. Tumor suppressor gene hypermethylation is frequently present in cervical cancer. It is unknown whether a cervical scraping reflects the methylation status of the underlying epithelium and it is therefore unclear whether QMSP on cervical scrapings could be used as a future screening method augmenting the current approach.

EXPERIMENTAL DESIGN

Cervical scrapings and paired fresh frozen cervical tissue samples were obtained from 53 cervical cancer patients and 45 controls. All scrapings were morphologically scored and analyzed with quantitative hypermethylation specific PCR (QMSP) for the genes *APC*, *DAPK*, *MGMT* and *GSTP1*. To adjust for DNA input, hypermethylation ratios were calculated against DNA levels of a reference gene.

RESULTS

Hypermethylation ratios of paired fresh frozen tissue samples and scrapings of cervical cancer patients and controls were strongly related (Spearman correlation coefficient 0.80 for *APC*, 0.98 for *DAPK* and 0.83 for *MGMT*, p<0.001). More cervical cancer patients than controls were *DAPK* positive (p<0.001). When cut-off levels for ratios were defined to be above the highest ratio observed in controls, QMSP in cervical scrapings identified 32 of 48 (67%) cervical cancer patients.

CONCLUSIONS

This feasibility study demonstrates that QMSP on cervical scrapings holds promise as a new diagnostic tool for cervical cancer. The addition of more genes specifically methylated in cervical cancer will further improve the assay.

INTRODUCTION

Cervical cancer is an important cause of death in women world wide.[1] There is a strong association between certain subtypes (high risk) of Human Papillomavirus (HPV) and cervical cancer.[2] However, it is clear that other factors are also involved in cervical carcinogenesis, because the majority of patients infected with HPV will not develop invasive cervical cancer.[3]

Cytomorphological examination of cervical smears is the most widely applied screening-method for cervical cancer and its precursors. The Pap-smear has false negatives rates of 2 - 40% due to a combination of sampling error, processing artifacts and the nature of subjective interpretation.[1,4,5] False-negative cytology may lead to a delay in the diagnosis of cervical cancer and can be found in about 50% of cases when previous negative smears are reviewed from the small proportion of screened women who develop invasive cancer.[5] Moreover, as many as 20% of all Pap-smears are interpreted as atypical squamous cells of undetermined significance (ASCUS) or borderline dyskaryotic, leading to increased surveillance frequency and more invasive tests in many of these patients.[4,6] Although it has been suggested that high risk HPV testing may well improve cervical cancer screening,[7,8] the specificity for high grade cervical neoplasia of high risk HPV testing is relatively low.[9] Therefore, new objective diagnostic methods are needed.

Silencing of tumor suppressor- or other cancer-associated genes by methylation of CpG islands, located in the promoter and/or 5'-regions of many genes, is a common feature of human cancer.[10] CpG island methylation is often associated with a transcriptional block and loss of the relevant protein.[10] In addition to the functional implications of gene inactivation in tumor development, these aberrant methylation patterns represent excellent targets for novel diagnostic approaches based on methylation sensitive PCR techniques.

Recently Dong *et al.* showed that promoter hypermethylation of at least one of the genes *P16, DAPK, MGMT, APC, HIC-1,* and *E-cadherin* occurred in 79% of cervical cancer tissues and in none of normal cervical tissues from 24 hysterectomy specimens.[11] Virmani *et al.* detected aberrant methylation of at least one of the genes *P16, RARß, FHIT, GSTP1, MGMT* and *hMLH1* in 14 of 19 cervical cancer tissue samples.[12] These experiments were carried out using conventional methylation specific PCR (MSP). An advancement of this technique is real-time quantitative MSP (QMSP) which permits reliable quantification of methylated DNA. This method is based on the continuous optical monitoring of a fluorogenic PCR. This PCR approach is more sensitive and more specific than conventional PCR and can therefore detect aberrant methylation patterns in human samples in the presence of normal DNA in a ratio of 1 to 10,000.[13]

Currently no data are available on whether cervical scrapings reflect the methylation status of the underlying cervical epithelium and it is unknown whether QMSP on

cervical scrapings could be used as a future screening method. In the present study we examined the promotor hypermethylation status of the tumor suppressor genes *APC*, *DAPK, GSTP1* and *MGMT* in cervical scrapings and paired fresh frozen tissue samples obtained from cervical cancer patients and controls.

PATIENTS AND METHODS

PATIENTS

CERVICAL CANCER PATIENTS

In the period March 2001 - August 2003 all patients referred because of cervical cancer or abnormal cervical cytology were asked to participate in our research program during their initial visit to the outpatient clinic of the University Hospital Groningen. In order to obtain a homogeneous population we chose for the present study to only analyze those patients diagnosed with squamous cell cervical cancer, in whom cervical cancer had not been fully removed by exconisation or loop excision before referral. Gynecologic examination under general anesthesia was performed in all cervical cancer patients for staging in accordance with the Fédération Internationale de Gynécologie - Obstétrique (FIGO) criteria.[14] During this procedure lesion size (largest diameter) and tumor spread beyond the cervix were estimated routinely.

All cervical scrapings were collected during the initial visit or before bimanual examination under general anesthesia. All tissue samples used for the study were collected during bimanual examination or at surgery, which was chosen as primary treatment for patients with stage IB1/IIA (tumor size ≤ 4 cm) cervical cancer.

CONTROLS

As controls served patients without a history of abnormal Pap-smears, who were planned to undergo a hysterectomy because of non-(pre)malignant disease in the same study-period. All samples used for the study were collected during surgery. Cervical epithelium of all control patients was confirmed to be normal on final histopathologic review.

The study was approved by the medical ethical committee of the University Hospital Groningen and all patients gave written informed consent.

SAMPLE COLLECTION

CERVICAL SCRAPINGS

The cervix of both cervical cancer and control patients was scraped with the blunt or pointed end of an Ayre's spatula and with an endocervical brush. The scraped cells were suspended in 5 mL ice-cold phosphate buffered saline and kept on ice until further processing. Of this cell suspension 1 mL was used for cytomorphological examination and 4 mL was centrifuged and washed with wash buffer, as described previously.[15] Subsequently a quarter of the pellet was snap frozen in liquid nitrogen and then stored at -80 °C until further use for DNA extraction. DNA was extracted using standard salt-chloroform extraction and ethanol precipitation for high molecular DNA and dissolved in 250 μL TE-4 buffer (10 mM Tris; 1 mM EDTA (pH 8.0))

TISSUE SAMPLES

For the present study fresh frozen tissue samples from the diagnostic or therapeutic

specimens were only taken when the pathologist was convinced it would not interfere with the diagnostic process. Fresh frozen tissue samples were snap frozen and then stored at -80 °C until further use for DNA extraction. Tumor tissue was selected from an area with > 75% malignant cells as determined on a hematoxylin and eosin stained slide. Normal cervical epithelium was selected from control patients samples by grossly dissecting it from underlying stromal tissue. DNA was extracted from 10 unstained 10 µm frozen sections of the tissue samples by standard salt-chloroform extraction and ethanol precipitation and subsequently dissolved in 250 µL TE-4 buffer.

REAL-TIME QUANTITATIVE METHYLATION SPECIFIC PCR

QMSP for *APC, DAPK, GSTP1* and *MGMT* was performed after bisulfite treatment on denatured genomic DNA[16] as previously reported for *APC* and *GSTP1*.[17,18] As internal reference gene the *ß-actin* gene was chosen. The amplicon sizes for the QMSP were 74bp for *APC* (position 761-834, deposited at GenBank as accession no. U02509), 101 bp for *DAPK* (5-102, X76104), 122 bp for *MGMT* (1029-1150, X61657) and 140 bp for *GSTP1* (1033-1172, M24485). The basis of primer design has been previously described.[17,19] For primer sequences, see Table 1.

Table 1 : Primer and probe sequences used for the QMSP and reference gene analysis

Gene	Forward primer (5' — 3')	Reverse primer (5' — 3')	TaqMan probe (6FAM5' — 3'TAMRA)
methylated *APC*	AACCAAAACGCTCCC CAT	TTATATGTCGGTTACG TGCGTTTATAT	CCCGTCGAAAACCCGC CCGATTA
methylated *GSTP1*	GTTGCGCGGCGATTT C	GCCCCAATACTAAATC ACGACG	GGTCGACGTTCGGGGT GTAGCG
methylated *MGMT*	CGAATATACTAAAACA ACCCGCG	GTATTTTTTCGGGAGC GAGGC	ATCCTCGCGATACGCA CCGTTTACG
methylated *DAPK*	GGATAGTCGGATCGA GTTAACGTC	CCCTCCCAAACGCCG A	TCGGTAATTCGTAGCG GTAGGGTTTGG
ß-actin	GGTGATGGAGGAGGT TTAGTAAGT	AACCAATAAAACCTAC TCCTCCCTTAA	ACCACCACCCAACACA CAATAACAAACACA

Amplifications were carried out in 384-well plates. As positive controls serial dilutions of in vitro CpG methylated DNA with Sss I (CpG) methylase (New England Biolabs. Inc., Beverly, MA) were used by which a calibration curve was constructed for each plate. The calibration curve was used to set a plate specific threshold for positivity and to determine DNA equivalents for the results obtained. Multiple water blanks were included as negative controls. Dilution experiments showed linearity of amplification down to a dilution of 1:10,000 for methylated promoter DNA, as well as for unmethylated *ß-actin* DNA. All samples were analyzed at least in duplicate. For quality control all amplification curves were visualized and scored without knowledge of the clinical data. Per analysis the methylation result was considered positive when the QMSP amplification curve crossed the set treshold before 50 cycles. However, amplification above threshold without an exponential curve was considered to be the

result of stochastic amplification and the results of such a single analysis was therefore disregarded. Samples that were only one time 'positive' or 'negative' in duplicate analyses after quality control were analyzed at least in quadruplicate. No samples had to be disregarded because of stochastic amplification since sufficient analyses were performed to obtain reproducable results. In further statistical analysis, for every single gene only a sample with sufficient DNA input (at least 225 pg β-actin DNA because in samples with DNA input below 225 pg stochastic amplification was too frequent) that was positive at least twice after multiple analyses was considered to be hypermethylation positive. A sample with sufficient DNA input that was negative at least twice and at most one time positive after multiple analyses was considered to be negative.

CYTOMORPHOLOGIC EXAMINATION

After scraping of the cervix 1mL of cell suspension was diluted with ethanol-carbowax (7% Polyethyleneglycol, 50% ethanol) and after resuspending centrifuged for 10 minutes at 1000 rpm. The cell-pellet was resuspended in ethanol-carbowax until an appropriate cell concentration was obtained. Hettich cytospins (Hettich centrifuge, Depex b.v., Veenendaal, the Netherlands) were made on Poly-L-Lysin (Sigma chemical c.o., St. Louis, MO) treated slides by centrifugation for 10 minutes at 1000 rpm. Cytospins were Papanicolaou stained and routinely classified by two independent pathologists without knowledge of the clinical data. In the Netherlands cervical smears are classified according to a modified Papanicolaou system in which borderline dyskaryosis corresponds well with the Bethesda classification ASCUS, mild dyskaryosis with low grade squamous intraepithelial lesion (LSIL) and moderate and severe dyskaryosis and carcinoma in situ with high grade intraepithelial lesion (HSIL).[20,21]

STATISTICAL ANALYSIS

QMSP values were adjusted for DNA input by expressing results as ratios between two absolute measurements ((average DNA quantity of methylated gene of interest / average DNA quantity for internal reference gene β-actin) x 10000) (more detailed information was described previously[13,17]). The correlation between methylation ratios as determined in cervical scrapings and in fresh frozen tissue samples was tested for all genes separately by the Spearman test. Differences in the heights of ratios between cancer patients and controls were tested with the Mann-Whitney U test for all genes. To evaluate the clinical value of QMSP in cervical scrapings 'screen positive' cutoff values were chosen above the highest ratio observed in controls for all genes separately.

Observed differences were considered to be significant when associated with $p \leq 0.05$. All analyses were carried out using the SPSS software package (SPSS 11.5, Chicago, IL, USA).

RESULTS

PATIENT POPULATION

In the period March 2001 - August 2003 108 patients met our inclusion criteria. Four patients refused to participate in the study; in two patients no cervical scraping was taken; in three the scraping taken was lost for analysis and one control patient was excluded because of complex atypical hyperplasia of the endometrium. The specimens of 98 patients, 53 squamous cell cervical cancer patients and 45 controls were used for further analysis. FIGO stage IA cervical cancer was diagnosed in 5 (9.4%); stage IB1 in 20 (38%); stage IB2 in 3 (5.7%); stage IIA in 5 (9.4%); stage IIB in 14 (26%); stage III in 5 (10%) and stage IV in 1 (1.9%) of the 53 cervical cancer patients. Indications for hysterectomy were uterine myomas in 20 (44%), uterine prolapse in 20 (44%), menometrorrhagia in 4 (8.9%) and severe dysmenorrhea in 1 (2.2%) of the 45 controls. The cervical cancer patients had a median age of 46 years (IQR 37-59), the controls of 49 years (IQR 44-60).

ADEQUACY OF CERVICAL SCRAPINGS TO REPRESENT THE HYPERMETHYLATION STATUS OF THE UNDERLYING CERVICAL EPITHELIUM

In order to assess whether cervical scrapings reflect the hypermethylation status of the underlying cervical epithelium we performed QMSP analysis on cervical scrapings and paired fresh frozen tissue samples. DNA quality was sufficient to perform QMSP in 89 (48 cervical cancer scrapings and 41 control scrapings) of the 98 available scrapings. Paired fresh frozen tissue samples with sufficient DNA quality were available of 39 patients (21 cervical cancer and 18 control samples); in 23 control cervices the epithelial orientation was insufficient to dissect epithelium from surrounding stromal tissue; in 21 cervical cancer patients no fresh frozen tissue sample was taken because of possible interference in the diagnostic process and in 13 an insufficient percentage (<75%) of cancer cells was present. The observed ratios between methylated DNA and reference DNA of cervical scrapings were compared with the ratios observed in fresh frozen tissue samples. In a total number of 156 (39 samples x 4 different genes) paired QMSP results the QMSP result of scraping and paired tissue corresponded in 152 (97%) analyzed pairs (Table 2).

Table 2: Correspondence of QMSP results in cervical scrapings with QMSP results in paired fresh frozen tissue samples.

Gene	Number of C[a]-Positives	Number of C-Negatives	Number of NC[b]- Positives	Number of NC-Negatives
APC	9	27	3	0
DAPK	14	25	0	0
MGMT	3	35	0	1
GSTP1	0	39	0	0
a	Corresponding			
b	Noncorresponding			

All three noncorresponding positive scrapings were found in controls and in two of these three *ß-actin* DNA was much higher for the scraping than for the corresponding tissue sample (25 ng more *ß-actin* DNA in the scraping than in the tissue sample in one sample and 11 ng more in the other sample). Spearman correlation coefficients were calculated for the genes with positive results in the 39 paired samples. High correlation coefficients were observed (0.80 (p<0.001) for *APC,* 0.98 (p <0.001) for *DAPK* and 0.83 (p<0.001) for *MGMT*), indicating that high hypermethylation ratios were present in scrapings from patients also harboring tumors with high hypermethylation ratios as illustrated for *APC* and *DAPK* in figure 1. Comparable correlation coefficients were obtained when cervical cancer samples and control samples were analyzed separately. It was concluded that the hypermethylation status of cervical epithelium is well represented by cervical scrapings.

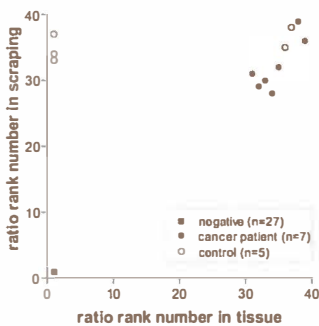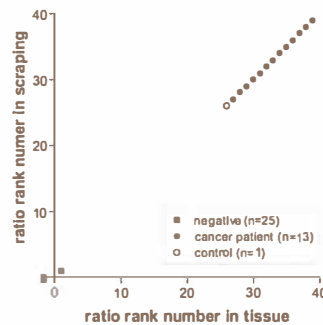**Figure 1A:** *APC*   **Figure 1B:** *DAPK*

The correlation between methylation levels in paired scrapings and in tissue samples for *APC* (A) and *DAPK* (B) expressed by rank numbers assigned. Rank numbers were assigned because the observed ratios were not normally distributed. Each circle represents a different sample. The square represents samples that are negative both in tissue and in scraping.

CLINICAL USE OF QMSP IN CERVICAL SCRAPINGS FOR CERVICAL CANCER DETECTION

In order to assess the possible clinical use of QMSP in cervical scrapings for cervical cancer detection, we performed QMSP on cervical scrapings and compared results obtained in cancer patients with results obtained in controls. All available 89 scrapings with sufficient DNA quality were included in further analysis since cervical scrapings represent the hypermethylation status of the underlying epithelium well. Methylated *APC* was detected in 26 (54%), *DAPK* in 35 (73%), *MGMT* in 5 (10%) and *GSTP1* in one (2%) of 48 cervical cancer scrapings. In 41 control scrapings methylated *APC* was amplified in 16 (39%), *DAPK* in 2 (4.9%, p<0.001), *MGMT* in 6 (15%) and *GSTP1* in none. Methylation ratios are illustrated in figure 2 for *APC* and *DAPK*.

The ratio cutoff value for each individual gene to be called 'screen positive' was arbitrarily defined to be above the highest ratio observed in controls. *APC* ratios were 'screen positive' in 6 (13%), *DAPK* ratios in 26 (54%), *MGMT* in two (4.2%) patients and *GSTP1* in one (2.1%). Three patients were 'screen positive' for more than one

gene, two for *DAPK* and *APC* and one for *DAPK* and *MGMT*. Overall 32 of 48 (67%) cervical cancer patients were 'screen positive' for at least one gene. When cutoff values for all genes were based on the 95th percentile observed in controls, 5 (12%) controls would be 'screen positive' for at least one gene and 35 (73%) of the cervical cancer patients. When a fixed cutoff value of 100 (average DNA quantity of methylated gene of interest / average DNA quantity for internal reference gene *ß-actin*) x 10000=100) for each gene was chosen, positives were only observed for *APC* (n=4 (8.3%)) and for *DAPK* (n=12 (25%)), and 15 (31%) patients were 'screen positive' for at least one of these two genes.

MORPHOLOGIC PAP-SMEAR CLASSIFICATION AND REAL-TIME QMSP

For comparison with cytomorphological screening we used the cutoff values as defined above the highest control, because this appeared to be the optimal balance between sensitivity and specificity.

CONTROLS

For the 45 controls a morphologic Pap-smear classification was not obtained for nine because the cervical scrapings of five controls were not available and another four scrapings were inadequate for cytomorphologic assessment. All four inadequate scrapings had sufficient DNA input to perform QMSP. Although the cervical epithelium of all controls was histologically diagnosed to be normal, six scrapings were cytomorphologically classified with borderline dyskaryosis (comparable to ASCUS)(Table 3). In five of these six DNA input was sufficient to perform QMSP and all these scrapings were negative (partly due to our definition). Three of the four scrapings with insufficient input DNA for QMSP were taken from control patients operated on because of prolapse uteri and one was taken from a control patient with myomas.

Table 3: Morphological Pap-smear classification related to hypermethylation results in cervical scrapings of control patients.

Morphologic classification	Number of patients	Sufficient[a] DNA	Screen positive[b]
No dyskaryosis	30	27	0
Borderline dyskaryosis	6	5	0
Inadequate	4	4	0
Not assessed	5	5	0
Total	45	41	0

a adequate DNA input, defined as *ß-actin* DNA above 225 pg.

b 'screen positive' is hypermethylation status defined as ≥ 1 of the analyzed tumor suppressor genes positive above the
   defined cut off value

CANCER PATIENTS

In the 53 cancer patients a morphologic Pap-smear classification was not obtained for ten because the cervical scrapings of two cancer patients were not available for classification and another eight scrapings were inadequate for cytomorphologic assessment. Of these eight cervical cancer scrapings seven had sufficient DNA input for QMSP of which three showed 'screen positive' ratios for at least one of the four genes

analyzed. Four cervical cancer scrapings yielded insufficient DNA input even though the sample was adequate for cytomorphological assessment. In three of these very few dysplastic cells were seen and in the other two scrapings many dysplastic cells accounted for the dysplastic morphological result. One cervical cancer patient was underdiagnosed by cytomorphological assessment which showed only borderline dyskaryosis (Table 4). In this borderline dyskaryotic scraping DNA quality was sufficient for QMSP, however, no 'screenpositive' ratio was observed for one of the four evaluated genes, although DAPK amplification was present in the scraping. Of this patient no tissue sample was available for analysis.

Table 4: Morphological Pap-smear classification related to FIGO stage and hypermethylation results in cervical scrapings of cervical cancer patients.

Morphologic classification	Number of patients	FIGO stage			Sufficient DNA input [b]	Screen positive [b]
		IA	IB/IIA	IIB-IV		
Borderline dyskaryosis	1	1	1	0		
Severe dyskaryosis/CIS	19	4	10	5	1B	11
Squamous cell cancer	23		12	11	20	17
Inadequate	B	1	4	3	7	3
Not assessed	2		1	1	2	1
Total	53	5	2B	20	48	32

a adequate DNA input, defined as B-actin DNA above 225 pg.

b 'screen positive' for hypermethylation is defined as ≥ 1 of the analyzed tumor suppressor genes positive above the defined cutoff value

DISCUSSION

Important drawbacks of conventional screening for cervical cancer have spurred the search for continued improvement of diagnostic accuracy of the Pap test. In this feasibility study we show that cervical scrapings can be used to detect hypermethylation of tumor suppressor genes in cervical cancer because the hypermethylation status of the tumor was well represented by cervical scrapings and 67% of the cervical cancer patients could be identified by QMSP for *APC, DAPK, MGMT* and *GSTP1*.

The four tumor suppressor genes analyzed in the present study were chosen because promoter hypermethylation and transcriptional repression of these genes might mediate tumorigenesis as demonstrated in cervical and other squamous cell cancers by conventional MSP.[11,12,19] *MGMT* is a DNA repair gene, *GSTP1* is a detoxifying gene, *DAPK* is a proapoptotic gene and potentially inhibits metastasis and *APC* mediates proliferative signals.[22-26] Analyzing paraffin embedded tissue samples by conventional MSP demonstrated hypermethylation of *APC, DAPK* and *MGMT* in 31%, 61% and 10% of squamous cell cervical cancers, respectively.[11] These percentages correspond well with our observations by analyzing cervical scrapings with QMSP. Adenocarcinoma subtypes of cervical cancer have distinct hypermethylation patterns.[11] *GSTP1* methylation was detected in 4 of 19 cervical cancer tissues of unknown histological cell type,[12] whereas in our series of 48 squamous cell cervical cancer patients *GSTP1* hypermethylation was demonstrated in only one patient. *GSTP1* may be especially associated with the adenomatous cell type, as *GSTP1* is frequently present in prostate adenocarcinoma, while it was demonstrated in only 3 of 73 transitional cell carcinoma samples of bladder cancer patients.[27,28]

Our study shows that cervical scrapings represent the hypermethylation status of underlying cervical epithelium well, given the high correlation coefficients between hypermethylation ratios of scrapings and tissue samples for all genes analyzed. No other data are available on a direct comparison between paired cervical scrapings and underlying tissue samples. Chan *et al.* showed frequent methylation of *RARß, DAPK, E-cadherin, P16, P15, GSTP1* and *MGMT* in urinary bladder cancer and 21 paired voided urine samples.[28] MSP analysis in voided urine resulted in no noncorresponding positives when compared to paraffin embedded tumors. However, noncorresponding negative results were frequent (17 (19%) of 88 paired MSP analyses). Still, MSP analysis for 7 genes was more sensitive for detection of bladder cancer than morphologic assessment of urine. Jéronimo *et al.* analyzed *GSTP1* hypermethylation in voided urine and frozen or paraffin embedded tissue samples of patients with prostate cancer by *MSP* and *QMSP* and showed that in urine no noncorresponding positives were observed, but noncorresponding negatives were frequent (50 (73%) and 42 (61%) of 69 paired analyses with *MSP* and *QMSP,* respectively).[27]

In our study on cervical scrapings no noncorresponding negatives were observed for *APC, DAPK* and *GSTP1* and only one noncorresponding negative was observed for *MGMT*. Testing cervical scrapings for cancer by molecular changes-based assays will likely continue to give rise to false negatives, especially due to sampling errors. Even with sensitive molecular assays, false negatives will occur if no tumor cells are collected by scraping the cervix. However, our data show that only a very low number of dysplastic cells appears to be necessary for an adequate QMSP since DNA input was sufficient for QMSP in seven of eight cervical cancer scrapings that were inadequate for cytomorphological assessment while three of these seven were 'screen positive'.

For *DAPK* no noncorresponding positives were observed in scrapings. *MGMT* and *GSTP1* analysis hardly contributed to the identification of cervical cancer patients by QMSP on cervical scrapings and for *APC* three noncorresponding positives were observed. For two of these three the explanation for the conflicting results may be the higher (>11 ng more ß-actin DNA input) *ß-actin* DNA levels in the scrapings than in the tissue samples. However, the three false positive results could also have been the result of technical failure in the tissue samples, meaning that the tissue samples were *APC* hypermethylation positive although not detected by QMSP. Another explanation may be that *APC* hypermethylation was positive in non cervical cancer cells. *APC* methylation has been demonstrated in several non-neoplastic tissues, as has also been shown for *DAPK*.[29-31] In the present study *DAPK* was the gene that was the most cervical cancer sensitive and specific gene despite its detection in two controls who both had normal cervical epithelium as confirmed by final histopathologic review. Cervical scrapings, apart from cervical epithelial cells, may also contain vaginal epithelial cells, cells of the endometrium and leukocytes. Although methylation in non-neoplastic cells may be the reason for some noncorresponding positive results or positive results in controls we still expect consequences for the use of QMSP as a screening tool to be low. Malignant cells usually have significantly higher methylation levels when positive than methylated non-malignant cells. By expressing methylation results as a ratio with a reference gene QMSP takes advantage of this characteristic.[32]

A key issue for molecular diagnosis is the ability to detect cancer cells missed by routine cytopathology. On direct comparison cytomorphologic assessment underestimated one case of stage IB disease in our study, which is not surprising since false-negative rates of the conventional Pap-smear are reported to be between 2 and 40%.[1,4,5] This morphologically false negative smear was not 'screen positive' for QMSP, because the *DAPK/ß-actin* ratio was below cutoff and methylation for none of the three other genes was detected. However, when more genes would have been available for analysis, QMSP might have been able to identify this cervical cancer case, because sample adequacy was sufficient for QMSP.

In our series of cervical cancer patients the frequency of the cytomorphologic results 'severe dyskaryosis/carcinoma in situ' may seem high (37%) in comparison to other studies with conventional Pap-smears. One of the consequences however, of

manufacturing Pap-smears by cytospin, as was performed in our study, is that mucus and debris are largely washed away. In conventional Pap-smears a background of blood and tumor debris distinguishes a cancer smear from a carcinoma in situ smear. Furthermore, smears classified as severe dyskaryosis/carcinoma in situ were not considered by us to be underestimates, because these results lead to immediate referral to a gynecologist.

Apart from the possible identification of cervical cancer patients, missed by cytology, QMSP on cervical scrapings may also have the advantage of hardly any false positive results in women with normal cervical epithelium. Although all cervical epithelia of control patients were confirmed to be normal, 13% of the paired cervical smears was cytologically classified with borderline dyskaryosis. All controls were 'screen negative' for QMSP. Because this 100% specificty was partly due to the definition we used to set cutoff values, future studies are warranted to assess whether the promise of few false-positives by QMSP in cervical cancer screening will hold, which of course will largely depend on how cutoff values will be defined. In our setting, analyzing patients already referred because of cervical cancer and controls, the choice of a cutoff for all genes tested above the highest ratio observed in controls represented the optimal balance between sensitivity and specificity. The distributions of the calculated ratios (figure 2) show that in the future, the choice for a 'screen positive' cutoff value may best be made separately for each gene. APC appears to be less sensitive and specific than DAPK, however, high (above 100) APC ratios may also be very specific for cancer. Defining the cutoff value for APC above 100 will improve specificty. However, sensitivity will be lower than of DAPK and the use of APC hypermethylation for the detection of cervical cancer remains questionable. For future population based screening purposes hypermethylation ratios should be determined by comparing large series of healthy women with series of cervical intraepithelial neoplasia (CIN) and cervical cancer patients and by constructing receiver operating characteristic curves in large preclinical populations.

Theoretically, QMSP appears to be very suitable as a cervical cancer detection technique because the PCR reaction is amenable to high-throughput techniques allowing the analysis of close to 400 samples in less than 2 hours without requirement for gel-electrophoresis. The identification of 67% of cervical cancer patients by QMSP for *APC, DAPK, MGMT* and *GSTP1* demonstrates that it will be necessary to identify other genes more specifically methylated in (cervical) cancer to compose a both sensitive and specific cervical cancer hypermethylation panel. The candidate tumor suppressor gene Tumor suppressor in lung cancer 1 (*TSLC1*) may be such an interesting candidate. Recently Steenbergen *et al.* showed that methylation of *TSLC1* was present in 59% of 49 cancer tissues, 35% of 20 high grade CIN lesions and in none of 11 low grade CIN lesions.[33]

QMSP on cervical scrapings is a promising new diagnostic tool for the detection of cervical cancer and will improve as more genes specifically methylated in cervical cancer are identified and added to the assay.
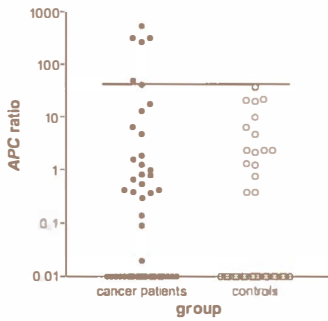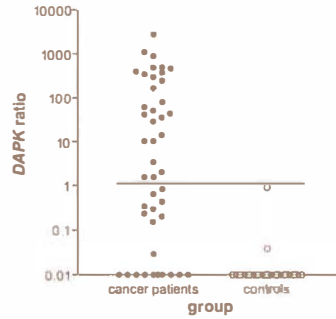
Distribution of APC (A) and DAPK (B) methylation levels in cervical scrapings of cervical cancer patients and controls. Each circle represents a different sample. Values diagrammed at 0.01 are zero values, which can not be plotted correctly on a log scale. The solid horizontal bars represent the defined cut-off values for the presented genes.

ACKNOWLEDGMENTS

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Death receptors and ligands in cervical carcinogenesis: an immunohistochemical study

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ABSTRACT

Increasing imbalance between proliferation and apoptosis is thought to be important in cervical carcinogenesis. The death ligands FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induce apoptosis by binding to their cognate cell-surface death receptors Fas or death receptor (DR) 4 and DR5. The aim of our study was to examine possible changes in expression of these death ligands and their receptors at different stages of cervical carcinogenesis. The immunohistochemical expression and localization of Fas/FasL and DR4/DR5/TRAIL were assessed in 11 normal , 15 cervical intraepithelial neoplasia (CIN) grade I, 15 CIN II, 13 CIN III and 25 (microinvasive) squamous cell cervical cancers. The number of apoptotic cells was determined by the use of morphological criteria and the number of proliferating cells by counting Ki-67 positive cells. A marked increase in proliferation as well as apoptosis percentage was found with increasing severity of neoplasia. In normal cervix and CIN I samples FasL, DR4, DR5 and TRAIL staining was mainly observed in the basal/parabasal layer, whereas Fas staining was localized in the superficial, more differentiated epithelial layer. Frequency of Fas positive staining decreased with increasing severity of CIN. In contrast, homogeneous FasL, DR4, DR5 and TRAIL expression throughout the lesions was more frequently observed in CIN III and cervical cancer. FasL, DR4, DR5 and TRAIL staining patterns were correlated, although TRAIL expression was more intense in low grade lesions. No association was found between death receptor or ligand expression with the percentage of apoptosis or proliferation. The loss of Fas and the deregulation of FasL, DR4, DR5 and TRAIL in the CIN-cervical cancer sequence suggest a possible functional role of these death ligands and receptors during cervical carcinogenesis. Their observed frequent expression present DR4 and DR5 as promising targets for innovative therapy modalities in cervical cancer.

INTRODUCTION

Cervical cancer represents the third most frequent gynecologic malignancy among women worldwide, with the highest incidence rates in less developed countries.[1] Premalignant cervical lesions or cervical intraepithelial neoplasia (CIN) are subdivided in low (CIN I), moderate (CIN II) or high (CIN III) grade lesions, based on morphologic criteria. Most CIN I lesions will regress spontaneously, while 20-45% of untreated CIN II/III lesions will progress to cervical cancer when left untreated.[2]

The primary risk factor for CIN and invasive cervical cancer is infection with oncogenic human papillomavirus (HPV). The oncogenic HPV E6 and E7 proteins are involved in cervical carcinogenesis by their potential to inactivate the tumor suppressor gene products p53 and pRb, respectively. E6 and E7 thereby prevent cell cycle arrest and intrinsic, p53-dependent apoptosis (programmed cell death), thus allowing uncontrolled cell-cycle progression, resulting in an imbalance between proliferation and apoptosis which indeed has been observed during cervical carcinogenesis.[3] Apart from many other cellular proteins involved in apoptosis, activation of apoptotic pathways may also occur by binding of apoptosis-inducing 'death ligands' such as Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to their cognate cell-surface death receptors, Fas and DR4/DR5, respectively.[4] When FasL binds to Fas an intracellular apoptotic cascade is activated.[5] The same accounts for TRAIL, but TRAIL can interact with four distinct receptors. DR4 (TRAIL-R1) and DR5 (TRAIL-R2/TRICK2) have apoptosis-inducing activity, whereas DcR1 (TRAIL-R3/TRID) and DcR2 (TRAIL-R4/TRUNDD), the so-called decoy receptors, can bind TRAIL but lack functional death domains and thus are unable to induce apoptosis.[6]

Expression of FasL, TRAIL and their receptors has been reported in many normal (epithelial) cells and neoplastic cells.[7-11] FasL has been implicated in immune evasion of cancer cells, while the physiological role of Fas extends beyond that exerted on immune cells and is involved in the induction of physiological apoptosis in non-immune cells.[12,13] The physiological function of TRAIL or TRAIL-receptors is less well understood. No data are available on the expression and localization of FasL, TRAIL and the receptors at different stages of cervical carcinogenesis in relation to proliferation and apoptosis. In the present study we investigated whether the imbalance between proliferation and apoptosis is related to death ligands and death receptors expression, in a series of CIN and cervical cancer samples, representing different stages of cervical carcinogenesis.

PATIENTS AND METHODS

TISSUE COLLECTION

Stored paraffin embedded tissue samples of patients treated in the University Hospital Groningen for cervical neoplasia between 1988 - 2001 were available for research. Eligible for our study were all specimens from patients treated in the University Hospital Groningen for an abnormal cervical smear showing at least borderline dyskaryosis (which is comparable to ASCUS (atypical squamous cells of undetermined significance) in the Bethesda classification system), classified according to a modified Papanicolaou system[14] and all specimens from patients treated for invasive squamous cell cervical cancer.

Tissue samples of patients with borderline, mild or moderate dyskaryosis were derived from either colposcopically directed biopsies or diathermic loop excision of the transformation zone following diagnosis of at least CIN II. Patients with severe dyskaryosis were treated with loop excision without preceding colposcopy taken biopsies. Cervical neoplasia was classified according to the most severe lesion found on histological examination of biopsy and loop excision specimens according to international criteria.[15] Patients with lesions of adenomatous or adenosquamous cell type were excluded from selection. Tissue samples of patients with squamous cell cervical cancer were derived from biopsies taken during gynecological examination under general anesthesia for clinical staging or from radical hysterectomy in patients with FIGO stage IB/IIA. Clinicopathologic characteristics of the patients from whom cervical cancer samples were analyzed, are summarized in Table 1.

Table 1: Characteristics of the studied cervical cancers

FIGO stage	Number of samples	Depth of invasion > 5 cm	Tumor size > 4mm	Poorly differentiated	Lymph node metastases
IA	4	0	0		
IB	17	10	9	4	5
IIA	2	2	0	2	1
IIB	2	2	2	1	

Clinical and histologic data of all patients were prospectively stored in a computerized database and from this database specimens from patients of the various groups were randomly selected for the present study: 11 normal cervices / CIN 0 (despite of an initial abnormal cervical smear no dysplasia was detected on histopathologic examination), 15 CIN I, 15 CIN II, 13 CIN III and 25 (microinvasive) squamous cell cervical cancer lesions.

IMMUNOSTAINING

For immunostaining 4 μm sections were cut and mounted on APES (amino-propyl-ethoxy-silan, Sigma-Aldrich, Diesenhofen Germany) coated glass slides and deparaffinized by a standard procedure. As a marker for proliferation Ki-67 expression

was determined.[16] Immunostaining for Ki-67, Fas, FasL, DR4, DR5 and TRAIL was performed as previously described.[8,17] In short: antigen retrieval was performed by autoclave (Fas, FasL and Ki-67) or microwave (DR5) treatment, while for TRAIL and DR4 staining no antigen retrieval was performed. All primary antibodies were diluted in PBS containing 1% BSA and 1% AB serum and applied for 1 hour at room temperature. Primary antibodies and dilutions were for Ki-67, mouse anti-MIB-1 monoclonal antibody (1:400, Immunotech, Marseille, France), for Fas mouse anti-Fas monoclonal antibody (1:100, clone CH-11, Upstate Biotechnology, Lake Placid, NY), for FasL mouse anti-FasL monoclonal antibody (1:160, Transduction Laboratories, Lexinton, KY), for DR4 goat anti-DR4 polyclonal antibody (1:100, clone C-20, Santa Cruz Biotechnology, Santa Cruz, CA), for DR5 rabbit anti-DR5 polyclonal antibody (1:100, Oncogene Research, Cambridge, MA), and for TRAIL goat anti-TRAIL polyclonal antibody (1:25, clone K18, Santa Cruz Biotechnology). Slides in which the primary antibody was substituted by PBS containing 1% BSA and 1% AB serum served as negative controls. As positive controls served normal liver tissue sections for Fas, DR4 and DR5 staining, normal testis sections for FasL staining and first trimester placenta sections for TRAIL staining.

SCORING OF IMMUNOSTAINING AND APOPTOSIS

For FasL, Fas, TRAIL, DR4 and DR5 staining intensity was semiquantitatively scored: no staining/negative (0), dubious (1), weakly positive (2), positive (3) or intense (4). For statistical analysis, samples with 0 or 1 staining intensity were regarded to be negative and samples with 2, 3, or 4 staining intensity were regarded to be positive. The cellular localization (nuclear, membranous or cytoplasmic), tissue localization (in CIN: basal, parabasal and superficial layer) and patterns of staining (homogeneous or heterogeneous) were also recorded. Cells were regarded positive for Ki-67 if nucleoplasm or nucleoli were unequivocally stained, regardless of intensity. Apoptosis was cytomorphologically scored on hematoxylin-eosin stained slides, as previously described.[17] In short: morphological characteristics included the presence of apoptotic bodies, nuclear condensation, cytoplasmic shrinkage and membrane blebbing.[17-21] Ki-67 positive cells and apoptotic cells were evaluated in 10 randomly selected high power fields (HPF) at 400x magnification. In case of smaller lesions at least 5 HPFs were evaluated. In CIN lesions full thickness of the epithelial layer was counted. For specimens from cancer patients HPFs were counted using an eyepiece grid. Per HPF, on ten consecutive horizontal lines, those cells that crossed the grid-lines were counted. The number of apoptotic and proliferating (Ki-67 positive) cells were both expressed as the percentage of the total number of epithelial cells counted.

STATISTICAL ANALYSIS

Data analysis was performed using SPSS 11.5 software package (SPSS Inc., Chicago, IL). Associations between staining positivity, intensity or patterns of staining and the severity of cervical neoplasia were evaluated by the Chi-square-test or Fisher's exact test, when appropriate. Associations between proliferation or apoptosis and severity of cervical neoplasia were evaluated by the Mann-Whitney-U-test. Differences associated with p-values ≤ 0.05 were considered significant.

RESULTS

### KI-67 STAINING AND APOPTOSIS IN CERVICAL NEOPLASIA

Data on Ki-67 positive cells and apoptotic cells are summarized in table 2. In CIN 0 Ki-67 staining was only observed in the parabasal layer. In CIN I-III lesions an increasing percentage of Ki-67 positive cells was associated with increasing grade of CIN (p<0.001). In cervical cancer the highest frequency of Ki-67 positive cells was found in undifferentiated area's. Overall, in cervical cancer lesions however there was a trend for a lower percentage of Ki-67 positive cells when compared to CIN III (p=0.054).

Table 2: Frequency of expression of death receptors and ligands and apotosis/proliferation in different stages of cervical neoplasia

Staining	Evaluable samples	CIN 0	CIN I	CIN II	CIN III	cancer
		n (%)	n (%)	n (%)	n (%)	n (%)
Fas	78	11 (100)	8 (53)	10 (67)	3 (25)[1]	112 (48)
FasL	79	9 (82)	12 (80)	9 (60)	9 (69)	20 (80)
DR4	72	8 (89)	10 (71)	10 (83)	10 (83)	24 (96)
DR5	71	9 (100)	14 (100)	9 (82)	11 (92)	25 (100)
TRAIL	71	8 (89)	12 (86)	8 (73)	9 (75)	17 (68)
		median	median	median	median	median
Proliferating cells (%)	73	16	25	40	48 [2]	35
Apoptotic cells (%)	77	0.4 [3]	1.8	1.8	2.0	3.4 [3]
Ratio	71	0.02	0.07	0.04	0.05	0.1 [4]

[1] X^2 for trend in CIN lesions, p=0.003

[2] X^2 for trend in CIN lesions, p<0.001

[3] X^2 for trend in CIN lesions and cervical cancer, p<0.001

[4]   (micro)invasive lesions vs. CIN lesions, p<0.001

In CIN I-III and cervical cancer lesions an increasing percentage of apoptotic cells was observed with increasing severity of neoplasia (p<0.001). However, in all lesions in which both apoptosis and proliferation percentages were assessed, the absolute number of Ki-67 positive cells far exceeded the number of scored apoptotic cells (fig. 1). Furthermore, figure 1 clearly illustrates a growing imbalance between the number of proliferating and apoptotic cells in higher grade CIN lesions and cervical cancer. When the ratio between apoptosis and proliferation was calculated separately for each of the samples, the calculated apoptosis/proliferation ratio was higher in cervical cancer than in the CIN lesions (p<0.001, table 2), indicating that the observed imbalance between proliferation and apoptosis was smallest in the cervical cancer samples. These results suggest that higher CIN lesions and cervical cancer have not lost their apoptotic ability but rather gained proliferative capacity.

figure 1
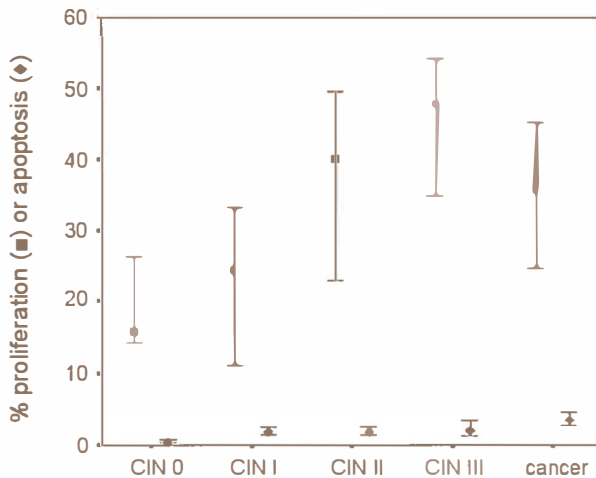
FAS AND FASL IMMUNOSTAINING IN CERVICAL NEOPLASIA

Data on Fas and FasL staining are summarized in table 3. Fas staining was mainly cytoplasmatic (fine granules). In CIN 0 and CIN I lesions Fas staining was especially localized in the superficial epithelial layer, comprising differentiating epithelial cells, while in the parabasal layer only occasionally (n=1) weak Fas positive cells were observed. Overall, in CIN lesions the mere staining of Fas decreased with increasing severity of the lesion (p=0.003). Fas staining was decreased in cervical cancer (48%) vs. CIN 0 (100 %) lesions (p=0.003), while the higher frequency of Fas staining in cervical cancer compared to CIN III lesions was not significant (table 2).

Table 3: Fas and FasL expression in the different layers of CIN and cancer

Staining pattern	CIN 0 n=11 n (%)	CIN I n=15 n (%)	CIN II n=15 n (%)	CIN III n=12 n (%)	Cancer n=25 n (%)
Fas					
(Para)basal*	1(9.1)	0(0)	2(13)	0(0)	3(12)
Superficial*	8(73)	7(47)	6(40)	0(0)	5(20) [1]
Homogeneous	2(18)	1(6.7)	2(13)	3(25)	4(16)
FasL					
(Para)basal	9(82)	12(80)	9(60)	5(42)	5(20) [2]
Superficial	0(0)	0(0)	0(0)	0(0)	1(4.0)
Homogeneous	0(0)	0(0)	0(0)	3(25)	14(56) [3]

* in cervical cancer samples (para)basal staining is scored in undifferentiated tumor parts and superficial staining is scored in differentiated tumor parts

1   X^2 for trend in CIN lesions and cervical cancer, p=0.004

2   X^2 for trend in CIN lesions and cervical cancer, p=0.001

3   CIN III and cervical cancer vs. CIN0, CIN I and CIN II, p<0.001

FasL was detected as a coarse granular cytoplasmatic staining. In CIN 0/I lesions FasL staining was only observed in the basal and parabasal layers (see fig. 2 and table 3). In FasL positive samples staining throughout the epithelial layer increased with increasing severity. FasL staining throughout the epithelial layer was observed in none of the CIN 0/I and CIN II samples, in 44% of CIN III and 70% of FasL positive cervical cancer samples (p<0.001). Fas and FasL were concurrently expressed in 17 of the 26 (65%) CIN 0 and I samples. In these 17 Fas and FasL positive CIN 0/I samples FasL showed a basal/ parabasal staining pattern, whereas Fas showed inverse staining with positivity in the superficial epithelial layer (fig. 2).

Fas   FasL

CIN 0

CIN III

cancer
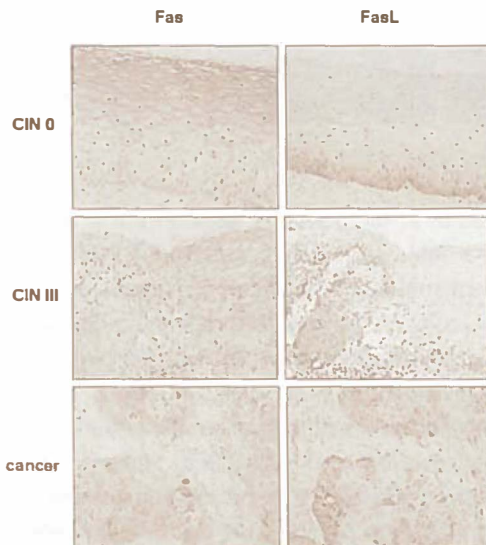
DR4, DR5 AND TRAIL IMMUNOSTAINING IN CERVICAL NEOPLASIA

DR4 (coarse granules) and TRAIL (fine granules) staining was mainly cytoplasmatic. DR5 (fine granules) staining was also cytoplasmatic but with stronger staining towards the nuclear membrane (fig. 3).

Figure 3
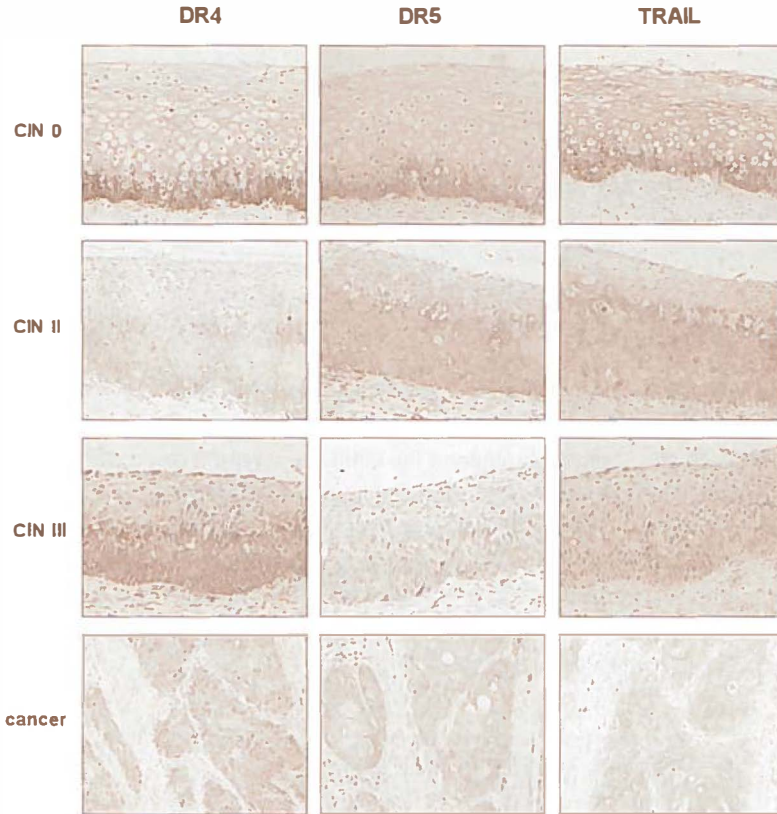
DR4 staining was present in 62 of 72 (86%), DR5 in 68 of 71 (96%) and TRAIL in 48 of 71 (65%) evaluable specimens (Table 2). In CIN 0/I lesions DR4, DR5 and TRAIL staining was associated with undifferentiated cells in the basal and parabasal layer (Table 4). In higher grade CIN lesions DR4 positive cells were more frequently observed throughout the lesions (p=0.049). There was no association between the percentage of DR4, DR5 or TRAIL positive lesions and severity of cervical neoplasia (Table 2), but a homogeneous DR4 and DR 5 staining pattern throughout the lesion was more frequently observed in cancer versus CIN lesions (p<0.0001 and p<0.0001). TRAIL staining was most intense in low grade lesions. In the 20 TRAIL-positive low grade CIN samples, intense staining was present in 7 (35%), whereas 1 of 34 (2.9%) high grade samples showed intense staining (p=0.003).

Table 4: DR4, DR5 and TRAIL expression in the different layers of CIN and cancer

Staining pattern	CIN 0 n=9 n(%)	CIN I n=14 n(%)	CIN II n=11 n(%)	CIN III n=12 n(%)	Cancer n=25 n(%)
DR4					
(Para)basal*	8(89)	9(64)	6(55)	6(50)	8(32)[1]
Superficial*	0(0)	1(7)	2(18)	0(0)	0(0)
Homogeneous	0(0)	0(0)	1(9)	4(33)	16(64)[2]
DR5					
(Para)basal*	6(67)	11(79)	7(64)	5(42)	4(16)[3]
Superficial*	0(0)	0(0)	0(0)	0(0)	0(0)
Homogeneous	3(33)	3(21)	2(18)	6(50)	21(84)[4]
TRAIL					
(Para)basal*	5(56)	8(57)	5(46)	4(33)	5(20)[5]
Superficial*	0(0)	0(0)	0(0)	0(0)	1(4)
Homogeneous	3(33)	4(29)	3(27)	5(42)	11(44)

* in cervical cancer samples (para)basal staining is scored in undifferentiated tumor parts and superficial staining is scored in differentiated tumor parts

[1] X^2 for trend in CIN lesions and cervical cancer, p=0.04

[2] CIN III and cervical cancer vs. CIN 0, CIN I and CIN II, p<0.001

[3] X^2 for trend in CIN lesions and cervical cancer, p=0.001

[4] CIN III and cervical cancer vs. CIN 0, CIN I and CIN II, p<0.001

[5] CIN III and cervical cancer vs. CIN 0, CIN I and CIN II, p=0.01

CORRELATION BETWEEN STAINING PATTERNS OF THE VARIOUS PROTEINS

The staining patterns of FasL, DR4, DR5 and TRAIL showed marked similarities. Overlapping staining patterns were observed for FasL and DR4 in 66%, for FasL and DR5 in 75% for FasL and TRAIL in 63%, for DR4 and DR5 in 76% and for DR4,DR5 as well as TRAIL in 61% of the 71 evaluable samples. The association between FasL and DR4, DR5 and TRAIL was also observed in CIN III and cervical cancer samples separately.

CORRELATION BETWEEN IMMUNOSTAINING AND APOPTOSIS/PROLIFERATION RATIO

Neither Fas, FasL, DR4, DR5, nor TRAIL staining were associated with apoptosis percentage, proliferation percentage or apoptosis/proliferation ratio.

DISCUSSION

In the present study marked changes were observed for Fas, FasL, DR4, DR5 and TRAIL staining in the different stages of cervical carcinogenesis. In low grade lesions positive staining for FasL, DR4, DR5 and TRAIL was predominantly observed in a parabasal layer and for Fas in the superficial layer comprising differentiated epithelial cells. The frequency of homogeneous staining of FasL, DR4 and DR5 throughout the epithelial layer increased with severity of cervical neoplasia, while Fas expression decreased with severity of CIN. Despite the suggested important role of death receptors and their ligands in apoptosis no relation was observed.

Cell turnover is a rapid process in cervical epithelium. A lifespan of four to five days is estimated for normal cervical epithelial cells and for dysplastic cells the lifespan is proposed to be even shorter.[22] We observed 35% proliferating cells in well to poorly differentiated cervical cancer compared to 48% in CIN III which confirms the data of a previous report.[23] Zanotti *et al.* observed higher proliferation in cancer samples compared to CIN III lesions, but only undifferentiated tumors were studied.[24] In normal human tissue the cell number is held constant by equal rates of cell proliferation and elimination. Physiologically, cells are eliminated by apoptosis which can be induced by various stimuli.[25] Apoptosis has not been assessed by morphological criteria in cervical neoplasia previously, although it is generally considered to be the reference standard.[18,20,26,27] Apoptosis percentages were between 1-4 % in cervical cancer by TUNEL staining and 2% in CIN III and 4% in cervical cancer with ISEL staining.[24,28] Although we used a distinct technique our results correspond well with these reports. In the present study the proportion of epithelial cells undergoing apoptotic cell death increased with increasing severity of neoplasia, which has been previously described for cervical epithelium and for epithelium of other origin.[8,17,24,26,28,29] The observed higher percentage of apoptosis in cervical cancer compared to CIN III may reflect an increase in DNA damage which makes cells more susceptible for apoptosis. However, the higher percentage of apoptosis in cancer may also reflect the absence of anoikis for tissue homeostasis. Anoikis is detachment-induced apoptosis which occurs in the top layer of epithelium.[25] In CIN lesions the percentage of apoptosis may have been underestimated because cells dying of anoikis are shed.[25] In general, highest proliferation percentages were observed in areas with undifferentiated cells. The relatively high proportion of well differentiated cancers, together with anoikis, may explain why the imbalance between proliferation and apoptosis seems to be more pronounced in CIN III than in cervical cancer samples.

Both FasL and TRAIL are death ligands that can activate apoptosis by binding to their cognate cell-surface death receptors, Fas and DR4/DR5. No associations between the mere expression of these proteins, nor with the staining patterns of these proteins and apoptosis or proliferation were observed. In other tissue types the expression of Fas/FasL or DR4/DR5/TRAIL and the degree of apoptosis was also uncorrelated.[8,17] Despite the lack of correlation between the expression of death ligands or receptors

and apoptosis or proliferation, we observed a change in Fas versus FasL staining pattern during cervical carcinogenesis. In normal epithelium or CIN I samples Fas and FasL showed inverse staining patterns, which was also observed by others in cervical lesions and has also been described for epithelium of different origin.[30,31] In concordance with its more frequent staining in differentiated cells, Fas expression decreased with increasing severity of CIN lesions. Interestingly, Fas was more frequently expressed in cancer lesions versus CIN III. Again, this last observation may be due to the relatively high proportion of well differentiated cancers in our study. By the loss of Fas expression in a subset of CIN III and cancer lesions and the high expression of FasL some of these lesions may evade induction of apoptosis. The resulting growth advantage may be overcome if Fas expression could be upregulated by modulating drugs such as interferon-gamma or cytotoxic drugs thereby inducing paracrine or autocrine apotosis via interaction with FasL[5]

In CIN 0 and CIN I proliferation was observed in the parabasal layer. The constitutive expression of FasL in the parabasal layer may therefore protect this layer from infiltrating Fas positive T cells and granulocytes as these cells rapidly undergo FasL induced apoptosis.[5] The increase in homogenous expression of FasL in CIN III and cervical cancer may play a functional role in escape from immune surveillance. FasL was more intensely stained in cervical cancer compared to lower grade lesions whereas the opposite was true for TRAIL. In light of these results, FasL upregulation seems more important for escape from immune surveillance than TRAIL upregulation. FasL is thought to be the key death factor in death receptor induced apoptosis in immune cells.[32] FasL is expressed on activated cytotoxic T cells and NK cells which can kill virally infected cells by activating available Fas receptors.[33] If HPV-infected tumor cells downregulate their expression of Fas, they may escape immune defence. Conversely, expression of FasL on the surface of tumor cells might lead to the killing of Fas-sensitive infiltrating lymphocytes. Studies determining a relation between FasL positivity of cervical squamous cell carcinomas and overall survival have not been published yet. However, patients with FasL expressing cervical adenocarcinomas had a significantly reduced survival time as compared to patients with low FasL expressing tumors.[34] Furthermore, all metastatic cervical adenocarcinomas were found to be FasL positive, suggesting a role for FasL in immune evasion, progression and metastasizing potential in cervical cancer.

In CIN 0 and CIN I samples expression of DR4, DR5 and TRAIL was mainly observed in the parabasal layer. Overlapping staining of TRAIL and its death receptors was also observed by others in cervical epithelium and in epithelium of different origin.[8,28] Several studies have demonstrated that the TRAIL/TRAIL receptor system may play an important role in the elimination of virus-infected cells, which may implicate persistent HPV-infected cervical basal and suprabasal cell layers. Cells infected by human immunodeficiency virus or cytomegalovirus revealed increased DR4, DR5 and TRAIL protein expression levels rendering them more sensitive to TRAIL-induced apoptosis by autocrine or T-cell derived TRAIL.[35,36] We found less intense TRAIL staining and a reduced

percentage of TRAIL-positive specimens with increasing severity of cervical lesions. From our results we can not conclude that loss of TRAIL expression plays a role in cervical carcinogenesis, but studies using TRAIL-deficient mice have demonstrated that TRAIL is important in controlling tumor growth.[37,38] The concomitant expression of TRAIL and TRAIL-receptors may also suggest that either TRAIL or TRAIL-receptors are not expressed on the cell surface or the presence of intrinsic resistance to TRAIL. The mechanisms of resistance of normal cells to TRAIL have been shown to vary between different cell types.[39] The cell killing properties of TRAIL and FasL have made their cognate cell-surface death-receptors exciting targets for drug development. For treatment of cervical neoplasia, Fas does not seem to be an interesting target because it was not expressed in the parabasal layer. DR4 and DR5 may be better targets as they were expressed in nearly all cervical neoplastic lesions, while the TRAIL decoy receptor genes appeared to be at least partially methylated in most cervical cancers.[40]

In conclusion, loss of Fas and deregulation of FasL, DR4, DR5 and TRAIL in the CIN-cervical cancer sequence suggest a possible functional role of these death ligands and receptors during cervical carcinogenesis. Their observed frequent expression present DR4 and DR5 as promising targets for innovative therapy modalities in cervical cancer.

ACKNOWLEDGMENTS

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Summary and future perspective
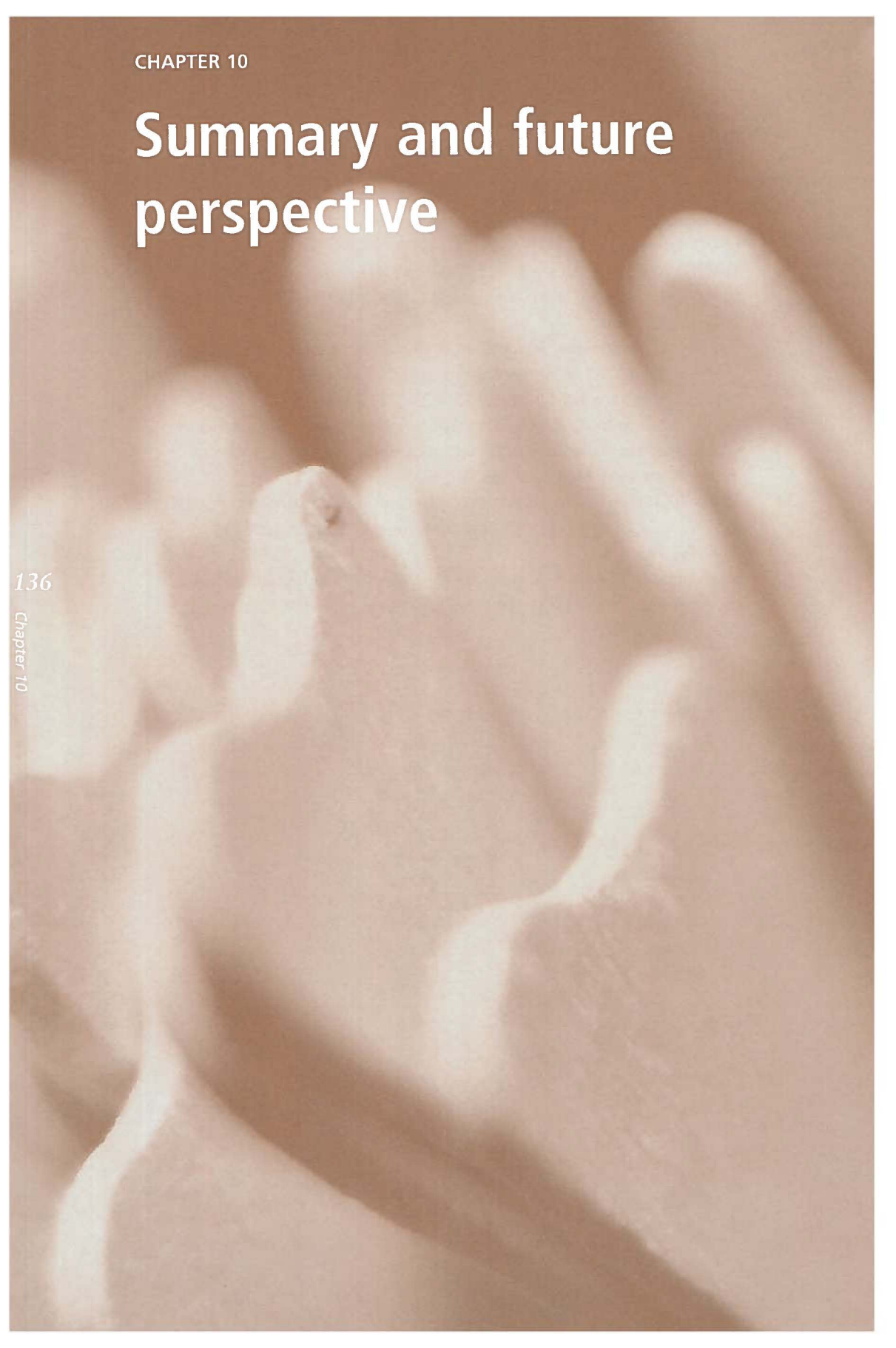
ABSTRACT

In chapter 10 the results of the thesis are discussed in the summaries of all chapters separately. Furthermore, the results of the thesis are put into a future perspective. Expected changes and developments in prevention, treatment and screening of cervical cancer are also discussed in the future perspective.

SUMMARY

Cervical cancer is an important cause of death in women worldwide. Infection with high-risk human papillomavirus (HPV) has been identified as the main etiologic factor in cervical carcinogenesis.[1] An estimated number of 370,000 – 470,000 new cases occur per year, and 230,000 women die of cervical cancer per year[2-4] thus being the third to second most common cancer in women with large incidence differences between developed countries with cervical cancer screening programs and less developed countries without such programs. The cumulative lifetime risk for a woman to develop cervical cancer varies from 0.4% in Israel to 5.3% in Colombia.[5] Cervical cancer develops from cervical intraepithelial neoplasia (CIN). A CIN-lesion can either regress, persist or progress towards (micro)invasive carcinoma. Most of the low grade CIN lesions (CIN I) will regress, while in the long-term 12% to 40% of high grade CIN lesions (CIN II/III) will progress to squamous cell carcinoma.[6,7] Because no markers exist that identify those lesions that will progress, clinicians have felt compelled to treat at least all CIN II/III lesions.

Cytomorphologic examination of cervical smears is the most widely applied screening-method for cervical cancer and its precursors. Disadvantages are high numbers of false-negative and false-positive cervical smears. Parameters in cervical scrapings are needed which more accurately predict the presence of higher grade CIN or cervical cancer lesions to improve cervical cancer screening.

In **Chapter 2** available data on new approaches to cervical cancer screening such as quantitative cytochemistry, and detection of HPV DNA, loss of heterozygosity, microsatellite alterations, telomerase activity and DNA methylation were summarized. The potential of these approaches to replace or augment current Pap-smear screening was discussed. It was concluded that to date no other diagnostic tools are available that can cost-effectively replace or augment current Pap-smear screening. However, improvements may lay in the future. Implementation of liquid based cytology would aid the development of new technologies that can be performed on cervical scrapings. HPV DNA detection stands closest to implementation in nation-wide screening programs of all markers reviewed, but may also have important drawbacks. An interesting new technology that should be further developed is the detection of promoter hypermethylation of tumor suppressor genes by quantitative methylation specific PCR (see Chapter 7).

Telomerase is an enzyme that can form new short stretches of repeat nucleotides at the telomeric ends of chromosomes that were lost with each round of replication. Studies in tumour cell lines, as well as in human tumour specimens have shown that, in contrast to normal somatic cells, the vast majority of malignant cells (>90%) are characterized by increased telomerase activity.[8,9] Therefore determination of telomerase activity has been suggested for early cancer detection. In **Chapter 3** we studied whether detection of either telomerase and its components, or high risk human

papillomavirus (HPV) are of value in predicting the presence of CIN II/III in patients referred because of cervical cytology reports showing at most moderate dyskaryosis. The cervical smears of 50 patients with cytological borderline, mild or moderate dyskaryosis were analyzed. Telomerase activity was assessed by a commercially available TRAP-assay and its components human telomerase RNA (hTR) and human telomerase reverse transcriptase (hTERT) by reverse transcriptase-PCR. HPV was detected by GP5+/6+ PCR enzyme immunoassay. Histological findings on colposcopy guided biopsies or excised cervical tissue were regarded as pathological final diagnosis. Sensitivity and specificity for detecting CIN II/III were calculated. Twenty-eight patients (46%) were diagnosed with CIN II/III. In the patients diagnosed with CIN II/III, telomerase activity was detected in none, hTR in 88%, hTERT in 23% and high-risk HPV in 79%. As a diagnostic test none of the described analyses combined a sensitivity of at least 90% with a specificity of at least 90%. Despite the small numbers, calculation of the 95% confidence intervals excluded a combined sensitivity and specificity of at least 90% for any of the evaluated parameters. Therefore neither detection of telomerase or its components, nor detection of high risk HPV seem suitable for the triage of patients with borderline, mild and moderate cytological dyskaryosis.

In order to prevent increased morbidity associated with double modality treatment, early stage cervical cancer patients should be offered radical surgery only when there is a low likelihood for adjuvant radiotherapy. **Chapter** 4 describes whether serum Squamous Cell Carcinoma-antigen (SCC-ag) analysis allows better preoperative identification of a group of patients with a low likelihood for adjuvant radiotherapy than currently used clinical parameters. In a historical cohort study, FIGO stage, tumor size and preoperative serum SCC-ag levels, as determined by enzyme immunoassay, were related to the frequency of postoperative indications for adjuvant radiotherapy in 337 surgically treated FIGO stage IB/IIA squamous cell cervical cancer patients. In patients with normal preoperative SCC-ag 16% of stage IB1 and 29% of stage IB2/IIA had postoperative indications for adjuvant radiotherapy, in contrast to 57% of stage IB1 and 74% of stage IB2/IIA patients with elevated (>1.9 ng/mL) serum SCC-ag (p<0.001). Serum SCC-ag was the only independent predictor for a postoperative indication for radiotherapy (odds ratio 7.1, 95% CI 4.2-12, p<0.001). Furthermore, in stage IB1 patients that did not have indications for adjuvant radiotherapy 15% of the patients with elevated preoperative serum SCC-ag levels recurred within 2 years in contrast to 1.8% of the patients with normal serum SCC-ag levels (p=0.02). It was concluded that determination of serum SCC-ag in early stage cervical cancer patients allows more refined preoperative estimation of the likelihood for adjuvant radiotherapy than current clinical parameters and simultaneously identifies patients at high-risk for recurrence when treated with surgery only. Therefore, preoperative serum SCC-ag analysis deserves implementation in clinical decision making.

Three different infectious agents of which a possible involvement in cervical carcinogenesis has been suggested in literature were studied. First, **Chapter 5** describes the prevalence of risk factors for and the impact on histologic changes of

bacterial vaginosis in patients with cytological abnormalities of the uterine cervix. Therefore 280 patients with dyskaryotic smears (mild, moderate or severe dyskaryosis) were asked to participate in the study, using a questionnaire to obtain data on smoking habits and sexual history. Bacterial vaginosis was diagnosed if the vaginal discharge produced a fishy odor upon alkalinization and if clue cells were seen in the wet smear. Furthermore, cervical scrapes were analyzed for the presence of HPV DNA, and cervical tissue specimens were analyzed for the presence and severity of (intraepithelial) neoplasia and the proliferation rate (mitotic index) of the lesion. *Chlamydia trachomatis* was identified by culture of an endocervical swab. Bacterial vaginosis was found in 56 (20%) of the patients. The presence of bacterial vaginosis was associated with the number of cigarettes smoked per day, age at first sexual intercourse, lifetime number of sexual partners, and current *Chlamydia trachomatis* infection. Number of cigarettes currently smoked per day and lifetime number of sexual partners were independent risk factors for the presence of bacterial vaginosis. There was no relation between the presence of bacterial vaginosis and HPV infection. Bacterial vaginosis did not influence the severity or the mitotic index of the neoplastic lesion found. In conclusion, in patients with dyskaryotic cervical smears, the prevalence of bacterial vaginosis is not increased, and bacterial vaginosis does not influence histologic changes. Bacterial vaginosis is therefore unlikely to be important in the etiology of cervical neoplasia, despite the similarity between its epidemiologic features and those of cervical HPV infection and cervical neoplasia.

Chlamydia trachomatis was the second infectious factor possibly involved in cervical carcinogenesis that was studied. In **Chapter** 6 it was explored whether the presence of *Chlamydia trachomatis* antibodies was associated with the severity of neoplastic lesions in patients with cervical dyskaryosis. In a cross-sectional study serum samples were analyzed for antichlamydial antibodies by enzyme immunoassay. Cervical neoplasia was graded histologically. In 296 eligible patients referred because of cervical dyskaryosis between 1988-1993 no association was found between increasing grade of CIN and the presence of antichlamydial antibodies. The proportion of patients with antichlamydial antibodies was higher in patients with (micro)invasive carcinoma (13/14 (93%)) than in patients with CIN (101/282 (36%)). As the high prevalence of antichlamydial antibodies in patients with cervical carcinoma was not consistent with prevalences reported in recent literature, a second group of patients was analyzed (331 eligible patients in the period 1995-1999) in which the high prevalence of antichlamydial antibodies in patients with cervical carcinoma was not confirmed. These results suggest that the presence of circulating antichlamydial antibodies is not associated with the severity of neoplastic lesions and it seems unlikely that *Chlamydia trachomatis* has a role in the progression of cervical neoplasia.

The last and most important infectious parameter studied was HPV. HPV infection is the first identified essential step in cervical carcinogenesis, meaning that cervical cancer, with very rare exceptions, can not develop without HPV infection. Over 100 HPV types have been identified of which more than 35 types can be found in the genital tract and

of which 18 are associated with cervical carcinogenesis. Because of the important etiologic role of HPV numerous studies have been performed to identify factors involved in HPV infection and persistence. Different HPV detection techniques are available and might influence the results of epidemiologic studies.

 A previous study comprising 304 patients referred because of abnormal pap smears showed that the prevalence of HPV rises with increasing histological severity of neoplasia, more cigarettes smoked per day and higher lifetime number of sexual partners in patients with cervical dyskaryosis using the GP5/6 primers based HPV PCR.[10] Data on the lifetime number of sexual partners and smoking behavior were obtained by questionnaire. HPV analysis was performed on cervical scrapes obtained at the enrolment visit. Some years after that initial epidemiologic study the highly sensitive SPF10 primers and Inno-LiPA (line probe assay) HPV prototype research assay became available for the detection and typing of HPV. In **Chapter 7**, using the SPF10-LiPA system, we re-analyzed the stored cervical scrapings of the earlier described study population. High-risk HPV was present in 288 (95%) patients. A total of 86 (30%) out of these 288 patients disclosed multiple types. HPV 16 occurred less often in multiple infections than was expected on the basis of chance alone. The grade of neoplasia was associated with the presence of oncogenic HPV, which depended on the presence of HPV type 16. No association was found between grade of neoplasia and the presence of multiple HPV types. Neither the lifetime number of sexual partners nor smoking were associated with high-risk HPV, the five most frequent HPV types separately or the presence of multiple types. We conclude that the association between the detection of HPV and the epidemiological risk factors, as found with the GP5/6 PCR in the past, could not be confirmed when using SPF10 PCR primers and LiPA HPV genotyping. We suggest that the number of sexual partners and smoking may be determinants of high HPV viral load rather than determinants of the presence of high-risk HPV per se.

It has become increasingly clear that silencing of tumor suppressor- or other cancer-associated genes by methylation of CpG islands is a common feature of human cancer including cervical cancer. In addition to the functional implications of gene inactivation in tumor development, these aberrant methylation patterns represent interesting targets for novel diagnostic approaches based on methylation sensitive PCR techniques which we tried to translate to clinical use in cervical cancer detection in **Chapter** 8. It was unknown whether a cervical scraping reflects the methylation status of the underlying epithelium. It was therefore unclear whether quantitative hypermethylation specific PCR (QMSP) on cervical scrapings could be used as a future screening method augmenting the current approach. The use of QMSP on cervical scrapings was explored by analyzing cervical scrapings and paired fresh frozen cervical tissue samples obtained from 53 cervical cancer patients and 45 controls. All scrapings were morphologically scored and analyzed with QMSP for the genes *APC, DAPK, MGMT* and *GSTP1. APC* mediates proliferative signals, *DAPK* is a proapoptotic gene and potentially inhibits metastasis, *MGMT* is a DNA repair gene and *GSTP1* is a detoxifying gene.[11 15] To adjust for DNA input, hypermethylation ratios were calculated against DNA levels of a reference gene.

Hypermethylation ratios of paired fresh frozen tissue samples and scrapings of cervical cancer patients and controls were strongly related. More cervical cancer patients than controls were *DAPK* positive (p<0.001). When 'screen positive' cutoff levels for ratios were defined to be above the highest ratio observed in controls, QMSP in cervical scrapings identified 32 of 48 (67%) cervical cancer patients. This feasibility study, in which cervical scrapings were analyzed for only four genes, demonstrates that QMSP holds promise as a new diagnostic tool for cervical cancer. The addition of more genes specifically methylated in cervical cancer are necessary to improve the sensitivity of the assay.

Increasing imbalance between proliferation and apoptosis is thought to be important in cervical carcinogenesis. The death ligands FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induce apoptosis by binding to their cognate cell-surface death receptors Fas or death receptor (DR) 4 and DR5. The aim of our study described in **Chapter 9** was to examine possible changes in expression of these death ligands and their receptors at different stages of cervical carcinogenesis. The immunohistochemical expression and localization of Fas/FasL and DR4/DR5/TRAIL were assessed in 11 normal, 15 cervical intraepithelial neoplasia (CIN) grade I, 15 CIN II, 13 CIN III and 25 (microinvasive) squamous cell cervical cancers. The number of apoptotic cells was determined by the use of morphological criteria and the number of proliferating cells by counting Ki-67 positive cells. A marked increase in proliferation as well as apoptosis percentage was found with increasing severity of neoplasia. In normal cervix and CIN I samples FasL, DR4, DR5 and TRAIL staining was mainly observed in the basal/parabasal layer, whereas Fas staining was localized in the superficial, more differentiated epithelial layer. Frequency of Fas positive staining decreased with increasing severity of CIN. In contrast, homogeneous FasL, DR4, DR5 and TRAIL expression throughout the lesions was more frequently observed in CIN III and cervical cancer. FasL, DR4, DR5 and TRAIL staining patterns were correlated, although TRAIL expression was more intense in low grade lesions. No association was found between death receptor or ligand expression with the percentage of apoptosis or proliferation. The loss of Fas and the deregulation of FasL, DR4, DR5 and TRAIL in the CIN-cervical cancer sequence suggest a possible functional role of these death ligands and receptors during cervical carcinogenesis. Their observed frequent expression present DR4 and DR5 as promising targets for innovative therapy modalities in cervical cancer.

FUTURE PERSPECTIVE

In the Netherlands a population-based cervical cancer screening program exists. Women aged 30-60 years are invited to be screened for cervical cancer once every five years. Cervical smears are taken by general practitioners. Patients are referred to a gynecologist for colposcopic examination if abnormalities are found during screening. Current morphology based cervical cancer screening is associated with significant false-positive and false-negative results. To date no other diagnostic tools are available of which is known that they can cost-effectively replace or augment current Pap-smear screening. In countries with low prevalence of cervical cancer, as in the Netherlands, it is hard to determine whether the decrease in cervical cancer deaths as a results of screening counterbalances the negative effects of screening. Cervical cancer screening will especially be of benefit in women in countries with high cervical cancer prevalences. Pap-smear screening seems the best screening tool currently available. HPV DNA detection stands closest to implementation in nation-wide screening programs of all currently known 'markers'. The main advantages of HPV screening would be the prolongation of the screening interval for HPV negative women and the possibility of triage for women with minor morphological abnormalities. In the next five years the results will become available of a Dutch radomized controlled trial that evaluates wether implementation of these two main advantages can improve the efficiency of cervical cancer screening.[16] However, low specificity for (progressive) high-grade CIN and cervical cancer as well as negative psychological effects of knowledge of HPV positivity are important drawbacks. Even if the trials that are underway will show cost-effectiveness of implementing HPV DNA testing in cervical cancer screening, new markers and technologies could theoretically still lead to major improvements.

If the high false-positive and false-negative numbers of cervical cancer screening would be reduced it will become more beneficial to screen women, even if they live in countries with a low cervical cancer prevalence. An interesting new cervical cancer detection approach is tumor suppressor gene hypermethylation analysis by QMSP. In this thesis we showed that it has theoretical advantages over current Pap-smear analysis and some other new screening tools based on molecular changes. Our first analysis in cervical scrapings was promising. Cervical scrapings reflect the hypermethylation status of underlying epithelium very well. Testing for only four genes, already 67% of cervical cancer patients were identified by QMSP. In prostate cancer *GSTP1* is methylated in more than 90% of the tumors and diagnostic tests are now being developed.[17] In light of these prostate cancer results we expect it to be possible that for cervical cancer a panel of genes can be identified, which will cover almost all cervical cancer cases. The available data and expectations should spur the search for more genes specifically methylated during cervical carcinogenesis. Once a (cervical) cancer sensitive and specific hypermethylation panel is composed it will be necessary to define 'screen positive' cutoff values for population-based screening purposes. Together with sensitivity and specificity of a screening test, the positive and negative predictive value of such a test in a population is determined by the prevalence of disease in that

population. It may therefore be a rational approach to let the QMSP cutoff values depend on the prevalence of cervical cancer in a country. Further studies should clarify wether QMSP can also be used to identify progressive CIN II/III lesions.

The prevalence of cervical cancer may show a drastic change in the future, because primary prevention of cervical cancer by effective prophylactic HPV vaccines may become possible.[18] When it would be implemented, HPV vaccination is supposed to lower the prevalence of cervical cancer and its precursor lesions dramatically. If sensitivity and specificity of our screening tools will not be changed in that situation, positive screen results will thereby be more likely to represent false positives. However, one may still want to screen for residual CIN/cervical cancer patients in the hypothetical scenario that HPV vaccination is implemented. Reasons for ongoing screening may be that immunization will fail in some women, women may refuse to be vaccinated and probably only vaccines against HPV 16 and 18 will be developed. It is hard to estimate the prevalence of cervical cancer once HPV 16 and 18 vaccination is implemented in our health care system. Even when this implementation lays in the near future it will however take at least decades before it will result in a decrease of cervical cancer cases.

In this thesis we show that it is possible to refine existing treatment protocols for cervical cancer by serum detection of SCC-ag. The pretreament level of serum SCC-ag in cervical cancer patients was already known to be of prognostic value. Determination of serum SCC-ag levels in patients with early stage cervical cancer also allows more refined preoperative estimation of the likelihood for adjuvant radiotherapy than current clinical parameters and simultaneously identifies patients at high-risk for recurrence when treated with surgery only. In our opinion preoperative serum SCC-ag analysis should now be implemented in clinical decision making.

The etiology of cervical neoplasia starts to be unraveled and new technologies may accelerate our understanding of cervical carcinogenesis. It will be an ongoing challenge to find ways by which these improvements will help us to refine screening and treatment policies for individual patients.

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Samenvatting en toekomstvisie
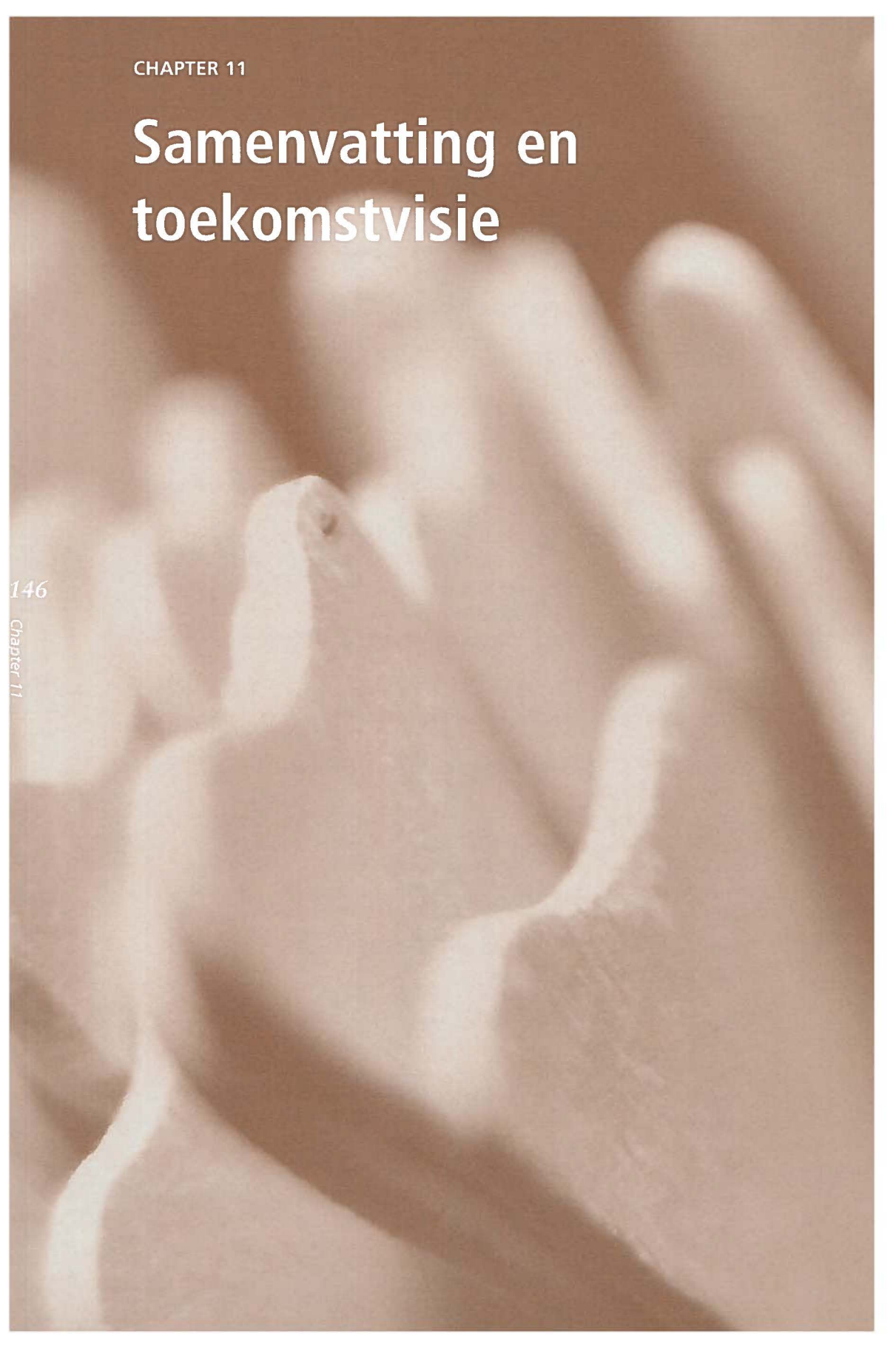
ABSTRACT

In Hoofdstuk 11 worden de resultaten van dit proefschrift bediscussieerd in de samenvattingen per hoofdstuk. Daarnaast worden de bevindingen in een toekomstperspectief geplaatst waarbij ook de te verwachten veranderingen in de preventie, screening en behandeling van cervixcarcinoom worden besproken.

SAMENVATTING

Cervixcarcinoom is wereldwijd gezien een belangrijke doodsoorzaak onder vrouwen. Infectie met hoog risico humaan papillomavirus (HPV) wordt als de meest belangrijke etiologische factor gezien voor cervicale carcinogenese.[1] Geschat kent het cervixcarcinoom jaarlijks 370,000 – 470,000 nieuwe gevallen en 230,000 doden.[2-4] waarmee het de derde tot tweede meest frequente vorm van kanker onder vrouwen is. Er bestaan grote verschillen in incidentie tussen 'westerse' landen met nationale screeningsprogramma's en ontwikkelingslanden zonder screening. Het cumulatieve lifetime risico voor een vrouw om cervixcarcinoom te krijgen varieert van 0.4% in Israël tot 5.3% in Colombia.[5] Cervixcarcinoom ontstaan uit voorloper afwijkingen, cervicale intraepitheliale neoplasie (CIN). CIN afwijkingen kunnen in regressie gaan, persisteren of progressie tot (microinvasief) carcinoom vertonen. De meeste CIN I afwijkingen gaan in regressie, terwijl op de lange termijn 12% tot 40% van de CIN II/III afwijkingen plaveiselcelcarcinoom van de cervix zal worden.[6,7] Omdat er geen markers bestaan die aan kunnen geven welke premaligne afwijkingen progressief zullen zijn, voelen de meeste gynaecologen zich genoodzaakt in ieder geval alle CIN II/III afwijkingen te behandelen.

Cytomorfologisch onderzoek van cervix uitstrijkjes wordt het meest gebruikt als screeningsmethode voor cervixcarcinoom en CIN. Nadelen hiervan zijn hoge vals positieve en negatieve uitslagen. Parameters die de aanwezigheid van progressieve CIN of cervixcarcinoom accurater aan kunnen geven en die bepaald kunnen worden in cervixschraapsels zijn noodzakelijk om de huidige cervixcarcinoom screening te verbeteren.

In **Hoofdstuk 2** werd een overzicht gegeven van de recente literatuur over het gebruik van nieuwe methoden om cervixcarcinoom screening te verbeteren, zoals kwantitatieve cytochemie, HPV DNA detectie, verlies van heterozygotie, microsatelliet veranderingen, telomerase activiteit en DNA methylatie. De mogelijkheden van deze benaderingen om de analyse van het huidige Pap-uitstrijkje te verbeteren of te vervangen werden bediscussieerd. De conclusie was dat er tot op heden nog geen diagnostische test beschikbaar is waarvan bekend is dat deze kosteneffectief geïmplementeerd kan worden in bestaande programma's voor cervix carcinoom screening. Verbeteringen zouden echter nabij kunnen zijn. De implementatie van liquid based cytology zou de verdere ontwikkeling vergemakkelijken van op cervix uitstrijkjes te gebruiken nieuwe technieken. Hoewel HPV DNA detectie belangrijke nadelen heeft staat deze techniek het dichts bij implementatie in landelijke screening programma's van alle methoden die werden samengevat. Een interessante nieuwe techniek die verder ontwikkeld zou moeten worden is de detectie van tumor suppressor gen hypermethylatie door middel van kwantitatieve methylatie specifieke PCR (zie Hoofdstuk 7).

Telomerase is een enzym dat in staat is om aan de uiteinden van telomeren stukjes DNA te synthetiseren die verloren waren gegaan tijdens de celvermenigvuldiging.

Zowel studies in tumorcellijnen als studies in menselijk tumormateriaal hebben laten zien dat in contrast met normale somatische cellen, de grote meerderheid van kwaadaardige cellen (>90%) een toegenomen telomerase activiteit heeft.[8,9] Vanwege die eigenschap is er voorgesteld om bepaling van telomerase activiteit te gebruiken voor de vroege opsporing van cervixcarcinoom. **Hoofdstuk 3** had tot doel te onderzoeken of het mogelijk was de aanwezigheid van CIN II/III te voorspellen bij patiënten die werden verwezen vanwege een uitstrijkje met atypie of geringe-matige dyskaryose. De uitstrijkjes van 50 vrouwen werden onderzocht. Als mogelijke markers voor CIN II/III gebruikten we telomerase activiteit, twee telomerase componenten en hoog risico HPV. Telomerase activiteit werd bepaald met behulp van een commercieel verkrijgbare TRAP assay. De componenten van telomerase, humaan telomerase RNA (hTR) en humaan telomerase reverse transcriptase (hTERT) werden bepaald met behulp van reverse transcriptase-PCR. HPV werd gedetecteerd door middel van de GP5+/6+ PCR enzymatische immunoassay. De histologische diagnose werd bepaald aan de hand van histopathologisch onderzoek van colposcopisch gerichte biopten of van het lisexcisiepreparaat. Sensitiviteit en specificiteit voor de detectie van CIN II/III werden berekend. Bij 28 vrouwen (46%) werd CIN II/III gediagnosticeerd. Er werd geen telomerase activiteit gevonden bij de vrouwen met CIN II/III. Wel werd hTR bij 88%, hTERT bij 23% en hoog risico HPV bij 79% van de vrouwen met CIN II/III gevonden. Ondanks de kleine aantallen sloten de berekende 95% betrouwbaarheidsintervallen een gecombineerde sensitiviteit en specificiteit van ten minste 90% uit voor alle bestudeerde tests. Ondanks het kleine aantal patiënten was de conclusie dat noch de detectie van telomerase activiteit of de telomerase componenten, noch de detectie van hoog risico HPV bruikbaar lijkt voor de triage van vrouwen met atypie, geringe of matige dyskaryose in hun uitstrijkje.

Om bij patiënten met een vroeg stadium cervixcarcinoom therapie geïnduceerde morbiditeit te voorkomen zouden bij voorkeur alleen de patiënten die waarschijnlijk geen adjuvante radiotherapie zullen krijgen middels radicale hysterectomie behandeld moeten worden. In **Hoofdstuk 4** werd geanalyseerd of met behulp van preoperatieve serum SCC-antigeen bepaling, beter dan met de huidig gebruikte klinische parameters, voorspeld kan worden wie een indicatie voor adjuvante radiotherapie zal hebben na operatie. Daarvoor werd in een historisch cohort van 337 FIGO stadium IB/IIA plaveiselcel cervixcarcinoom patiënten die een primair chirurgische behandeling ondergingen de relatie tussen FIGO stadium, tumor grootte, preoperatieve serum SCC-ag waarde en de postoperatieve indicatie voor adjuvante radiotherapie bepaald. Van de patiënten met een normale preoperatieve SCC-ag waarde had 16% van de stadium IB1 en 29% van de stadium IB2/IIA patiënten een postoperatieve indicatie voor adjuvante radiotherapie. Bij patiënten met verhoogd serum SCC-ag (>1,9 ng/mL) bleek 57% van de stadium IB1 en 74% van de stadium IB2/IIA patiënten een indicatie te hebben voor adjuvante radiotherapie (p<0,001). Serum SCC-ag was de enige onafhankelijke voorspeller voor een postoperatieve indicatie voor radiotherapie (odds ratio 7,1, 95% betrouwbaarheidsinterval 4,2 - 12, p<0,001). Van de stadium IB1 patiënten zonder indicatie voor adjuvante radiotherapie bleek 15% van de patiënten met verhoogd

preoperatief serum SCC-ag binnen twee jaar een recidief te ontwikkelen. Datzelfde gebeurde bij 'slechts' 1,8% van de patiënten met een normale serum SCC-ag waarde (p=0,02). De conclusie was dat het preoperatief bepalen van serum SCC-ag een scherpere voorspelling van de noodzaak voor adjuvante radiotherapie mogelijk maakt dan dat momenteel kan met behulp van de klassieke FIGO parameters. Tegelijkertijd kan serum SCC-ag analyse een groep patiënten identificeren met een hoog risico op het krijgen van een recidief wanneer zij alleen operatief zouden worden behandeld. Preoperatieve serum SCC-ag analyse verdient daarom implementatie in de klinische beleidsbepaling bij patiënten met een vroeg stadium plaveiselcel cervixcarcinoom.

Drie infectieuze (co)factoren die volgens de literatuur mogelijk betrokken zouden zijn bij cervicale carcinogenese werden bestudeerd. In **Hoofdstuk 5** werd de prevalentie van bacteriële vaginose bepaald in 280 patiënten die verwezen waren vanwege cytologische afwijkingen (geringe, matige of ernstige dyskaryose). We onderzochten risico factoren voor het krijgen van bacteriële vaginose en de betekenis voor histologische veranderingen. De deelnemende patiënten gaven informatie over rookgedrag en seksuele voorgeschiedenis door middel van een gestructureerde vragenlijst.

Bacteriële vaginose werd gediagnosticeerd wanneer er in de vaginale afscheiding een vissige geur ontstond door toediening van KOH en wanneer clue cells gezien werden in een direct preparaat met fysiologisch zout. Verder werden de cervixschraapsels op de aanwezigheid van HPV DNA getest en werd de aanwezigheid en ernst van (intraepitheliale) neoplasie en de proliferatie graad (mitose index) van de laesie bepaald. *Chlamydia trachomatis* werd geïdentificeerd door kweek van endocervicaal afgenomen fluor. Gebruik makende van deze methoden en definities werd bacteriële vaginose vastgesteld bij 56 (20%) van de 280 patiënten. De aanwezigheid van bacteriële vaginose bleek geassocieerd met dagelijkse sigaretten consumptie, aantal seksuele partners, sexarche en recente *Chlamydia trachomatis* infectie. Dagelijkse sigaretten consumptie en het aantal verschillende seksuele partners waren onafhankelijke voorspellers voor de aanwezigheid van bacteriële vaginose. Er bestond geen associatie tussen de aanwezigheid van bacteriële vaginose en HPV infectie. Bacteriële vaginose had geen invloed op de ernst van de gediagnosticeerde neoplastische laesie noch op de mitotische index. De conclusie was dat het onwaarschijnlijk is dat bacteriële vaginose een belangrijke etiologische rol speelt bij cervicale neoplasie, ondanks de overeenkomsten in de epidemiologische kenmerken van bacteriële vaginose en HPV bij vrouwen met een afwijkend uitstrijkje.

De tweede infectieuze factor die werd bestudeerd is *Chlamydia trachomatis*. **Hoofdstuk** 6 beschrijft of de aanwezigheid van *Chlamydia trachomatis* antilichamen in serum geassocieerd was met de ernst van neoplastische cervix laesies, gediagnosticeerd bij vrouwen die werden verwezen vanwege een afwijkend uitstrijkje. In twee onderzoeksperioden analyseerden we serum monsters van vrouwen die werden verwezen vanwege een uitstrijkje met geringe, matige of ernstige dysplasie. In die

serum monsters werd de aanwezigheid van antichlamydia antilichamen bepaald door middel van een enzymatische immunoassay.

Bij 296 patiënten, doorverwezen tussen 1988 en 1993 bestond geen associatie tussen toenemende ernst van CIN en de aanwezigheid van antichlamydia antilichamen. Het percentage vrouwen met antichlamydia antilichamen was echter hoger in vrouwen met (micro)invasief carcinoom (13/14 (93%)) dan in de vrouwen met CIN (101/282 (36%)). Aangezien deze geobserveerde hoge prevalentie van antichlamydia antilichamen bij vrouwen met cervixcarcinoom niet overeenkomt met de in de literatuur gerapporteerde prevalenties analyseerden we een tweede groep patiënten. Bij 331 patiënten, doorverwezen tussen 1995 en 1999, konden we de hoge prevalentie inderdaad niet bevestigen. Deze resultaten suggereren dat de aanwezigheid van circulerende antichlamydia antilichamen niet geassocieerd is met de ernst van cervix neoplastische laesies en het lijkt onwaarschijnlijk dat *Chlamydia trachomatis* een rol speelt in de progressie van cervicale neoplasie.

De laatst bestudeerde infectieuze factor in dit proefschrift en de meest belangrijke, is HPV. In de literatuur wordt voorgesteld om HPV als de eerst geïdentificeerde noodzakelijke oorzaak van cervix carcinoom aan te merken. Dit houdt in dat men aanneemt dat cervixcarcinoom, op zeldzame uitzonderingen na, niet kan ontstaan zonder voorafgaande HPV infectie. Meer dan 100 verschillende HPV types zijn geïdentificeerde, waarvan 35 types in het genitaal kanaal gevonden kunnen worden. Van die 35 genitale types zijn er 18 geassocieerd met cervixcarcinogenese. Vanwege de belangrijke rol binnen de etiologie van het cervixcarcinoom is in vele studies geprobeerd factoren te identificeren die van belang zijn bij HPV infectie en persistentie. Verschillende HPV detectie technieken zijn beschikbaar, welke mogelijk de uitkomsten van epidemiologische studies beïnvloeden.

In een eerdere studie in 304 patiënten, verwezen vanwege een afwijkend uitstrijkje werd HPV analyse verricht op cervixschraapsels die waren afgenomen bij het eerste bezoek aan de polikliniek.[10] De op GP5/6 primers gebaseerde HPV PCR werd gebruikt. De prevalentie van HPV bleek toe te nemen met de ernst van cervixneoplasie, het aantal seksuele partners en de dagelijkse sigaretten consumptie. Een aantal jaren na deze epidemiologische studie kwam de uiterst sensitieve op SPF10 primers gebaseerde Inno-LIPA (line probe assay) HPV prototype research assay beschikbaar voor de detectie en typering van HPV. Gebruik makend van deze nieuwe assay werd in **Hoofdstuk 7** dezelfde onderzoekspopulatie nogmaals geanalyseerd. Hoog risico HPV bleek aanwezig bij 288 (95%) patiënten. In totaal waren 86 (30%) van deze 288 patiënten geïnfecteerd met meerdere HPV types tegelijk. HPV 16 werd minder vaak gevonden in infecties met multipele types dan te verwachten op basis van kans alleen. De ernst van de gediagnosticeerde neoplasie was geassocieerd met de aanwezigheid van hoog risico HPV. Deze associatie werd volledig gedragen door de associatie tussen de ernst van neoplasie en de aanwezigheid van HPV 16. Er werd geen associatie gevonden tussen het totale aantal seksuele partners of roken en HPV, noch voor één van de vijf meest voorkomende HPV types apart, noch voor de aanwezigheid van multipele types.

De conclusie was dat de associatie tussen de detectie van HPV en de epidemiologische risico factoren, zoals gevonden met de GP5/6 PCR in het verleden, niet bevestigd konden worden met de SPF10 PCR primers en LiPA HPV genotypering. Het vermoeden bestaat dat het aantal seksuele partners en roken surrogaat determinanten van hoge HPV virus belasting zouden kunnen zijn in plaats van determinanten van de aanwezigheid van HPV op zich.

Van epigenetische mutaties, veroorzaakt door de hypermethylatie van promotor regionen van tumor suppressor genen, werd het laatste decennium bekend dat ze een belangrijke plaats innemen bij het ontstaan van maligne (cervix)afwijkingen. Onduidelijk was nog of cervixschraapsels een goede afspiegeling kunnen zijn van de hypermethylatie status van onderliggend cervicaal epitheel. Daarom was het ook niet in te schatten of QMSP (kwantitatieve methylatie specifieke PCR) op cervixschraapsels in de toekomst gebruikt zou kunnen worden ter verbetering van de huidige manier van screenen. In **Hoofdstuk** 8 werd dit verduidelijkt met behulp van cervix schraapsels en gepaard vers gevroren cervixepitheel verkregen van 53 cervixcarcinoom patiënten en van 45 controles. Alle schraapsels werden cytomorfologisch gescoord en geanalyseerd met QMSP voor de genen *APC, DAPK, MGMT* en *GSTP1*. *APC* medieert proliferatieve signalen, *DAPK* is een proapoptotisch gen en bezit misschien de mogelijkheid metastasen te voorkomen, *MGMT* is een DNA reparatie gen en *GSTP1* is een detoxificerend gen.[11-15] Om te corrigeren voor de gebruikte hoeveelheid DNA werden de hypermethylatie resultaten uitgedrukt als ratio met een referentie gen. Bij cervixcarcinoom patiënten en controles waren hypermethylatie ratio's van gepaarde cervixweefsels en cervixschraapsels sterk geassocieerd. *DAPK* was frequenter positief in cervixcarcinoom patiënten dan in controles (p<0,001). Wanneer we afkapwaarden voor ratio's definieerden boven de hoogste ratio die werd gevonden in controles, konden we met de analyse van slechts vier genen reeds 32 van de 48 (67%) cervixcarcinomen identificeren met QMSP op cervix schraapsels. Deze studie laat zien dat QMSP bruikbaar is op cervix schraapsels en dat de techniek de potentie heeft om een nieuwe screeningstest voor de detectie van cervixcarcinoom te worden. Wel zal het noodzakelijk zijn meer genen te identificeren die specifiek gemethyleerd raken tijdens cervicale carcinogenese en deze genen dienen toegevoegd te worden aan de test ten einde een sensitiviteit en specificiteit van ten minste 90% te bereiken.

Er wordt gedacht dat een toegenomen dysbalans tussen proliferatie en apoptose belangrijk is voor het ontstaan van cervixcarcinoom. De death-liganden FasL en tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induceren apoptose door te binden aan hun corresponderende death-receptoren: Fas voor FasL en DR4 en DR5 voor TRAIL. Het doel van de studie beschreven in **Hoofdstuk 9** was om te onderzoeken of er veranderingen in de expressie van deze death-liganden en hun receptoren ontstaan binnen de verschillende stadia van cervicale carcinogenese. De expressie en localisatie van Fas/FasL en DR4/DR5/TRAIL werd immunohistochemisch bepaald op coupes van cervixepitheel (11 keer normaal cervixepitheel, 15 keer CIN I, 15 keer CIN II, 13 keer CIN III en 25 keer (microinvasief) plaveiselcel carcinoom). Het aantal apoptotische

cellen werd op basis van morfologische criteria bepaald en het aantal prolifererende cellen werd bepaald door het aantal Ki-67 positieve cellen te tellen. Toegenomen ernst van de neoplasie ging gepaard met een duidelijke toename in zowel proliferatie als apoptose. In normaal cervicaal weefsel en in CIN I werd FasL, DR4, DR5 en TRAIL voornamelijk aangekleurd in de basale en parabasale cellagen, terwijl Fas in dezelfde coupes met name werd aangekleurd in de oppervlakkige, meer gedifferentieerde cellagen. De frequentie van Fas detectie nam toe met de ernst van de CIN graad. FasL, DR4, DR5 en TRAIL aankleuring van alle cellagen van het epitheel werd daarentegen vaker in CIN III en in cervixcarcinoom geobseveerd. De patronen van aankleuring van FasL, DR4, DR5 en TRAIL waren gecorreleerd. De intensiteit van TRAIL expressie was hoog in laag gradige afwijkingen. Er werd geen associatie gevonden tussen death-receptor of ligand expressie en het percentage proliferatie of apoptose. Verlies van Fas aankleuring en deregulatie van het aankleuringspatroon van FasL, DR4, DR5 en TRAIL bij de toename van CIN naar cervixcarcinoom suggereert een mogelijke functionele rol bij cervixcarcinogenese. De geobserveerde frequente expressie van DR4 en DR5 maakt deze receptoren tot interessante targets voor innovatieve cervix carcinoom therapie.

TOEKOMSTVISIE

In Nederland bestaat een landelijk georganiseerd programma voor cervixcarcinoom screening. Vrouwen worden in de leeftijd van 30-60 jaar eens in de vijf jaar uitgenodigd om zich te laten screenen. Het hiervoor noodzakelijke uitstrijkje wordt in principe door een huisarts afgenomen. Patiënten worden doorverwezen naar een gynaecoloog voor colposcopisch onderzoek wanneer tijdens de screening afwijkingen worden gevonden. De huidige op morfologie gebaseerde manier van screenen voor cervixcarcinoom, kent een belangrijk percentage fout positieve en fout negatieve resultaten. Op dit moment zijn er nog geen andere diagnostische tests beschikbaar waarvan bekend is dat ze kosten-effectief de Pap smear screening kunnen vervangen of aanvullen. In landen met een lage prevalentie van cervixcarcinoom, zoals Nederland, kan het moeilijk zijn te bepalen of de daling in het aantal cervixcarcinoom doden ten gevolge van de screening opweegt tegen de negatieve kanten die deze screening ook heeft. De voordelen zijn veel duidelijker voor vrouwen in landen (met name ontwikkelingslanden) met een hoge prevalentie van cervixcarcinoom. Pap smear screening lijkt de beste test die op dit moment gebruikt kan worden. HPV DNA detectie is van alle bekende 'markers' het dichtst bij toepassing binnen cervixcarcinoom screeningsprogramma's. De twee grote voordelen van HPV screening zouden kunnen zijn het verlengen van het screeningsinterval voor HPV negatieve vrouwen en triage van vrouwen met milde cytologische afwijkingen. In de komende vijf jaar zullen de resultaten bekend worden van een Nederlandse gerandomiseerde studie die evalueert of het implementeren van deze twee voordelen van HPV DNA detectie ook zou leiden tot verbeterde efficiëntie van het screeningsprogramma.[16] Echter, HPV detectie heeft een relatief lage specificiteit voor (progressieve) CIN II/III en cervixcarcinoom en er zitten ook belangrijke psychologische nadelen aan het invoeren van deze test. Zelfs als de HPV DNA test kosten-effectief de cervixcarcinoom screening kan verbeteren, zal er ruimte blijven voor vooruitgang.

Wanneer het hoge aantal fout positieve en negatieve uitslagen van cervixcarcinoom screening verminderd kunnen worden zal dat het voordeel van cervixcarcinoom screening vergroten, ook voor de vrouwen uit landen met een lage prevalentie van cervixcarcinoom. Een interessante nieuwe benadering van cervixcarcinoom detectie is de analyse van tumor suppressor gen hypermethylatie analyse door middel van QMSP. In dit proefschrift beschreven we de theoretische voordelen van QMSP ten opzichte van Pap smear analyse en een aantal andere, nieuwe screening methodes, die gebaseerd zijn op moleculaire veranderingen. Onze eerste QMSP resultaten in cervix schraapsels waren veelbelovend. Cervix schraapsels gaven een goede weerspiegeling van de hypermethylatie status van het onderliggende cervicale epitheel. Terwijl de cervixschraapsels voor slechts vier genen werd geanalyseerd werd reeds 67% van de cervixcarcinoom patiënten geïdentificeerd met behulp van QMSP. In prostaat carcinoom is *GSTP1* gemethyleerd in meer dan 90% van de tumoren en op basis van deze bevinding worden momenteel diagnostische tests ontwikkeld.[17] Gezien de resultaten die behaald worden bij het prostaat carcinoom verwachten we dat het mogelijk moet zijn om voor cervixcarcinoom een panel van genen te identificeren waarvan tenminste één gen

gehypermethyleerd zal zijn in nagenoeg alle cervixcarcinoom gevallen. De beschikbare gegevens en deze uitgesproken verwachtingen zouden het onderzoek naar meer genen die specifiek gemethyleerd raken tijdens de cervicale carcinogenese aan moeten sporen. Als er een sensitief en specifiek cervixcarcinoom hypermethylatie panel samengesteld zou zijn wordt het tijd voor het definiëren van de 'screen-positieve' QMSP afkapwaardes voor screeningsdoeleinden. Samen met de sensitiviteit en specificteit van een screenings test wordt de positief en negatief voorspellende waarde van een test in een populatie bepaald door de prevalentie van de op te sporen ziekte in die populatie. Het zou daarom een verstandige aanpak kunnen zijn om de hoogte van de QMSP afkapwaarden per land af te laten hangen van de cervixcarcinoom prevalentie. Verdere studies zouden moeten verduidelijken of QMSP ook gebruikt zou kunnen worden om progressieve CIN II/III lesies op te sporen.

De prevalentie van cervixcarcinoom en CIN zou wel eens drastisch kunnen gaan veranderen als in de toekomst primaire preventie van cervixcarcinoom door HPV vaccinatie mogelijk wordt.[18] Als HPV vaccinatie wordt ingevoerd, mag natuurlijk een belangrijke daling in de prevalentie van cervixcarcinoom en voorloper afwijkingen worden verwacht. Wanneer in het hypothetische geval van geïmplementeerde HPV vaccinatie de sensitiviteit en specificiteit van de gebruikte screeningstechniek niet wordt aangepast, zal een positieve test uitslag vaker berusten op een fout positief resultaat. Misschien zal het ook na invoering van HPV vaccinatie van voordeel zijn om te blijven screenen op cervixcarcinoom omdat het ontstaan van cervixcarcinoom niet volledig is te voorkomen. Immunisatie zal soms mislukken, vrouwen kunnen weigeren om te worden gevaccineerd en het lijkt waarschijnlijk dat er alleen tegen de oncogene HPV types 16 en 18 zal worden gevaccineerd.

Het is moeilijk in te schatten hoe hoog de prevalentie van cervixcarcinoom zal blijven als HPV vaccinatie geïmplementeerd zou zijn in ons gezondheidszorg systeem. Zelfs als zo'n implementatie nabij is zal het zeker decennia duren voor het effect op het aantal nieuwe gevallen van cervixcarcinoom zichtbaar wordt.
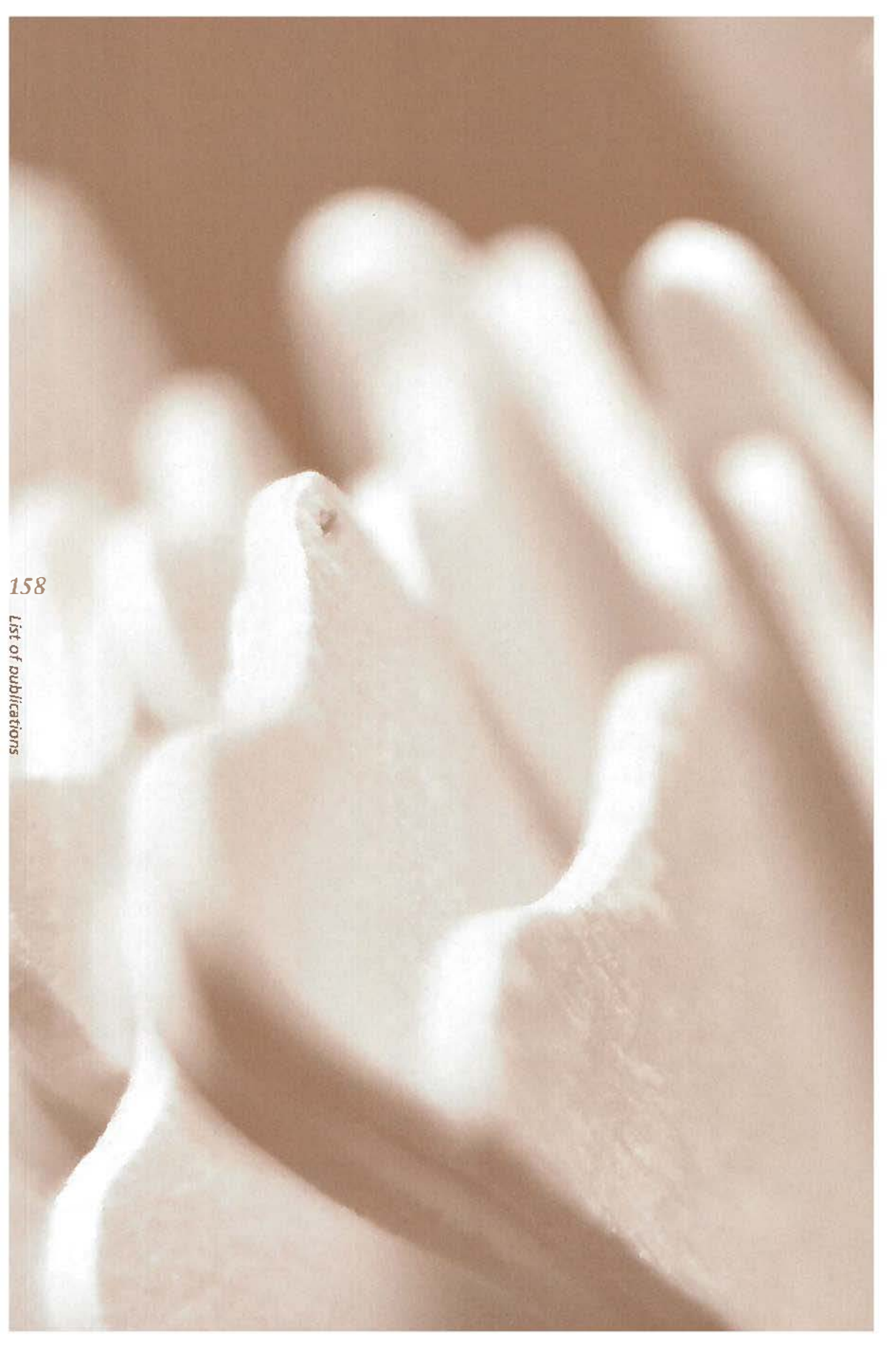
Dit proefschrift beschrijft dat het mogelijk is om bestaande behandelingsprotocollen voor cervixcarcinoom te verfijnen door gebruik te maken van SCC-ag serum detectie. SCC-ag was al bekend als prognostische marker bij cervixcarcinoom. Met het bepalen van SCC-ag in preoperatief afgenomen serum bij patiënten met een vroeg stadium cervixcarcinoom is nauwkeuriger dan met de in gebruik zijnde klinische parameters, per patiënt in te schatten wat het risico is op postoperatieve radiotherapie. Tegelijkertijd is met serum SCC-ag analyse een groep patiënten te identificeren met een hoog risico op het krijgen van een recidief, wanneer ze alleen chirurgisch worden behandeld. Naar onze mening zou serum SCC-ag analyse nu ingevoerd moeten gaan worden in de klinische beleidsbepaling bij vroeg stadium cervixcarcinoom patiënten.

De etiologie van cervix neoplasie wordt steeds duidelijker en nieuw ontwikkelde technologieën zullen misschien leiden tot snelle toename van kennis over cervicale carcinogenese. Het zal een uitdaging blijven om deze nieuwe inzichten te vertalen naar een verfijning van de screening en behandeling van de individuele patiënt.

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LIST OF ABBREVIATIONS

AGUS	Atypical glandular cells of undetermined significance
APC	Adenomatous Polyposis Coli
APES	3-Aminopropylethoxysilan
ASCUS	Atypical squamous cells of undetermined significance
bp	Base pairs
BSA	Bovine serum albumine
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CIS	Carcinoma in situ
CISOE-A	Composition-Inflammation-Squamous epithelium-Other and endometrium-Columnar epithelium endocervix / Adequacy
DAPK	Death associated protein kinase
DcR	Decoy Receptor
DEIA	DNA enzyme immuno assay
DNA	Deoxyribo Nucleic Acid
DR	Death Receptor
EDTA	Ethylenediamine tetra-acetic acid
EIA	Enzyme Immuno Assay
FHIT gene	Fragile Histidine Triad gene
FIGO	International Federation of Gynecology and Obstetrics / Fèdèration Internationale de Gynécologie - Obstétrique
GAPDH	Glyceraldehyde-3-phosphatase dehydrogenase
GSTP1	Glutathione S-transferase P1
GT	Guanidine isothiocyanate
Gy	Gray
HIC-1	Hyper methylated in cancer 1
hMLH1	Human mutL homolog 1
HPF	High power fields
HPV	Human Papillomavirus
HSIL	High grade squamous intraepithelial lesion
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA
IQR / IQ-range	Inter Quartile Range
kB	Kilobase
KCL	Potassium Chloride
KOH	Potassium Hydroxide
(L)LETZ	(Large) Loop Excision of the Transformation Zone
LiPA	Line probe assay
LOH	Loss of heterozygosity
LSIL	Low grade squamous intraepithelial lesion
Mcm5	Minichromosome maintenance 5 protein
MGMT	O6-methylguanine DNA methyltransferase
(M)IC	(micro) Invasive carcinoma
(m)RNA	(messenger) Ribo nucleic acid

MSI	Microsatellite instability
MSP	methylation specific PCR
NIH	National Institutes of Health
Pap(-smear)	Papanicolaou(-smear)
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
pRb	Retinoblastoma protein
QMSP	Quantitative methylation specific PCR
RARß	Retinoic acid receptor-beta
ROC	Receiver operating characteristic
rpm	Rounds per minute
RT	Radiotherapy
rt- PCR	Reverse transcription-polymerase chain reaction
SCC-ag	Squamous cell carcinoma-antigen
SCJ	Squamo-columnar junction
SD	Standard deviation
SIL	Squamous Intraepithelial Lesion
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAP	Telomeric repeat amplification protocol
TSLC1	Tumor suppressor in lung cancer 1

List of abbreviations
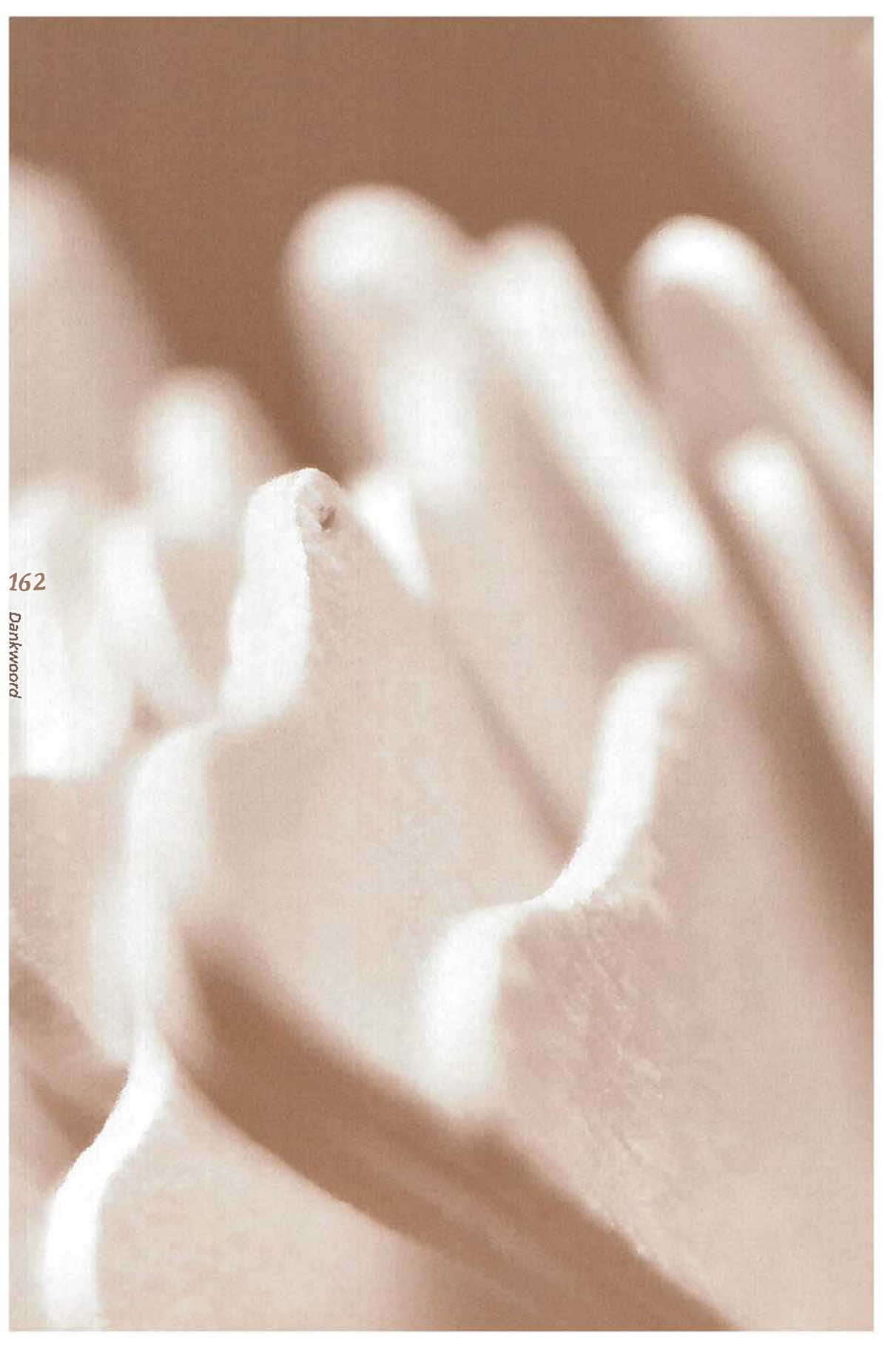
Dankwoord

In de vierde of vijfde klas van de lagere school besloot ik dat ik een boek zou gaan schrijven. Het zou gaan over een walvis en een meisje dat Indira heette. En hoewel ik al wel had bedacht dat ik (dieren)arts wilde worden had ik toen nooit kunnen denken dat het boekje uiteindelijk over afwijkingen aan de baarmoedermond zou gaan, laat staan dat ik het pas in 2004 af zou hebben. Maar goed, áf is het!

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op een manuscript de vinger op de zere plek te leggen, zowel epidemiologisch als anderszins. Je steun in de algemene begeleiding vond ik erg plezierig. Ik had graag nog veel meer met je willen overleggen. Gelukkig wilde Marike Boezen als 'verlengde-arm' van je optreden toen ik naar Groningen ging en kon ik toch mijn vragen kwijt en mijn epidemiologie opleiding afronden. Beste AMC onderzoekers (Judith, Madelon, Marianne, Marja, Moira, Neriman, Saskia, Wessel), we waren een heerlijke club en het was goed om tijdens lunch en soms thee (onderzoekers) lief en leed te delen. Toen ik naar Groningen zou gaan wist ik dat ik dat zou gaan missen. Na mijn werk als arts-onderzoeker in Groningen wachtte de start van de opleiding gynaecologie in de Isala klinieken. Gynaecologen, verpleging en collega's uit Zwolle, dank voor het begrip voor mijn 'vermoeide' toestand en voor jullie interesse. Ik hoop dat jullie de reis naar het Noorden zullen wagen. Tja en op het grensvlak van werk en privé wil ik nog noemen de twee studenten die hun wetenschappelijke stage 'bij mij' kwamen doen en die goede vriendinnen werden. Justine, ik hoop (mede uit eigenbelang) dat je ook de opleiding in komt en dat je artikel op een goede plek gepubliceerd zal worden. Nynke, dat je mijn paranimf bent zegt denk ik wat onze vriendschap voor me betekent.

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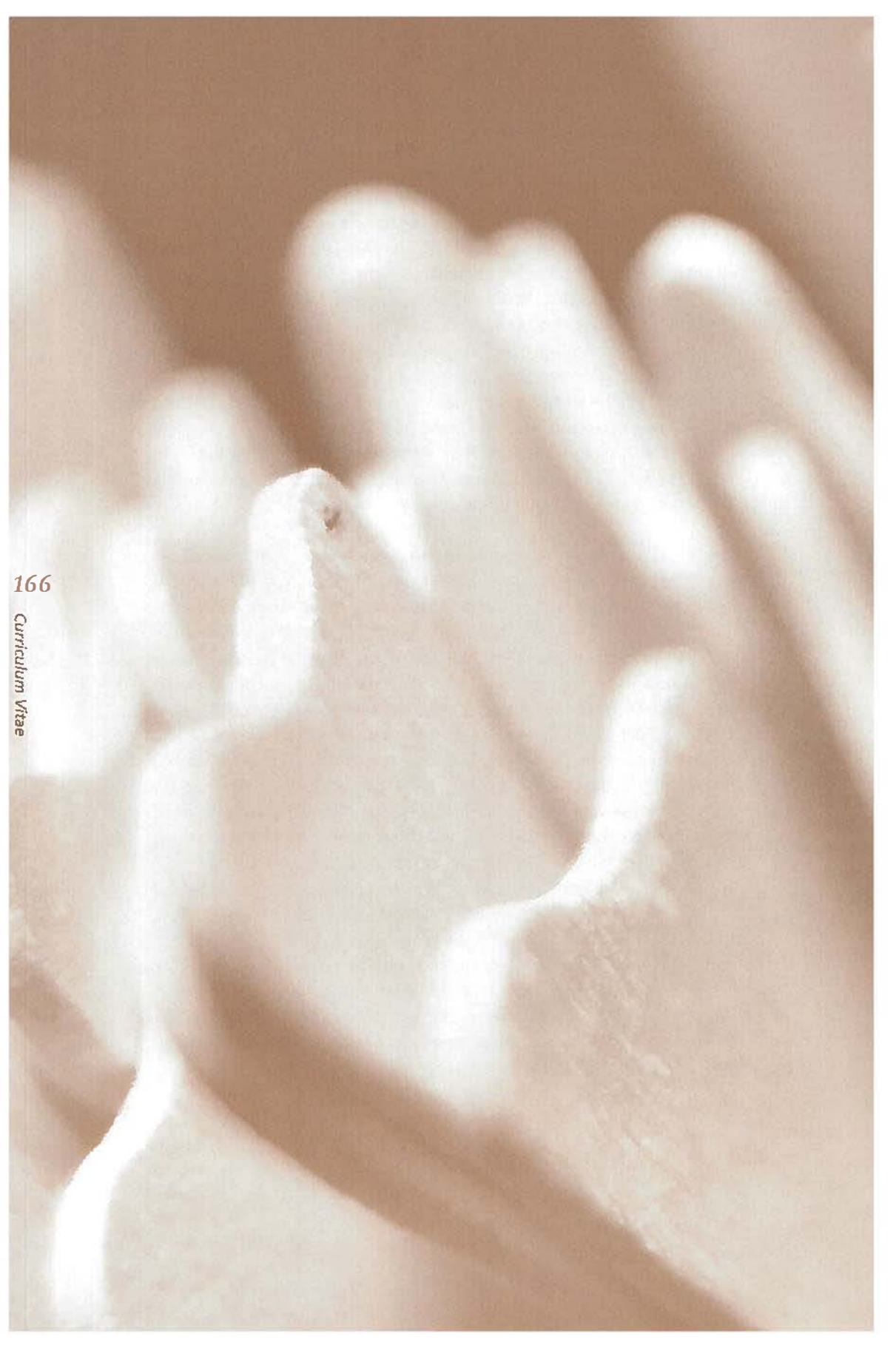
CURRICULUM VITAE

Nathalie Reesink-Peters werd op 24 februari 1972 geboren in Sleen als jongste dochter van Ankie en John Peters. Als jong meisje groeide ze samen met haar zus Daniëlle op in Aalden, Drente. In 1990 behaalde zij haar VWO diploma aan de Gemeentelijke Scholengemeenschap van Emmen. Ze startte datzelfde jaar met de studie geneeskunde in Groningen en behaalde het doctoraalexamen in april 1994. Tijdens de studie was ze lid van de jaarvertegenwoordiging. Na het behalen van het doctoraalexamen volgden de co-schappen in het Universitair Ziekenhuis Antwerpen en in het Academisch Ziekenhuis Groningen. De co-schappen werden voor een deel parttime gevolgd omdat zij betrokken was bij het ontwikkelen van het onderwijsprogramma Medisch Professionele Vorming voor het curriculum 2000 van de studie geneeskunde aan de RUG. In april 1997 slaagde zij cum laude voor het artsexamen.

Na het artsexamen werkte zij ruim $2^1/2$ jaar als AIO bij de Universiteit van Amsterdam en had ook een poliklinische taak in het AMC. In het AMC werd gestart met de opleiding tot epidemioloog B aan de afdeling Klinische Epidemiolgie en Biostatistiek. De AMC-AIO-periode werd niet afgerond, omdat zij overstapte naar het AZG waar ze vier jaar werkte als arts-onderzoeker. Haar onderzoeksgebied betrof (pre)maligne cervix aandoeningen en vanwege haar epidemiologische achtergrond was zij ook bij een aantal andere projecten betrokken. Klinisch was ze werkzaam op de CIN-polikliniek en de oncologische dagbehandeling. In deze vier jarige periode werd de opleiding tot epidemioloog B vervolgd, die met de promotie zal worden voltooid. Verder werkte ze in de laatste twee jaar samen met het researchlaboratorium-KNO van David Sidransky aan de John Hopkins University en ze werkte ook kortdurend ter plaatse in Baltimore, USA.

Op 1 januari 2004 werd gestart met de opleiding Obstetrie en Gynaecologie in het opleidingscluster Groningen. Momenteel is zij werkzaam in de Isala klinieken, Zwolle, voor het eerste, perifere deel van de opleiding.

Met Gerard Reesink heeft zij twee kinderen, dochter Maite (1998) en zoon Jonne (2001) en is zij in verwachting van hun derde kind. Samen wonen ze in Niezijl, Groningen.