

CERVICAL NEOPLASIA: CAUSES AND CONSEQUENCES

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ABBREVIATIONS

ACIS	Adenocarcinoma in situ
AGUS	Atypical glandular cells, uncertain significance
ASC-H	Atypical squamous cells, high-grade squamous intraepithelial lesions cannot be ruled out
ASC-US	Atypical squamous cells- uncertain significance
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CLC	Cervical laser conisation
DNA	Deoxy ribonucleic acid
HPV	Human papilloma virus
HSIL	High-grade squamous intraepithelial lesions
LEEP	Loop electrosurgical excision procedure
LSIL	Low-grade squamous intraepithelial lesion
OR	Odds ratio
PCR	Polymerase chain reaction
pPROM	Preterm premature rupture of membranes
RNA	Ribonucleic acid

LIST OF INCLUDED PAPERS

Paper I

Katrine D. Sjøborg, Ingvild Vistad, Siv S. Myhr, Rune Svenningsen, Christine Herzog, Arild Kloster-Jensen, Gunhild Nygård, Sølvi Hole, Tom Tanbo.

Pregnancy outcome after cervical cone excision: a case-control study.

Acta Obstet Gynecol Scand 2007; 86:423-428.

Paper II

Katrine D. Sjøborg, Anne Eskild.

Vaccination against human papillomavirus-an impact on preterm delivery? Estimations based on literature review.

Acta Obstet Gynecol Scand 2009; 88:255-260.

Paper III

Ameli Tropé*, Katrine D. Sjøborg*, Anne Eskild, Kate Cuschieri, Tormod Eriksen, Steinar Thoresen, Martin Steinbakk, Vigdis Laurak, Christine M. Jonassen, Unni Westerhagen, Morten B. Jacobsen, Agnes Kathrine Lie.

Performance of human papillomavirus DNA and mRNA testing strategies for women with and without cervical neoplasia.

J Clin Microbiol 2009; 47:2458-64.

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Paper IV

Katrine D. Sjøborg, Ameli Tropé, Agnes Kathrine Lie, Christine M. Jonassen, Martin Steinbakk, Mona Hansen, Morten B. Jacobsen, Kate Cuschieri, Anne Eskild.

HPV genotype distribution according to severity of cervical neoplasia.

Gynecol Oncol 2010; 118:29-34.

INTRODUCTION

Cervical carcinoma develops through precursor lesions in the cervix. Treatment of the premalignant lesions of the cervix is important in secondary prevention of cervical carcinoma. However, most of the premalignant lesions of the cervix regress spontaneously. Today one cannot distinguish between lesions that will progress to invasive carcinoma from the vast majority of the premalignant lesions that will spontaneously regress.

As prevention of cancer has high priority, women diagnosed with moderate to severe premalignant cervical lesions are generally recommended to be treated by cervical cone excision. The majority of the treated women are in their reproductive age. It is now known that cervical cone excision is associated with increased risk of preterm delivery in subsequent pregnancies. Since the premalignant lesion will regress in many women, specific prognostic markers of progression to cervical carcinoma are needed.

Persistent infection with high-risk human papillomavirus (HPV) is the primary cause of cervical carcinoma. HPV infections are very common and the majority of HPV infections will spontaneously regress without clinically disease. To improve the identification of women at risk for developing cervical carcinoma, we need a better understanding of the natural course of the different HPV infections and biomarkers that can detect the premalignant lesions that will progress to invasive carcinoma.

The objective of this thesis was to investigate

- I) the impact of cervical cone excision on the outcome of subsequent pregnancies,
- II) to estimate the number of preterm deliveries that may be prevented by an HPV16/18 vaccination programme,
- III) to compare HPV mRNA testing and HPV DNA testing for detection of cervical neoplasia,
- IV) to study the HPV genotype profile and presence of multiple infections according to severity of cervical neoplasia.

BACKGROUND

CERVICAL ANATOMY

The cervix (form Latin “neck”) is the name of the most inferior portion of the uterus, protruding into the upper vagina. The protruding part is referred to as the portio vaginalis or ectocervix. The opening of portio is called the external os. The passage between the external os and the uterine cavity is referred to as the endocervix which ends at the internal os which is the opening to the uterine cavity (Figure 1). The length and width of the cervix varies, but it is approximately three cm in length and between two and three cm in width in reproductive women.

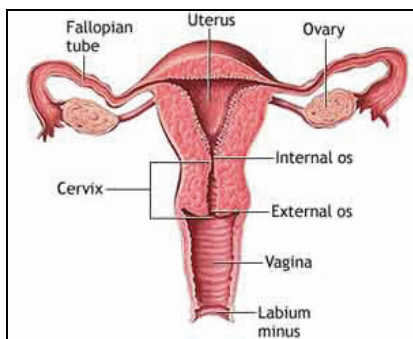


Figure 1. The human uterus. Adapted from www.clarian.org/ADAM/doc/graphics/images/en/19263.jpg

The cervix is composed of a mixture of connective tissue, muscular and elastic tissue of which the connective tissue is the predominant component. The portio vaginalis is lined by multi-layered squamous epithelium while the endocervix is lined by columnar epithelium. At the portio vaginalis, the squamous epithelium of the portio meets the columnar epithelium of the endocervix in the squamocolumnar junction or transformation zone (Figure 2). Most neoplastic lesions develop from the squamous epithelium in the transformation zone.¹

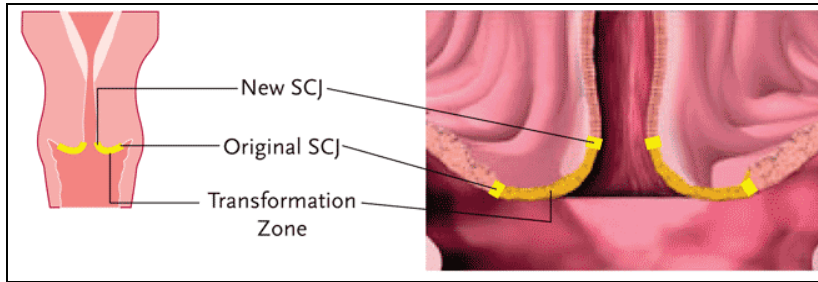


Figure 2. The transformation zone. Adapted from www.prn.org/images/uploads/Palefsky-fig3-680.gif

The central function of cervix during pregnancy is to keep the foetus in utero. During pregnancy, the cervix must therefore remain unyielding and reasonably rigid. With the prelude to labour, the cervix must soften and yield. The cervical modifications during the first phase of labour involve mainly changes in the connective tissue. The results of these changes are cervical thinning, softening, and relaxation, which allow the cervix to initiate dilatation. The dilatation of the cervix will proceed until the cervix is fully dilated (about ten cm) and allow passage of the foetus. In preterm deliveries, these cervical modifications start premature.

OCCURRENCE OF DISEASE

Occurrence of cervical carcinoma

Cervical carcinoma is the second most common cancer among women in the world. Almost 500 000 women are diagnosed with invasive cervical carcinoma each year and 288 000 women die of cervical carcinoma every year, of whom 80% in developing countries.^{2,3} In Norway, as in many Western countries, the incidence and prevalence of cervical carcinoma decreased after implementation of cytological screening programmes. In 2008, 270 women were diagnosed with cervical carcinoma and in 2007, 84 women died of cervical carcinoma in Norway.⁴

Occurrence of pre-invasive cervical lesions

About ten million women are diagnosed with high-grade cervical lesions (CIN2+) and about 30 million women are diagnosed with low-grade cervical lesions every year world wide.² In Norway, 5288 women were diagnosed with high-grade cervical lesions and 17031 were diagnosed with low-grade lesions by cytological examination of cervix in 2008.⁴ In Norway, about 80% of the women with histological verified high-grade cervical lesions (CIN2+) were in reproductive age (Table 1).

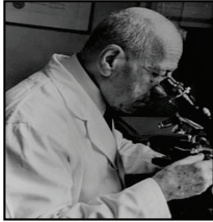
Table 1. Number of women with histological verified CIN2+ in Norway based on data from The Cancer Registry of Norway, 2007.

Age	CIN2	CIN3	ACIS	Total
0-19	11	18	0	29
20-24	71	191	5	267
25-29	107	498	20	625
30-34	82	566	29	677
35-39	72	430	22	524
40-44	57	334	15	406
45-49	42	173	7	222
50-54	31	87	5	123
55-59	26	68	8	102
60-64	11	34	2	47
65-69	8	31	5	44
70-74	4	18	3	25
75+	2	17	3	22
Total	524	2465	124	3113

Identification of women at risk for cervical carcinoma

Cervical cancer screening programmes were introduced in order to identify women at risk of developing cervical carcinoma for treatment of pre-cancerous lesions and thereby prevent cancer development. The International recommendations for cervical cancer screening suggest that screening should start before 35 years of age with no less than three-year interval.^{2,5} The Norwegian Cervical Cancer Screening Programme started in 1995.⁴ All women between 25 and 70 years of age living in Norway are invited to participate, and cervical cytological sampling is performed in primary health care. In Norway, 429790 cervical cytological examinations and 21735 histological examinations of cervical biopsies were performed in 2008.⁴

DIAGNOSIS OF CERVICAL NEOPLASIA



Cervical neoplasia comprises premalignant and malignant lesions in the cervix and is diagnosed by colposcopy, cytological and histological examinations of specimens from the cervix.

Cytology was developed by Papanicolaou in the 1930s. His method is referred to as the Pap smear and is still used in the cervical cancer screening programmes.⁶

George N. Papanicolaou

Cells from the ecto- and endocervix are collected for conventional light microscope examination. Histological examinations are based on colposcopically directed cervical biopsies from the transformation zone, endocervical curettage and cone specimens of the cervix.

Cytological classification of cervical neoplasia

Precancerous lesions in cytological samples are classified according to the Bethesda system.⁷ The squamous lesions are classified as low-grade squamous intraepithelial lesions (LSIL) (Figure 3) and high-grade squamous intraepithelial lesions (HSIL). A precancerous lesion in the glandular cells is not graded but classified as adenocarcinoma in situ (ACIS). The Bethesda system also opens for doubt, using the terms atypical squamous cells- uncertain significance (ASC-US), atypical squamous cells- cannot exclude HSIL (ASC-H) and atypical glandular cells of uncertain significance (AGUS). The sensitivity of cytology testing for detection of cervical neoplasia varies between laboratories, ranging from 30 to 87%.⁸ The glandular lesions are more often missed by cytology than the squamous lesions. According to the Norwegian guidelines women diagnosed with HSIL, ACIS, ASC-H, and AGUS are referred to a gynaecologist for colposcopy, cervical biopsy and endocervical curettage.⁹

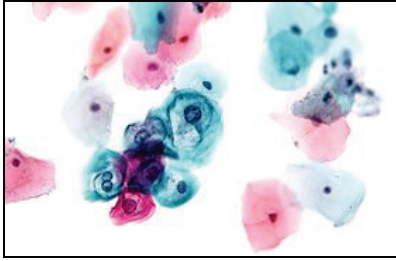


Figure 3. Pap smear showing a low-grade squamous intraepithelial lesion (LSIL). Adapted from Wikipedia, www.wikipedia.org/wiki/Papanicolaou_stain

Histological classification of cervical neoplasia

The squamous cervical lesions are now classified by the cervical intraepithelial neoplasia (CIN) terminology described by Richart in 1973.¹⁰ This system, which is based on the severity of atypia and the distribution of mitoses in the squamous epithelium, are graded into CIN1, 2 and 3 (Figure 4). CIN1 corresponds to mild dysplasia in the old WHO classification; CIN2 corresponds to moderate dysplasia and CIN3 to severe dysplasia and carcinoma in situ. Precancerous lesions in glandular cells are not graded and are classified as adenocarcinoma in situ (ACIS). When the basement membrane is breached by the neoplastic cells allowing for local spread and also distant metastasis, it is diagnosed as invasive cervical carcinoma.

The majority of malignant tumours in the cervix are carcinomas which originate in the squamous or the glandular epithelium via premalignant lesions. The predominant histological type is squamous cell carcinoma (77%), and the adenocarcinomas comprise approximately 15%.^{3,4}

In this thesis we focused on pre-invasive lesions of the cervix.

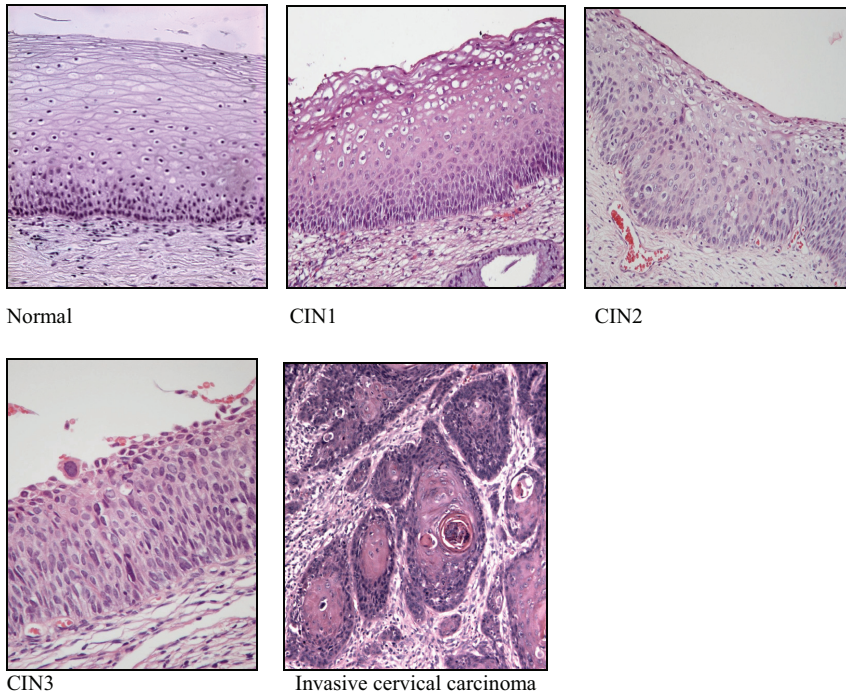


Figure 4. Histological classification of cervical neoplasia. With permission from A. K. Lie.

THE NATURAL HISTORY OF CERVICAL NEOPLASIA

Most cervical neoplastic lesions develop from the transformation zone of cervix where the tall columnar cells are constantly being transformed into flat squamous cells. This metaplastic change occurs in all women of reproductive age. When the process becomes abnormal, it may lead to the development of precancerous lesions of the squamous epithelium in the cervix. These lesions are characterised by abnormal maturation, high mitotic activity, nuclear enlargement and atypia and are called cervical intraepithelial neoplasia (CIN) (Figure 4). Neoplastic lesions in glandular cells develop from the columnar epithelium in endocervix.

The risk of CIN progressing into invasive carcinoma of the cervix depends on the severity of the lesion (Figure 5). Ostor concluded that a proportion of 1%, 5% and > 12% for CIN1, CIN2 and CIN3 respectively, develop into cervical carcinoma (Table 2).¹¹ His classical review is based on studies published between 1950 and 1990. The follow-up time in the included studies varied between 0.5 -10 years.

Table 2. Natural history of CIN.¹¹

	Regress	Persist	Progress to CIN3	Progress to cervical carcinoma
CIN1	57%	32%	11%	1%
CIN2	43%	35%	22%	5%
CIN3	32%	<56%	-	>12%

At the National Women’s Hospital, Auckland, New Zealand, treatment of CIN3 was withheld from a substantial number of women between 1965 and 1974 as part of an unethical clinical study.¹² A judicial inquiry referred all women included in this study for independent clinical review in 1988, resulting in recorded follow-up of 1229 women, and this is the most recent publication on this subject.¹³ Among women with CIN3, the cumulative incidence of invasive cervical carcinoma was 31.3% (95% CI 22.7–42.3) 30 years after the diagnosis, while the incidence was 50.3% (37.3–64.9) among the women who had persistent CIN3 in at least 24 months.

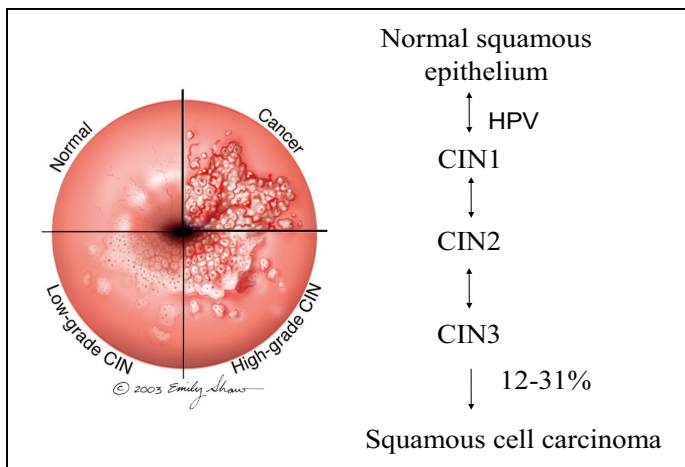


Figure 5. The risk of CIN progressing into cervical carcinoma. Adapted from The CIBA Collection of Medical Illustrations.

Cytological and histological examinations cannot reliably distinguish the women with high-risk precursor lesions that will progress to invasive carcinoma from the vast majority of those

precursor lesions that will spontaneously regress. Since few premalignant lesions progress to invasive carcinoma, there is a need to find biomarkers to identify women at risk for such progression.

THE CAUSES OF CERVICAL NEOPLASIA



Harald zur Hausen

Harald zur Hausen received the Nobel Prize in Physiology and Medicine in 2008 for his pioneering work more than 30 years ago, concerning the role of human papillomavirus (HPV) in the development cervical carcinoma.¹⁴ Certain high-risk genotypes of HPV have now been established as etiologic agents for the development of high-grade cervical neoplasia and cervical carcinoma.¹⁵⁻¹⁸

Most women who get infected with HPV will, however, never develop high-grade cervical neoplasia. A number of cofactors are therefore likely to be involved in the carcinogenesis, or in some women protective factors are present. Several co-factors of progression to neoplasia in HPV infected women have been suggested: environmental or exogenous cofactors including hormonal contraceptives, tobacco smoking, diet and co-infections with other sexually transmitted agents or immunodeficiency viruses, and host co-factors including parity, genetic factors and host immune response.^{17, 19-30}

THE NATURAL HISTORY OF HUMAN PAPILLOMAVIRUSES (HPV)

More than 100 different HPV genotypes have been identified, and at least twelve of these have been linked to the development of cervical neoplasia and therefore classified as high-risk genotypes.³¹

Based upon epidemiological studies HPV viruses are classified as high-risk, probably high-risk and low-risk types (Table 3).³¹

Table 3. Classification of HPV genotypes according to their oncogenic potential.³¹

Classification	HPV genotypes
High-risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Probably high-risk	26, 53, 66, 68, 73, 82, IS39
Low-risk	6, 11, 40, 42, 54, 61, 70, 72, 81, CP 6108
Not yet classified	55, 62, 64, 67, 69, 71, 83, 84

The vast majority of HPV infections are asymptomatic and transient, especially in the young population, and more than 90% of incident infections will resolve within two years.³²

Low-risk genotypes, such as HPV6 and 11, are most likely to resolve, whereas high-risk genotypes such as HPV16 and 18 have slower rates of clearance.³² Some women resolve the infection quite rapidly, within months, and others take up to three years. The duration of persistence of high-risk HPV infection required for development of CIN3+ appears to vary between one to 10 years.³²

Papillomaviruses are non-enveloped, epitheliotropic, double-stranded DNA viruses, approximately 55nm in diameter. The genomic organisation of each papillomaviruses is remarkably similar and can be divided into three functional regions (Figure 6). The HPV genome contains a non-coding area, the late genes L1 and L2, which regulate viral coat proteins (with L1 being the major coat protein and also used to make virus-like particles used in the vaccines). The so-called early proteins (E1, E2, and E4-E7) are necessary for the replication of the viral DNA and for the assembly of newly produced virus particles within the infected cells. Both sets of genes are separated by a control region that does not code for proteins but contains cis-elements required for regulation of gene expression, replication of the genome, and its packaging into virus particles.

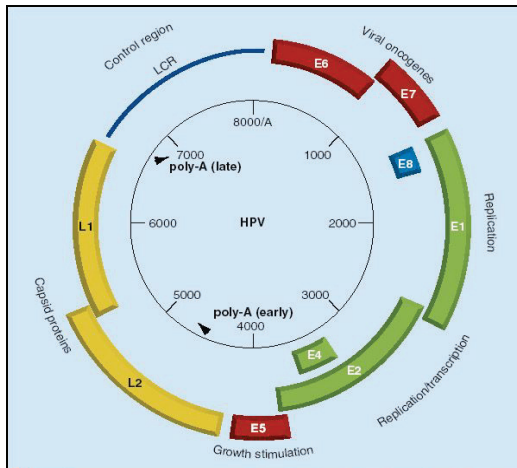


Figure 6. Genome organization of the papillomavirus. Adapted from Thomas Iftner, <http://img.medscape.com/fullsize/migrated/585/223/erm585223.fig2.jpg>

Papillomaviruses are perfectly adapted to their natural host tissue, the differentiating epithelial cell, and exploit the cellular machinery for their own purpose.³³ The natural history of HPV starts with infection of the epithelial basal cells. Access to the basal cells is thought to be due to a cut, tear or inflammation. The replication cycle within the epithelium can be divided into two parts. First, the viral genome is replicated to a copy number of about 100 and maintained for varying periods of time at this low copy number within the initially infected, but still replicating, competent cells. Second, once the basal cells are pushed to the suprabasal compartment, they lose their ability to divide and instead initiate the terminal differentiation program. As the cells travel up through the epithelium, different HPV proteins are expressed. In the upper layer of the epithelium, the late genes (L1 and L2) are expressed, and HPV DNA is packaged into the viral capsid. When cells are normally desquamated, infectious virion are released (Figure 7).

Progression to high-grade intraepithelial lesions and invasive carcinomas is associated with a persistent high-risk HPV infection, integration of the HPV genome into the host chromosomes, loss or disruption of E2 and subsequent up regulation of E6 and E7 expression (Figure7).³³⁻³⁵ The critical molecules in the process of virus replication are the viral proteins E6 and E7, which are the oncogenes of the virus. Continuous expression of these genes is required for malignant transformation. E6 and E7 interact with a number of cellular proteins.

Among others, E6 and E7 mediate binding and degradation of the tumor suppressor genes *TP53* and *RB1*, respectively, and interfere with cell-cycle regulation.^{33,34} Constant activity of E6 and E7 leads to increasing genomic instability, accumulation of oncogene mutations, further loss of cell-growth control, and ultimately development of carcinoma.³⁶ During the development of invasive carcinoma, the viral genomes integrate into the host chromosome, which results in a constant level of E6/E7 proteins via stabilisation of mRNA, probably by loss of negative regulation of transcription mediated by the viral E2 protein.³⁵

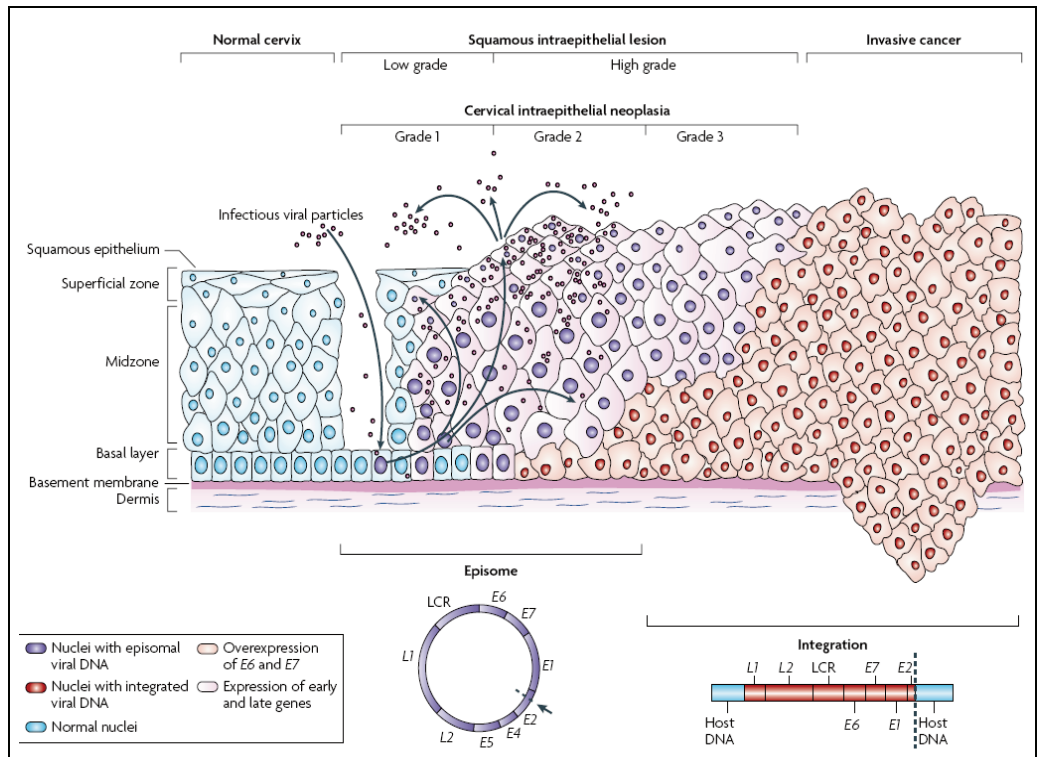


Figure 7. HPV mediated progression to carcinoma. Adapted from Woodman et al 2007.³⁴

There are differences between the E6/E7 proteins of high-risk and low-risk HPV genotypes, but these are often of a quantitative rather than qualitative nature.³⁷ E6 and E7 proteins from low-risk types are less competent in interfering with p53 and pRb functions than E6/E7 proteins from high-risk genotypes.^{38,39} The expression of other viral proteins such as E4 result

in cytoskeleton changes resulting in perinuclear halos which is the hallmark of the koilocytic cell. Infections with low-risk genotypes are associated with benign proliferations, such as genital warts and low-grade intraepithelial lesions prone to regress.

PRESENCE OF HPV

HPV is the most common sexually transmitted infection among men and women, and it has been estimated that 70% of sexually active women will acquire an HPV infection at some point during their lifetime.⁴⁰ Prevalence of HPV varies between geographic locations and age groups. The prevalence of HPV is high in young women. The prevalence declines in the middle-age groups and a second rise in prevalence is observed in women 35-54 years old (Figure 8).⁴¹

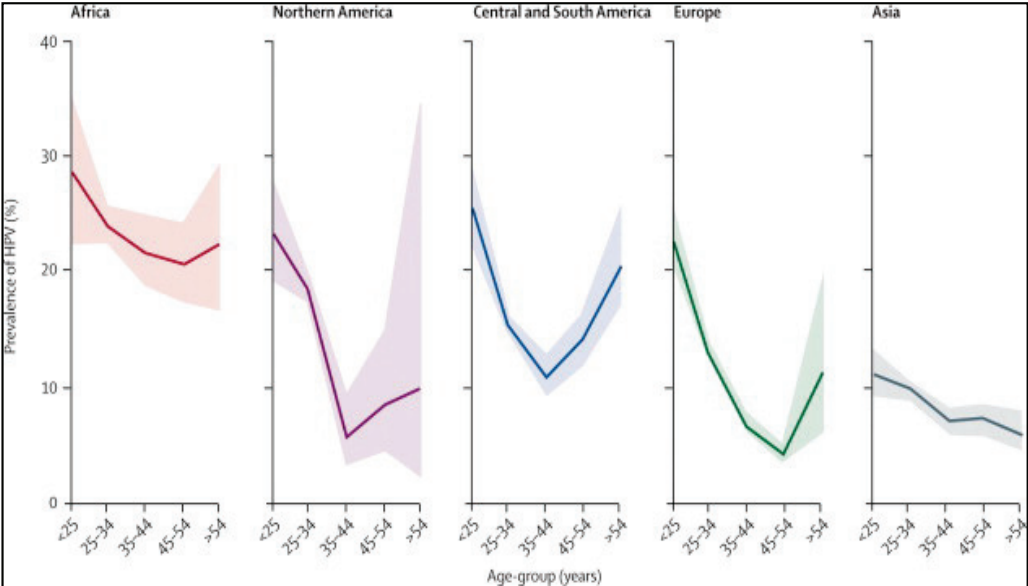


Figure 8. Age-specific HPV prevalence. Adapted from de Sanjose et al 2007.⁴¹

Prevalence of HPV in women with normal cervical cytology

The most comprehensive available data on the HPV prevalence in women with normal cervical cytology derives from a large, global meta-analysis of the literature published in 2007 compiled by the World Health Organisation.⁴² The study includes publications from 1999 up to early 2005 on 157 897 women, and only women with reported normal cytology were included. The results indicate that 10.4% (95% CI 10.2-10.7) of the women worldwide are positive for HPV DNA in cervix. HPV prevalence is higher in less developed regions (13.4%, 95% CI: 13.1-13.7) than in more developed regions (8.4%, 95% CI: 8.3.-8.6). Similar results were observed in an International Agency for Research on Cancer (IARC) population-based survey conducted on 15613 women aged 15-74 years from eleven countries around the world. In all continents, HPV16 is the most common HPV genotype with an estimated point prevalence of 2.6% (95% CI: 2.5-2.8) worldwide.⁴³

HPV genotype distribution in women with pre-invasive cervical lesions

Beyond HPV16, the relative importance of the different HPV genotypes for the development of cervical neoplasia remains insufficiently understood.^{15, 44-48} The distribution of HPV genotypes according to severity of cervical neoplasia can help us gain insight into the oncogenic potential of the different HPV genotypes.

The HPV genotype distribution in women with pre-invasive cervical lesions has been described in several cross sectional studies.^{49, 50} Across all five continents, HPV16 has been reported to be the most common genotype in high-grade cervical intraepithelial neoplasia (CIN2+) with a contribution ranging from 33.3% in Oceania to 51.8% in Europe.^{49, 51-53} In an analysis of pooled data of more than 7000 women diagnosed with high-grade squamous intraepithelial lesions (HSIL) worldwide, the most common HPV genotypes worldwide were HPV16, 31, 58, 18, 33, 52, 35, 51, 56 and 45.⁴⁹

HPV genotype distribution in women with invasive carcinoma

Also in cervical carcinomas, HPV16 is the most common HPV genotype detected in 53-57% of women with invasive cervical carcinoma (Figure 9).^{54, 55} The results from "Pooled analysis of the IARC cervical cancer series",² an updated meta-analysis of 14500 women with invasive cervical carcinoma⁴⁹ and the ICO survey⁵⁴ are consistent in identification of HPV16 and 18 as

the two most prominent genotypes, followed by HPV45, 31 and 33 with small variability. HPV18 is more closely associated with cervical adenocarcinoma, which is more difficult to detect by cervical screening than squamous cell carcinoma.⁵⁶

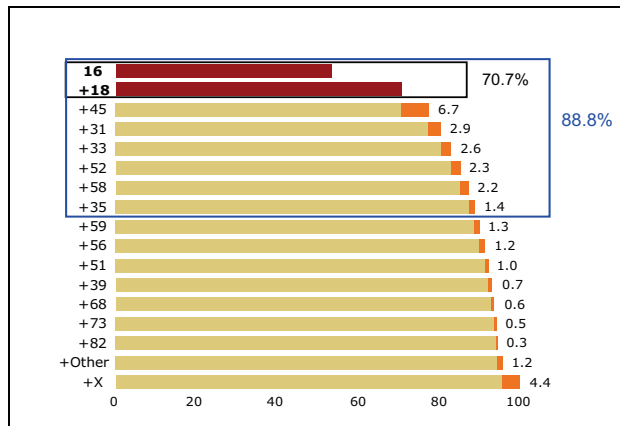


Figure 9. Worldwide prevalence of different HPV genotypes in invasive cervical carcinoma. Adapted from Clifford, 2006.⁵⁵

HPV16, 18, 33 and 45 have been detected more frequently in invasive cervical carcinomas compared to premalignant lesions.^{49, 57} But an increasing prevalence of HPV33, 39, 52 and 58, and a decreasing prevalence of HPV45 and HPV18 have also been reported.⁵⁸

The natural history and oncogenic potential of different HPV genotypes is not sufficiently understood. Only follow-up studies of a large number of women with incident HPV infection can give reliable estimates of genotype specific prognosis. Such studies are costly and time consuming. In cross sectional studies, the distribution of HPV genotypes according to severity of cervical intraepithelial neoplasia (CIN) can help us gain insight into the oncogenic potential of the different HPV genotypes.

The magnitude of increased risk for one specific HPV genotype compared to a reference HPV genotype as well as the role of multiple HPV infections in the progression of cervical neoplasia to carcinoma remains uncertain.^{46, 47, 59} Such knowledge may be essential to identify women at high-risk of disease progression from CIN2 to invasive carcinoma.

In a cross-sectional study, we therefore wanted to study the association between different HPV genotypes and presence of multiple HPV infection according to the severity of cervical lesion.

METHODS OF HPV DETECTION

Since HPV cannot be cultured, HPV has to be diagnosed by DNA, RNA or proteins in the infected tissue.⁶⁰ The most commonly used HPV tests are based on direct hybridization or DNA-based amplification techniques.

DNA-based amplification techniques

Polymerase chain reaction (PCR) is regarded as the most sensitive technique and allows testing on samples with less tissue or cells, poorer DNA quality and fewer viral copies. The PCR tests are based on consensus or type-specific assays. The most commonly used consensus PCR targets the highly conserved L1-region. After PCR, the amplicon can be used for genotyping with genotype specific probes. L1-based PCR tests can give false-negative results in screening since integration of the HPV genome into the human chromosomes may result in loss of the L1 region.

Commercial HPV assays based on L1 or E1 PCR for high-risk HPV DNA detection and genotyping are now available from different companies: among others Amplicor and Linear Array (Roche Molecular Systems, CA, USA), INNO-LiPA (Innogenetics, Ghent, Belgium) PapilloCheck (Greiner Bio- One GmbH, Germany) and Multiplex HPV genotyping kit (Multimetrix GmbH, Heidelberg, Germany). Limited clinical validation exists only for the Amplicor test.⁶¹⁻⁶⁴

Direct hybridization

In situ hybridization by chromogenic or fluorescence techniques is based on the complementary pairing of a labelled probe to HPV antigens or nucleic acids (DNA or mRNA) within either paraffin embedded tissue biopsies or cervical smears. Hybrid Capture 2 (HC2; Digene Corporation, MD, USA) is a signal-amplified hybridization microplate-based assay,

and at present the only US FDA-approved HPV test. This is the most widely used and clinically validated assay on the market.⁶⁵⁻⁶⁷ Hybrid Capture 2 can detect 13 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Limitations of this test are lack of internal control for the amount of input of DNA and cross-reactivity with HPV types not included in the probe mix resulting in false-negative and false positive results.^{53, 68, 69} Hybrid Capture 2 cannot identify specific HPV types, hence other techniques have to be used for genotyping.

RNA-based amplification techniques

HPV E6/E7 mRNA is easier to detect than the viral proteins E6 and E7. Detection of HPV E6/E7 mRNA can be performed by reverse-transcriptase (RT)-PCR or by nucleic acid sequence-based amplification (NASBA).^{70, 71} Presence of E6/E7 mRNA transcripts represent oncogene activity in cervical specimens (Figure 10).

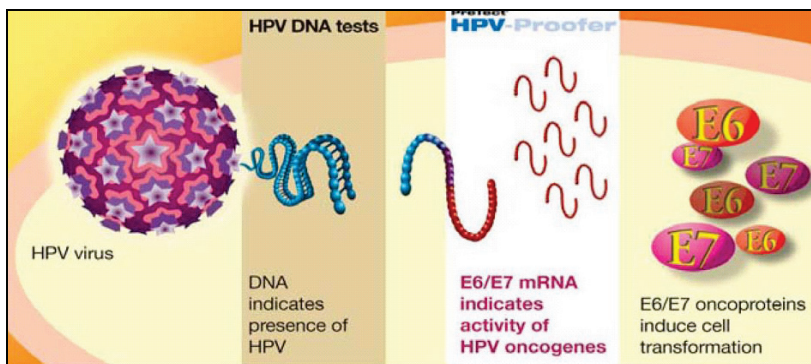


Figure 10. HPV mRNA testing. Adapted from NorChip.

A NASBA-based assay detecting E6/E7 transcripts from the five most common high-risk HPV types in cervical carcinoma (16, 18, 31, 33 and 45) is now commercially available (same product, marketed under different brand names: PreTect HPV Proofer, NorChip AS, Klokkarstua, Norway and NucliSENS EasyQ, BioMerieux SA, France). The advantage with

PreTect HPV-Proofer and NucliSENS EasyQ is that both HPV detection and genotyping are performed in the same reaction.

Gen-Probe is currently developing the APTIMA HPV Assay, targeting E6/E7 mRNA from 14 carcinogenic HPV genotypes; a prototype of this assay has been evaluated in one cross-sectional study.⁷² Meta-analyses, randomized clinical trials and expert reviews reveal that HPV DNA testing for the identification of women at risk of cervical neoplasia is more sensitive but less specific than cytology, and the positive predictive value is low.^{66, 73, 75-78, 136}

Since only a minority of HPV infected women develop cervical neoplasia, identification of HPV infected women by HPV DNA testing could result in follow-up of women with a clinically insignificant infection, resulting in increased costs and patient anxiety.

It has been suggested that the detection of viral gene expression rather than the presence of HPV DNA, may be a better indicator to identify women at risk of developing high-grade cervical dysplastic lesions and cervical cancer (CIN2+). Expression of E6 and E7 mRNA have been found to increase with lesion severity,^{53, 70, 72, 79} therefore the detection of E6/E7 mRNA may be of higher prognostic value and may improve the specificity and positive predictive value compared with HPV DNA testing in screening.^{52, 53, 80-86} However, no population studies have reported the predictive values of mRNA testing for developing CIN2+ compared to cytology or HPV DNA testing.

The marketing of the commercially available DNA and mRNA molecular based HPV tests has been offensive. There are, however, no population-based studies that compare the predictive values for detection of cervical neoplasia of these tests in screening. Comparisons between different HPV tests methods with regard to sensitivity and specificity for detection of HPV and also for the prediction of women whom will develop cervical neoplasia remains insufficiently studied. Few studies have compared HPV mRNA and HPV DNA testing with regard to detection of cervical neoplasia.

Therefore we wanted to compare three different commercial available HPV assays with regard to the detection of HPV, and to compare the strength of the association between a positive test result and the severity of the cervical lesion between the HPV-test methods.

TREATMENT OF PRE-INVASIVE CERVICAL LESIONS

Treatment of pre-invasive cervical lesions is an important measure in the secondary prevention of cervical carcinoma. Women with CIN2 or more severe lesions are generally recommended to be treated by cervical cone excision.^{9, 87-90} Basically, two treatment options exist: cervical cone excision or ablative treatment without excisional surgery. Cervical cone excision can be performed by cold knife, laser, or loop electrosurgical excision procedure (LEEP) (Fig.11). In contrast to ablative procedures such as electrocautery, laser or cryosurgery, cone excision gives a specimen for evaluation of radicality and final histological staging. No technique seems to be superior to another with regard to avoiding recurrent disease.⁸⁸

According to Norwegian guidelines, cold knife excision is not recommended, except when invasive disease is suspected.⁹ LEEP and laser cone excision are less invasive procedures than cold knife excision and are performed as out-patient treatment. Laser cone excision involves special training and is more costly than LEEP procedures.

Cone excision is commonly performed in countries with a cervical cancer screening programme. In the European Union 163 000 cervical cone excisions are estimated to be performed yearly.² In Norway about 3 000 cone excisions are performed yearly.⁴

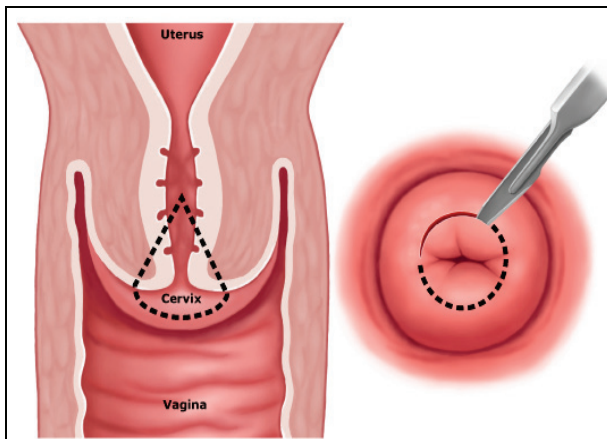


Figure 11. The principal of cervical cone excision. Adapted from http://www.nycosmetics.com/assets/8/Cone_biopsy.jpg

Consequences of cervical cone excision for subsequent pregnancies

Lund and Bjerkedal showed, as early as in 1986, that cold knife cone excision had an adverse effect on subsequent pregnancies, with a relative risk of perinatal mortality of 3.4 in deliveries occurring after cone excision compared to deliveries prior to such treatment in women who gave birth before as well as after cone excision. Compared to deliveries in women without any cone excision, they found a relative risk of perinatal mortality of 11.4.⁹¹

Until recently, it has been argued that LEEP and laser cone excision, to a lesser extent than cold knife cone excision, influences subsequent pregnancy outcome. However, results have been uncertain, and the risks associated with LEEP and laser cone excision were not well known.⁹²⁻⁹⁷ We therefore wanted to estimate risks associated with LEEP and laser cone excision of perinatal death, preterm delivery, low birth weight and preterm premature rupture of membranes (pPROM).

PRIMARY PREVENTION OF CERVICAL CANCER

In the early 1990s work started on the development of prophylactic vaccines against specific HPV genotypes. The vaccines were created from the L1 major capsid proteins of virus-like particles of specific HPV genotypes. These particles are non-infectious and do not contain viral genetic material.

Two prophylactic HPV vaccines are now commercially available. Gardasil is a quadrivalent vaccine which protects against HPV6, 11, 16 and 18 and is developed by Merck and C. Inc (West Point, Pennsylvania, USA). Cervarix is a bivalent vaccine which protects against HPV16 and 18 and is developed by GlaxoSmithKline Biologicals (Rixensart, Belgium). Both vaccines are approved by the US Food and Drug Administration (FDA) and have been licensed in Europe.

Studies on the effectiveness of these vaccines have been encouraging. Both vaccines have undergone double-blind, placebo-controlled phase III clinical trials in North America, Latin America, Europe and the Asia-pacific region. After three doses of either the quadrivalent or bivalent vaccine, almost 100% of women aged 15-26 had detectable antibodies to each HPV genotype, with levels being 10-104 times higher than those seen in natural infections⁹⁸⁻¹⁰⁰

For the quadrivalent vaccine, 12167 women aged 16-26 at enrolment were vaccinated with either the vaccine or placebo. The endpoints measured were CIN2/3, ACIS, cervical carcinoma and genital warts. In the 5305 vaccinated women who had no evidence of past or present infections with HPV16/18, and who received all vaccine doses, the vaccine efficacy was 98% (95% CI: 86-100) against CIN2+ related to HPV16/18 after a mean follow up period of three years. If the women with less than perfect compliance also were included, the vaccine efficacy was 95% (95% CI: 85-99) for the same endpoints. The quadrivalent HPV vaccine has shown cross protections against non-vaccine HPV genotypes, most notable for HPV31.^{101, 102}

For the bivalent vaccine, 18644 women aged 15-25 at enrolment were vaccinated with either the vaccine or placebo. The endpoints measured were CIN2/3, ACIS and cervical carcinoma. In the final analysis of phase III trials of the bivalent vaccine, the vaccine efficacy against CIN2+ associated with HPV16/18 was 98.1% (95% CI: 88.4-100) in HPV negative women at baseline. Vaccine efficacy against CIN2+ irrespective of HPV genotype in lesions was 70.2% (95% CI: 54.7-80.9) in women who were HPV negative at baseline. Corresponding results for CIN3+ were 87.0% (95% CI 54.9-97.7) in HPV negative women at baseline.¹⁰³

HPV vaccination - an impact on preterm delivery?

Based on the vaccine efficacy against CIN2+, a vaccination programme against HPV16/18 is likely to reduce the need for cervical cone excision. Since cone excision is likely to cause preterm delivery, an HPV16/18 vaccination programme may therefore also prevent some preterm deliveries. Numbers of preterm deliveries that may be prevented by an HPV vaccination programme has, to our knowledge, never been estimated. Therefore, we wanted to estimate a possible range of preterm deliveries per 100 000 pregnancies that may be prevented by an HPV16/18 vaccination programme.

AIMS OF THE STUDIES IN THIS THESIS

I) To investigate the risks associated with cervical laser conisation or loop electrosurgical excision procedure of perinatal death, preterm delivery, low birth weight and preterm premature rupture of membranes (pPROM) (Paper I).

II) Estimate the number of preterm deliveries per 100 000 pregnancies that may be prevented by an HPV16/18 vaccination programme (Paper II).

III) To compare HPV mRNA testing and HPV DNA testing with regard to detection of HPV in women with and without cervical neoplasia. We also wanted to compare the association between positive test results by the different HPV assays used and the severity of the cervical lesion (Paper III).

IV) To study HPV genotype distribution and the presence of multiple HPV infections in women with high-grade precancerous lesions. We also wanted to identify the HPV genotypes more prevalent in CIN3+ than in CIN2 and to estimate the odds ratios of CIN3+ for infections with different HPV genotypes and combinations HPV infections (Paper IV).

MATERIALS AND METHODS

PAPER I

Study design and study samples

The study “Pregnancy outcome after cervical cone excision: a case-control study” was a multi-centre study within a cohort of women who gave birth at nine different hospitals in the Southern part of Norway (Table 4). The study included 742 women who had been treated with loop electrosurgical excision procedure (LEEP) or cervical laser conisation (CLC) and 742 women who had not been treated with cervical cone excision. Women were included from the following hospitals: Østfold Hospital Trust, Sørlandet Hospital Trust Arendal, Sørlandet Hospital Trust Kristiansand, Vestfold Hospital Trust, Telemark Hospital Trust, Bærum Hospital, Buskerud Hospital Trust, Ringerike Hospital and Rikshospitalet University Hospital.

The study in Paper I was classified as a case-control study. This is, however, not the case. It was a retrospective cohort study. In case-control studies individuals are included in the study based on whether they do (cases) or do not (controls) have the condition in question. The groups with and without disease are compared with regard to prevalence of exposures. In cohort studies subjects are followed from a certain point of time, usually the exposure defining event, until the development of disease or censoring/end of the follow-up period.

In our study, treatment by LEEP or CLC was the exposure. The exposed women were identified through the participating hospital’s patient records, and followed through delivery. Also women who had not been treated with cervical cone excision were identified from the hospitals’ patient registries and followed through delivery. Pregnancy outcomes according to exposure were compared. Our study should therefore be classified as a retrospective cohort study, rather than a case-control study as stated in the paper.

Identification of women treated with cervical cone excision

We identified all women who had undergone either LEEP or CLC by using the participating hospital’s patient’s records during the period from January 1, 1990 through to December 31, 1999. Women who were 40 years of age or younger at the time of conisation, were contacted with a postal letter with study information and a request for permission to collect information

about their obstetrical history from their medical records. Women, who consented to participate and delivered an offspring after 16 weeks of pregnancy at one of the participating hospitals subsequent to cone excision, were included in our study.

Of the 742 included women treated with cervical cone excision, 419 women had given birth before conisation. The occurrence of the pregnancy outcomes; perinatal mortality, preterm delivery, birth weight and preterm premature rupture of membranes (pPROM) was compared before and after conisation in women who had undergone such treatment and also compared to women who had not undergone treatment.

Identification of women not treated with cervical cone excision

Women without cervical cone excision were identified from the respective participating hospital's birth registries as the first woman who delivered, after the index women (the women treated with conisation) and whom had the same age (+/- 2 years), parity and plurality.

Table 4. Study samples and study design of included papers.

Study	Study samples	Study design	Enrolment to study
Paper I	742 women who gave birth after cervical cone excision* 742 women without cervical cone excision*	Multi-centre, retrospective cohort study	From January 1990 through December 2003
Paper II	Literature from 1999-2008	Estimations based on modeling	Literature search in PubMed and MEDLINE from January 1980 through September 2007
Paper III	643 women with CIN2+** 736 with normal cytology	Cross-sectional study	From January 2005 through December 2006
Paper IV	643 women with CIN2+**	Cross-sectional study	From January 2005 through December 2006.

*Women were recruited from the following hospitals: Østfold Hospital Trust, Sørlandet Hospital Trust Arendal, Sørlandet Hospital Trust Kristiansand, Vestfold Hospital Trust, Telemark Hospital Trust, Bærum Hospital, Buskerud Hospital Trust, Ringerike Hospital and Rikshospitalet University Hospital.

**Women were recruited from the following hospitals: Østfold Hospital Trust, Akerhus University Hospital and Innlandet Hospital Trust.

Variables

Dependent variables

Our dependent variables were perinatal mortality, gestational duration, birth weight and preterm premature rupture of membranes (pPPROM). Perinatal mortality was defined as number of deaths among all still-and live births after 22 weeks of gestation, including neonatal deaths during the first week after delivery.

Estimations of gestational age were calculated from ultrasound due date (scan performed between 17 and 19 weeks) and used in the data analyses as both a continuous variable (days) and as the following categories: delivery before 37, 32 and 28 weeks (yes/no). Birth weight was used as a continuous variable in grams and as the following categories: birth weight less than 2500, 1500 and 1000 grams (yes/no). PPRM was defined as rupture of membranes before 37 completed weeks of gestation and before onset of labour.

Independent variables

Independent variables were treatment by cervical cone excision (yes/no). Cervical cone excision was done by either laser conisation or LEEP. Treatment by LEEP or CLC depended on the choice of operating procedure routinely used at the respective hospital. Also depth of the cone was used as the independent variable. The cone depth was measured in millimetres as the vertical depth of the cone after fixation.

Potentially confounding variables

Marital status, levels of education and smoking habits were included in the data analyses as confounding variables. Marital status was categorised as married/co-habitant or single/divorced/widow. Smoking habits was categorised as smokers or non-smokers during pregnancy. Level of education was categorised as <12 years or >12 years of education.

Statistical analysis

Differences in perinatal mortality, preterm delivery, birth weight and pPPROM in women with and without cervical conisation were compared using chi-squared and Fisher exact tests. Differences in perinatal mortality, preterm delivery, birth weight and pPPROM before and after conisation in the same woman were estimated by using McNemars test. Continuous data were

compared with t tests. To study the association of cervical conisation and preterm delivery also odds ratios were estimated with 95% confidence intervals in logistic regression analyses. Adjustment was made for potentially confounding factors; smoking during pregnancy, marital status and educational level. The relationship between depth of the cone excised and the risk of preterm delivery was estimated by Spearman product-moment correlation. Continuous data were given as mean \pm standard deviation (SD). The statistical analyses were conducted using SPSS software, version 13.0.

PAPER II

Study design and study samples

In the study “Vaccination against human papillomavirus-an impact on preterm delivery? Estimations based on literature review”, we made estimations on the number preterm deliveries that may be prevented by HPV16/18 vaccination based on a mathematical model.

The number of prevented preterm deliveries depends on the number of preterm deliveries caused by cervical cone excision (extent of the health problem), and the proportion of this health problem that could be prevented by a vaccination programme. The number can be estimated as follows:

The extent of the health problem was defined as: $a(b-c)$;

a = the proportion of pregnant women treated with cervical cone excision,

$(b-c)$ = the proportion of preterm deliveries attributable to cervical cone excision = the proportion of preterm deliveries in women treated with cone excision (b) minus the proportion of preterm deliveries in women not treated with cone excision (c).

The preventable proportion was defined as; $d \cdot e$

d = the proportion of cervical cone excisions that can be prevented by vaccination,

e = the proportion of childbearing women who have been vaccinated.

The number of preterm deliveries prevented by a vaccination programme can thereby be estimated as the extent of the health problem; $a(b-c)$ multiplied with the preventable proportion; $d \cdot e$.

We obtained values on the proportion of preterm deliveries attributable to cervical cone excision from the scientific literature. To identify relevant studies, a search in PubMed and MEDLINE from January 1980 through September 2007 was performed using the search words: “pregnancy” and “loop electrosurgical excision procedure “ (“LEEP”, “LETZ”, “LLETZ”), “loopexcision”, “cervical cone excision”, “conization” or “conisation”. Reference lists in the identified publications were searched manually to identify additional relevant studies. Two meta-analyses and five population-based registry studies were included.¹⁰⁴⁻¹¹⁰

We obtained values on the preventable proportion of cervical cone excision in vaccinated women from the scientific literature.^{100, 101, 111-115}

Study factors used in the estimations

The proportion of pregnant women treated with cone excision. In our model, we included three different assumptions of the proportion of women who had been treated with cone excision prior to delivery; one, two and four percent. (Nohr B, personal communication)^{107, 116, 117}

The proportion of preterm deliveries attributable to cervical cone excision.

A range of probable proportions were obtained from the scientific literature. Only studies estimating the risk of preterm deliveries associated with LEEP or CLC were considered. We ensured that the studies giving the highest and the lowest risks for preterm delivery after cervical cone excision with adequate power and design were included.

The proportion of cervical cone excisions that can be prevented by HPV vaccination.

This proportion was set to be 65%.^{100, 101, 111-113}

The proportion of pregnant women who are vaccinated.

This proportion was set to be 90%.^{114, 115}

PAPER III AND PAPER IV

Study design and study samples

The studies “Performance of human papillomavirus DNA and mRNA testing strategies for women with and without cervical neoplasia” and “HPV genotype distribution according to severity of cervical neoplasia” were cross-sectional studies (Table 4).

Participants were identified through the Cervical Cancer Screening Programme of Norway.⁴ Enrolment took place from January 1, 2005 through December 31, 2006. Included in the study III were 736 women with normal cervical cytology and 643 women with CIN2+. Included in the study IV were only the 643 women with CIN2+.

Identification of women with normal cervical cytology

We included 736 women, 30 years or older, who had been through a routine gynaecological examination including cytological sample from the cervix. The women were recruited from general practitioners and gynaecologists in private practices who sent Pap smear samples to be evaluated at the Department of Pathology at Akershus University Hospital. The included women had normal Pap smear cytology, normal cytological results from the preceding two years and no history of treatment for cervical neoplasia. To be included in the study women were cross-checked with The Cancer Registry of Norway to ensure no prior history of cervical neoplasia or abnormal cytology. The median age was 51 years (range 31-82 years).

Identification of women with CIN2+

We included 643 women (no age criteria imposed) with histological confirmed CIN2+ recruited from a source population of 424,143 women in Health Region East, and who were diagnosed at one of the following hospitals: Østfold Hospital Trust, Akershus University Hospital and Innlandet Hospital Trust. Of all women with CIN2+ (n=655), twelve women were excluded since their HPV tests were not evaluable. The median age was 35 years (range 17-76 years).

Cytological samples

Cervical specimens for cytological examinations were collected from the ecto-and endocervix with Cytobrush Plus (Medscan Medical AB, Sweden). CellPath CytoFixx (Mochdre Enterprise, Newton UK) was used to prepare slides for cytological analysis. Cytological

diagnoses were defined according to the Bethesda nomenclature system.⁷ Normal cytology was defined as normal Pap smear cytology at inclusion time, normal cytological results from the preceding two years and no previous history of treatment for cervical neoplasia.

Cervical biopsies and cones

Cervical neoplasia was diagnosed with histological analyses of colposcopically directed biopsies and of cone specimens, according to the WHO classification of cervical neoplasia.¹¹⁸ Most biopsies and cone specimens were primarily evaluated by light microscopy by one experienced pathologist (Lie, AK). If not, the specimens were re-evaluated by Lie, and at disagreement, Lie's diagnoses were included in the study. The diagnosis was based on the most severe lesion seen in the biopsy or the cone specimen. Histology revealed CIN2 in 21.0% of the women (135/643), CIN3 in 73.7% (474/643) and adenocarcinoma in situ (ACIS) in 3.3% (21/643). Invasive carcinoma was diagnosed in 2.0% (13/643) of the women. CIN2+ included CIN2, CIN3, ACIS and invasive carcinoma. In paper IV, we calculated risk of CIN3+ versus CIN2. CIN3+ included CIN3, ACIS (either isolated or together with CIN2/3) and invasive carcinoma.

Detection of HPV

Collection of specimens for HPV testing

HPV testing was performed on cell suspension from cervix. For the normal cytological group a conventional Pap smear was taken first and the brush was transferred to a PreServ Cyt vial (Cytoc Corporation, USA) for HPV testing. For the women with CIN2+, specimens from the cervix were obtained with Cytobrush Plus collected at the time of conisation or at the time of biopsy taken within two months before conisation, and the brush was transferred directly to the PreServ Cyt medium. Cells were stored in PreServ Cyt medium for up to 21 days at room temperature or at 4°C before HPV testing.

HPV DNA testing and genotyping

The presence of HPV DNA was detected by Amplicor and Linear Array. The Amplicor HPV test (Roche Diagnostics, Switzerland) detects the following HPV DNA genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The Amplicor test does not include genotyping, and

a positive result of the test was interpreted as presence of one or more of the above genotypes. HPV DNA genotyping was performed with the Linear Array HPV assay (Roche Diagnostics, Switzerland) in women with normal cytology and positive HPV test and in all women with histologically confirmed CIN2+. This assay detects 37 different genotypes (Table 3). This was done retrospectively using the same extracts used for the Amplicor HPV test and PreTect HPV-Proofer test. The analyses were performed according to the manufacturer's recommendation.

HPV mRNA testing

The presence of E6/E7 mRNA was detected by PreTect HPV-Proofer (Norchip, Norway) which detects E6/E7 full-length mRNA transcripts from HPV 16, 18, 31, 33 or 45. Samples that were HPV mRNA negative, internal control negative or internal control indeterminate (signal between 1.4-1.7), as well as samples that were HPV indeterminate were re-extracted and reanalysed as recommended by the manufacturer (Norchip, PreTect HPV-Proofer user guide version 1105 720001 and earlier). Samples that tested indeterminate twice were considered negative if the internal control was positive.

Presence of HPV genotypes, Paper III

Presence of HPV was defined as: HPV DNA detected with Amplicor (yes/no), presence of HPV DNA detected with Linear Array (yes/no) and presence of E6/E7 mRNA detected with PreTect HPV-Proofer (yes/no). In addition, the presence of HPV16, 18, 31 and 33 as detected by both PreTect HPV-Proofer and Linear Array were presented.

Presence of HPV genotypes, Paper IV

HPV DNA genotyping was performed with the Linear Array HPV assay. HPV genotypes were classified as high-risk, probably high-risk, low-risk and not yet classified (Table 3). We studied the presence of each high-risk HPV genotype separately. In addition, in a regression analyses, presence of HPV genotypes were categorized as follows: single infection with HPV16, 18, 31 or 33, co-infections with HPV16+31, HPV16+33, HPV16+18, HPV16 + any other HPV genotype, HPV33 + any other HPV genotype and HPV co-infections not including HPV16, 18, 31 or 33. Single HPV infection (except infection with HPV16, 18, 31 or 33) was used as the reference category. Presence of multiple HPV infections were categorised as 1 genotype, 2-4 genotypes and >4 genotypes.

Statistical analysis

In Paper III, statistical analyses were performed using 2x2 contingency tables with two-sided *p*-values calculated with Pearson's Chi-square test. Fisher's exact test and McNemar's test were used for comparison of paired proportions. *P*-values <0.05 were considered statistically significant. Cohen's kappa statistics were used as indicators of concordance; κ values of <0.20 indicate poor agreement, 0.21-0.40 fair agreement, 0.41-0.60 moderate agreement, 0.61-0.80 substantial agreement and >0.80 indicate nearly perfect agreement. Kappa values were calculated for agreement between assays on detection of HPV16, 18, 31, 33 or 45.

In paper IV, the prevalence of one specific HPV genotype in CIN3+ versus CIN2 was presented and compared by applying the Pearson's chi-square test. The associations of HPV genotypes with CIN3+ were estimated as crude and adjusted odds ratios with 95% confidence intervals by applying logistic regression analyses. Age was included as a potentially confounding variable and coded as ≤ 30 year and > 30 year. The statistical analyses were performed by applying SPSS software, version 16.0.

ETHICAL ASPECTS

Prior to start of study I, written informed consent was obtained from all women to collect relevant information from the medical records in the respective hospitals. Data regarding the control group was extracted anonymously from birth registries based on matching properties, and written consents were not collected for the controls. The Regional Committee for Ethics in Medical Research, Region South, Norway (S-01151) approved the study on beforehand.

Prior to start of study III and IV, written informed consent was obtained from all study participants. The Regional Committee for Ethics in Medical Research, Region East, Norway (676-04239), the Norwegian Health Directorate (05/163) and the Norwegian Data Inspectorate (07/00975-2/SVE) approved the study on beforehand.

SYNOPSIS OF INCLUDED PAPERS

Paper I

Objective. To investigate the effect of loop electrosurgical excision procedure (LEEP) or cervical laser conisation (CLC) on the outcome of subsequent pregnancies.

Methods. Multi-centre, retrospective cohort study which included a cohort of 742 women whom, after treatment by LEEP or CLC, gave birth or suffered second trimester miscarriage. Control women (n=742) were extracted from the respective hospital birth registries and matched by age and parity. Outcome measures were perinatal mortality, length of gestation, birth weight and preterm premature rupture of membranes (pPROM).

Results. There was no significant difference in perinatal mortality among women having undergone LEEP or CLC compared to the control group, 6/742 versus 2/742: OR= 3.1 (95% CI 0.6-15.2). Pregnancies after conisation were shorter than among the control group. The mean length of gestation was 270.6±25.5 days in the first pregnancy after conisation, compared to 279.7 ±12.7 days in the control group; t =8.5, p<0.001. Excluding second trimester miscarriages, odds ratio for giving birth before week 37, 32 and 28 after conisation compared to the control group were 3.4 (95% CI 2.3 – 5.1), 4.6 (95% CI 1.7-12.5) and 12.4 (95% CI 1.6-96.1) respectively, after adjusting for smoking habits during pregnancy, marital status and educational level. Adjusted odds ratio of birth weight <2500g, <1500g, <1000g after conisation compared to the control group were 3.9 (95% CI 2.4-6.3), 4.4 (95% CI 1.5-13.6) and 10.4 (95% CI 1.3-82.2) respectively. The adjusted odds ratio for pPROM was 10.5 (95% CI 3.7-29.5). 419 of the women had given birth before conisation as well as after conisation. The duration of gestation was shorter in the first pregnancy after conisation compared to the last pregnancy prior to conisation. The mean length of gestation was 271.0 ±25.0 days in the first pregnancy after conisation compared to 277.6 ±16.3 days before conisation, t=5.0; p<0.001. Odds ratio for giving birth before week 37 and 32 after conisation compared to prior to conisation were 2.6 (95% CI 1.4-4.5) and 4.5 (95% CI 1.0-20.1) respectively. There was an inverse correlation with deeper cone excisions associated with shorter pregnancy duration: r = -0.12, n=599, p=0.01, corresponding to a 7% increased risk of preterm delivery for each millimetre excised.

Conclusion. Treatment by LEEP and CLC increases the risk of preterm delivery, low birth weight and pPROM in subsequent pregnancies.

Paper II

Objective. Cervical cone excision increases the risk of preterm deliveries. Vaccination against human papillomavirus 16/18 (HPV16/18) will probably prevent the development of high-grade cervical intraepithelial neoplasia and thereby reduce the need for cervical cone excisions. An HPV16/18 vaccination programme may therefore also prevent some preterm deliveries. The aim of this study was to illustrate how different parameters influence the number of preterm deliveries that may be prevented by an HPV16/18 vaccination programme. In a model we included different values of these parameters and estimated a range of possible preventable preterm deliveries.

Methods. We identified the parameters influencing the effect of an HPV16/18 vaccination programme on preterm deliveries, and estimated a possible range of preventable deliveries before the 37th week of pregnancy. The number of preterm deliveries prevented by HPV16/18 vaccination programme will depend on the number of preterm deliveries related to cervical cone excision (extent of the health problem), and the proportion of this health problem that could be prevented by a vaccination programme. We obtained values of the parameters used in the estimations from the scientific literature.

Results. If 2% of childbearing women are treated with cervical cone excision, between 60 and 220 preterm deliveries/100 000 births may be related to such treatment. Close to 60% (between 35 and 128 preterm deliveries) could be prevented by an HPV16/18 vaccination programme, if the programme coverage was 90%. If 4% of women are treated with cone excision, between 70 and 257 preterm deliveries/100 000 births could be prevented.

Conclusion. HPV16/18 vaccination programmes may reduce the number of preterm deliveries through reducing the need for cone excision.

Paper III

Objective. To compare HPV mRNA testing and HPV DNA testing with regards of detecting HPV in women with and without cervical neoplasia. We also wanted to compare the association between positive test results by the different HPV assays used and the severity of the cervical lesion.

Methods. We included 643 women with high-grade cervical neoplasia (135 cases of CIN2, 495 cases of CIN3/ACIS and invasive carcinoma in 13 cases) and 736 women with normal cytology. HPV was detected using the Amplicor and PreTect HPV-Proofer assays. In addition, genotyping was performed with Linear Array in women with normal cytology and a positive HPV test and in all women with histological confirmed CIN2+.

Results. In women with normal cytology; 8.3% (61/736) were Amplicor positive and 3.3% (24/736) were PreTect HPV-Proofer positive ($p < 0.001$). Concordant results between Amplicor and PreTect HPV-Proofer were present in 90.3% (665/736). In women with CIN2+ lesions 96.4% (620/643) were positive by Amplicor, 98.4% (633/643) by Linear Array and 64.1% (412/643) by PreTect HPV-Proofer. Concordant results for the three HPV assays were present in 63.8%. The genotype profile detected by Linear Array and PreTect HPV-Proofer showed substantial agreement for HPV16, 18, 33 and 45. HPV types 16 and/or 18 were detected in 58.8% (378/643) of the women with high-grade neoplasia. Detection of E6/E7 mRNA by PreTect HPV-Proofer increased with severity of the cervical lesion. Detection of HPV DNA, however, was not associated with histology grade.

Conclusion. The detection of HPV varied according to the assay used, and the concordance between the tests were poor. Our results indicate that mRNA testing may be a biomarker for progression of cervical neoplasia, but the optimal genotype mix remains to be determined.

Paper IV

Objective. To analyse the HPV genotype profile and the presence of multiple HPV infections according to severity of cervical neoplasia.

Methods. From a population of 424,143 women in Norway, we included all women ($n=643$) with histologically confirmed cervical intraepithelial neoplasia grade 2 or higher (CIN2+) and

evaluative HPV test during 2005 and 2006. Histology revealed CIN2 in 135 women, CIN3/ACIS in 495, and invasive carcinoma in 13 women. HPV genotyping was performed on cell suspensions from cervix by linear array which differentiates 37 HPV genotypes.

Results. HPV was detected in 98.4% (633/643) of the women, of whom 52.5% (338/643) were infected with more than one HPV genotype. HPV16 was most common, being detected in 51.2% (329/643) of all cases, followed by HPV31, 33, 52, 18, and 51. Overall, HPV16 or 18 were detected in 58.0% (373/643), with 34.7% (223/643) without concurrence of other high-risk genotypes. HPV16 and HPV33 as single infections were more common in women with CIN3+ as compared to CIN2 (age-adjusted odds ratio=5.93, 95% CI=2.73–12.87, and age-adjusted odds ratio=4.53, 95% CI=1.42–14.46, respectively). Concurrent infections with other HPV genotypes did not significantly alter the associations to CIN3+ for HPV16 or HPV33. A single HPV infection, other than HPV16, 18, 31, or 33, was used as the reference. HPV18 or multiple HPV infections not including HPV16 or HPV33 were not associated with the severity of cervical neoplasia.

Conclusion. HPV16 and HPV33 appear to have a higher oncogenic potential than other HPV genotypes.

DISCUSSIONS

PAPER I

In Paper I, we estimated risks associated with LEEP and CLC on perinatal death, preterm deliveries, low birth weight and preterm premature rupture of membranes (pPROM).

In our study, exposed women were matched with non-exposed women by age (\pm 2 years), parity, plurality and time of delivery. Matching was done in order to increase the power of the study by reducing the number of potential confounding variables. The matching variables could therefore not be investigated as possible risk factors for perinatal death, preterm delivery, low birth weight and pPROM. Logistic regression analyses were used to adjust for other confounding variables such as smoking during pregnancy, marital status and educational level. There may however have been other potential confounding factors, such as infections, that we have not adjusted for in our analyses.

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Women with and without cone excision were identified through different patient records. Women treated with cone excision were identified through patient records of patients whom had undergone surgery and followed to delivery through their medical records. Women without cervical cone excision were identified through birth records at the maternity wards. Although it is common for women in second trimester to deliver at the maternity ward, some women may, however, have delivered in other departments. Hence, they may not have been registered in the birth records at the maternity ward and therefore not included in our study. Therefore, an underestimate of second trimester abortions may have occurred in the non-exposed group in our study. In our study, 0.1% of the non-exposed women delivered between 16 and 28 weeks of gestation. In a Norwegian population based study by Albrechtsen et al, 0.4% of the women not treated by cervical cone excision delivered between 24-27 weeks of gestation.¹²² This difference suggests that an underestimation of second trimester abortions in the non-exposed women may have occurred in our study.

Our results showed an increased risk of low birth weight after cervical cone excision. Low birth weight is associated with preterm birth. In our analyses, differences in gestational age in offspring born by women with and without cervical conisation could explain most of the

differences in birth weight between the groups (data not shown). Therefore, our results with regard to low birth weight should be interpreted as a consequence of preterm delivery.

We had limited statistical power to study differences in perinatal mortality between the women with and without cervical cone excision. Also, our estimated risk for delivery before 28 weeks of gestation associated with cervical cone excision is uncertain.

Studies published before 2002 on the risk for preterm delivery associated with LEEP or CLC were contradictory. Resents studies, however, are in agreement with our results, and an increased risk of preterm delivery after treatment by LEEP and CLC has been found.^{104, 106-109, 122-124} Our results and later publications have shown that the relative risk of preterm delivery attributed to cervical cone excision increases with decreasing gestational age.^{107, 122, 123} The risk of preterm delivery was most pronounced in the early gestational age groups in which the clinical significance is the highest.

Our results showed an association between cone depth and increased risk of preterm delivery. This is in agreement with other studies.^{108, 125-127} The proportion of the total cervical volume or endocervical canal removed may be more determinant of risk, than the actual depth of excision. In cold knife cone excisions, more cervical tissue is excised than in loop excisions. However, in loop excisions the cones may vary from superficial and low volume cones to deep and large volume cones. Most cervical cone excisions in young women with fully visible transformation zones need to be only 1 cm deep, and this should protect against serious obstetric outcomes.¹²³ Caution should be exercised when treating fertile women who may wish to become pregnant in the future. Also, ablative treatments may be considered if the transformation zone is fully visible.^{110, 128-131}

Several mechanisms have been proposed to explain the increased risk of preterm delivery after cervical cone excision. Removal of part of the cervix might compromise its functions, leading to lack of mechanical support in the cervix in future pregnancies. A reasonable hypothesis would be that the degree of obstetric morbidity noted might be related to the amount of the cervical tissue removed. As discussed above, several investigators have described a positive association between depth of excision and risk of adverse obstetric events. Others suggested that pathophysiological mechanisms might also be mediated by the different composition of the quality of collagen in the regenerated cervix or other

immunological factors, such as impairment of antimicrobial defence mechanisms after removal of cervical glands and thereby alteration of cervicovaginal bacterial flora.

PAPER II

In Paper II we made estimations on the number preterm deliveries that may be prevented by HPV16/18 vaccination based on a model. Our model was very simple and more advanced modelling could have been applied. Such model could have included a range of possible values of the parameters included and robustness of the model could have been tested. We had, however, no competence to use or define such advanced modelling tools. Our model has, however the advantage of being easy to understand, easy to use, and also errors are easy to detect.

The values of the parameters that we used to calculate the numbers of preventable preterm deliveries by an HPV vaccination programme are uncertain. However, the values in the model may be changed according to updated and better knowledge.

The proportion of pregnant women treated with cone excision

In most countries, the proportion of women treated with cone excision prior to delivery is not known. We included three different assumptions one, two and four percent. In a recent Norwegian study on all deliveries from 1967 to 2003, 2.6% of the women gave birth after cervical cone excision.¹²²

Our assumptions were based on results from a Danish and a Finnish study where respectively 2.3 - 3.04% and 0.8 % of the women had been treated with cervical cone excision prior to delivery (Nohr B, personal communication).^{107, 108, 116, 117} The differences in the proportion of pregnant women treated with prior cervical cone excision may represent country-specific differences or changes in clinical practice over time. In Finland the public cervical screening programme includes women ≥ 30 years. The proportion of childbearing women treated with cone excision may therefore be higher in countries with women screened at younger age. The proportion of pregnant women treated with cone excision may be increasing in many developed countries since both the incidence of HPV infection¹³² and the mean age at delivery are increasing.^{122, 133}

The proportion of preterm deliveries attributable to cervical cone excision

We used point estimates from seven studies in our model. It may have been better to use a plausible range of values, and not simply show the point estimate for each included study. Of the included studies in our model, the study by Jacobsson¹⁰⁷ has a sample size about 30 times larger than the remaining studies combined. Based on this, we may also have used only the Jacobsson study in our model, and used the confidence intervals from that study as a plausible range for the proportion of preterm deliveries attributable to cervical cone excision. However, this would not change our estimates significantly.

The proportion of all cervical cone excisions that can be prevented by vaccination

The effect of HPV16/18 vaccination on CIN2+, regardless of the causal HPV genotype, remains uncertain. We assumed a 65% vaccine effect on CIN2+, based on the reported distribution of HPV16/18 in CIN2+ lesions¹¹³ and the estimated 100% vaccine effect against these HPV genotypes and some additional cross-protection against other oncogenic HPV genotypes.^{100, 101, 111, 112} These assumptions may be too optimistic. The Future I and II Study Group estimated a 27% (CI 95%; 4-44%) overall reduction of CIN2+ in an intention-to-treat analysis.¹⁰¹ Not all women in the vaccine group received the three doses and some women may have been infected with HPV genotypes other than HPV16/18. If we assume a 27% HPV16/18 vaccine effect on CIN2+ and 80% coverage of the vaccination programme, the range of prevented preterm deliveries will be between 7 and 95 per 100 000 births. However, in the final analysis of phase III trials of the bivalent vaccine published in 2009, the vaccine efficacy against CIN2+ irrespective of HPV DNA in lesions was 70.2% (95% CI: 54.7-80.9) in women who were HPV negative at baseline.¹⁰³

The proportion of pregnant women who are vaccinated

In our estimations we assumed 90% vaccination coverage, as is common in public childhood vaccination programmes in developed countries. However, vaccination coverage of the three recommended HPV vaccine doses in 12 year olds girls may be lower; hence our results of the number of preventable preterm deliveries may be overestimated. The last annual HPV vaccine coverage in United Kingdom for 12-13 years old girls was 88.6% for the first dose, 86.6% for the second dose and 80.9% for all three doses. (Morkved JH, Samofi Pasteur MSD, personal communication) The quadrivalent vaccine has been implemented in the National Vaccine Programme in Norway for 12 years old girls from the autumn of 2009. By March 17th 2010, the overall vaccine coverage in Norway was 57%. However, vaccination against HPV has

been delayed in many municipalities in Norway due to vaccination of the pandemic influenza A (H1N1), and in ten percent of the municipalities in Norway, the vaccination against HPV have not yet started. It is therefore too early to draw conclusions about the vaccine coverage in Norway.¹¹⁵

The surgical procedure may influence the risk of preterm delivery. In our model we included risk estimates after laser or loop electrosurgical excision only. The negative impact of cone excision may, however, be most prominent after cold knife excision.^{91, 123} If cold knife excisions are performed in childbearing women on a large scale, the number of preterm deliveries related to cone excision would have been underestimated in our study and consequently more preterm deliveries may be prevented by a vaccination programme.

Up to 10% of all deliveries in developed countries are preterm.¹³⁴ Only a fraction of all preterm deliveries can be attributed to cervical cone excision. In the study by Albrechtsen et al, the proportions of preterm delivery attributable to cervical cone excision before 37, 33 and 28 weeks of gestation were 1.2%, 1.7% and 2.0% respectively.¹²² However, preterm delivery may cause serious disability for the child. Each prevented preterm delivery may therefore save the child and the family from suffering.

To illustrate the potential effect of the HPV16/18 vaccination programme, a 70% reduction of cervical cancer incidence in Norway, will be from 9.5 to 2.9 cases per 100 000 women.⁴ As compared to the number of preventable cases of cervical cancer through a vaccination programme, the number of preventable preterm deliveries may be considerable. When estimating healthy years of life gained by an HPV16/18 vaccination programme, the effect through reducing preterm delivery should also be considered.

PAPER III

In Paper III we compared HPV mRNA testing and HPV DNA testing with regard to detection of HPV in women with and without cervical neoplasia. We also compared the association of positive test results with severity of cervical lesion according to the HPV test used.

The prevalence of HPV varied according to the assay used, and the concordance between the tests was low. There are several explanations for the differences in test results. The different assays are not uniform with regards to the number of HPV genotypes the tests are addressed to detect and the tests differ in detection of HPV mRNA and DNA.

PreTect HPV-Proofer was the HPV mRNA test we used, and it detects transcripts and oncogene activity from 5 out of the 13 HPV genotypes included in the Amplicor test (DNA test used). The Linear Array test, which was the other HPV DNA test we used, detects 37 different genotypes. This may to some extent explain the differences in HPV prevalence estimated with these different HPV test methods.

PreTect HPV-Proofer is based on nucleic acid sequence-based amplification (NASBA) whereas Amplicor and Linear Array are based on PCR that target the L1 region in the HPV genome. L1- based PCR tests can give false negative results since integration of the HPV genome into the human chromosomes may result in loss of the L1 region. PreTect HPV-Proofer tested positive in 2.3% of the Amplicor negative cases. This could be caused by false negative DNA tests due to break point in the L1 region during HPV integration, or false positive mRNA test.

The agreement between Linear Array and PreTect HPV-Proofer in detection of the five genotypes included in both test was substantial for HPV16, 18, 33 and 45, and poor to moderate for HPV31. HPV mRNA-negative test results in HPV DNA-positive samples may be interpreted as HPV infections without active viral transcription or it may be that transcriptional activity occurs but at levels insufficient for PreTect HPV-Proofer detection.

In women above the age of 30 with normal cytology, 3.3% tested positive with PreTect HPV-Proofer and high-risk HPV DNA was detected in 8.3% with Amplicor. These prevalence's are in agreement with another Norwegian study including 4000 women above the age of 30 with normal cytology, in this study PreTect HPV-Proofer tested positive in 2.4% of the women and

high-risk HPV DNA was detected in 9.3% of the women.¹³⁵ In a study from South Carolina, USA in 2007 where the APTIMA HPV assay (detecting E6/E7 mRNA for 14 high-risk HPV types) was used, 8.0% of the women with normal cytology tested positive.⁷² It could be argued therefore that the smaller HPV genotype range of the PreTect HPV-Proofer explains lower prevalence.

In our study 96.4% of women with CIN2+ tested positive with Amplicor which is in accordance with the large POBASCAM and ARTISTIC trials where HPV DNA testing was performed with PCR or Hybrid Capture 2.^{77, 78}

The number of high-risk HPV genotypes that should be included in an HPV test in order to achieve sufficient sensitivity and specificity for development of cervical neoplasia remains to be documented. The natural history of the different HPV genotypes is not yet known. There will have to be a compromise between including low prevalent or less oncogenic HPV genotypes to maximise sensitivity or to include high prevalent HPV genotypes with high oncogenic potentials to increase the specificity. HPV tests detecting different panels of HPV genotypes may also be necessary to use in different parts of the world, since the prevalence of HPV genotypes is dependent on the geographic region. Moreover, as HPV vaccination embeds and the HPV prevalence of vaccine types may change, there will be a requirement to re-consider/recalibrate HPV assays in line with the shifting dynamics of HPV genotype specific prevalence and associated disease.

A prognostic test for identification of the women who will develop cervical carcinoma and not only pre-cancerous lesions is warranted. With such test, numerous of unnecessary treatments with cervical cone excision could be prevented. An increased understanding of the oncogenic potentials of the different HPV genotypes and also increased understanding of expressions of oncogenic transformations may in the future enable development of prognostic tests with high specificity in detection of women with true risk of cancer development.

Population based, randomized, clinical trials have shown that HPV DNA testing is more sensitive than cytology for detection of CIN2+.^{66, 75, 76, 136} The type-specific persistence of oncogenic HPV is considered to be the true precursor of neoplastic progression,¹³⁷ whereas the expression of the E6/E7 oncogenes is necessary for the malignant transformation and maintenance of the neoplastic state.³⁵ Therefore, the detection of the E6/E7 mRNA of the

respective HPV genotypes may serve as a better prognostic test than mere DNA detection for the development of high-grade cervical lesion.^{53, 135}

In our study, detection of E6/E7 mRNA by PreTect HPV-Proofer increased by severity of the cervical lesion, where as detection of HPV DNA was not associated with histology grade. This is in agreement with other studies.^{84, 135, 138} Detection of HPV oncogene activity, through the detection of mRNA transcripts may therefore be a better indicator of HPV infection associated with increased risk of progression to neoplasia, than detection of HPV DNA. However, the mRNA-based test was negative in 35.9% of the women with CIN2+ in our study. Whether they represent CIN2+ lesions associated with regressing lesions or not, will be impossible to confirm since Norwegian women with CIN2+ lesions are routinely treated. It is not clear whether the increased specificity of the PreTect HPV-Proofer for detection of cervical neoplasia is driven truly by detecting transcripts or by detecting a more limited range of HPV types. DNA and mRNA testing may be applied together, to take advantage of the higher sensitivity and specificity of these respective tests. In such situations mRNA testing may act as a biomarker for progression of disease, but further data are needed to consolidate this. Only large follow-up studies of women with incident HPV infection can confirm whether mRNA testing is a marker to identify women at risk for progression of cervical neoplasia.

PAPER IV

In Paper IV we present the association of different HPV genotypes and presence of multiple HPV infections with severity of the cervical lesion in a cross sectional study.

In cross-sectional studies exposure and outcome is measured simultaneously and the association between exposure and outcome cannot be confirmed. In spite of the cross-sectional design, our results on the distribution of HPV genotypes according to severity of cervical neoplasia may help us gain insight into the oncogenic potential of the different HPV genotypes.

Infection with HPV16 and HPV33 were associated with higher prevalence of CIN3+. The importance of HPV16 in development of high-grade cervical lesions has been documented in several other studies.¹³⁹⁻¹⁴¹

In our study, HPV18 was not associated with severity of cervical neoplasia. The lack of association may be explained by the limited statistical power in our study. HPV18 has shown to be more closely related to ACIS and adenocarcinoma than CIN.⁵⁶ Of the included women in our study, only three percent were diagnosed with ACIS and two percent with invasive carcinoma, which may to some extent, explain our results regarding HPV18. Prior studies have also reported that the risk posed by HPV33 to induce CIN3 seemed higher than that of HPV18 and 45.^{139, 142}

Our study included all cases of CIN2+ during a two year period from a large source population. Still, we had limited statistical power to distinguish between effects of uncommon HPV genotypes; hence type 2 errors may have occurred. Our categorisation of HPV genotypes in the estimation of odds ratios was therefore determined by the prevalence of HPV genotypes in our study sample and also by the associations with CIN3+ observed in the data analyses when we studied each HPV genotype separately. In studies of HPV genotype specific risk of cervical neoplasia, there has been a lack of uniform reference category, making comparison between studies difficult

It is still unclear whether multiple HPV infections with certain HPV genotypes will exert a synergistic effect on malignant transformation, or if cervical neoplasia can arise at multiple sites in the cervix. In our study, multiple HPV infections were detected in more than half of

the included women with CIN2+. Our results do not support an association of multiple infections with increased severity of cervical neoplasia, which is in agreement with other cross sectional studies.^{15, 44, 45, 48} Results from the Guanacaste Cohort Study, however, suggest that multiple infections may increase the risk of high-grade lesions and cervical carcinoma.⁴⁶ That study has the advantage of being prospective.

Our study suggests differential risk of cervical neoplasia according to HPV genotype. Knowledge of HPV genotype specific prognosis may be essential to identify women at high-risk of pre-cancerous cervical lesions, and important to better understand disease progression from CIN2 to invasive carcinoma. With such knowledge, HPV genotyping may be an important tool in cervical screening programmes.

Based on the distribution of HPV genotypes in our study, vaccines against HPV16/18 have the potential to prevent at least 34.7 percent of CIN2+ lesions. If HPV16/18 are causal also in the presence of other high-risk HPV genotypes, the preventative potential may be 58.0 percent or higher, if reported cross-protection against other high-risk HPV genotypes are taken into account.

IMPLICATION OF FINDINGS AND SUGGESTIONS FOR FUTURE RESEARCH

We found that cervical laser conisation and loop electrosurgical excision procedures are associated with increased risk of preterm delivery, low birth weight and pPROM in subsequent pregnancies. The risk of preterm delivery was most pronounced in the early gestational age groups in which the clinical significance is highest. About one-third of the women diagnosed with CIN2+ in Norway are younger than 29 years. The mean maternal age in primiparous is increasing and was 28 years in Norway in 2007.^{133, 143} Hence, a large proportion of the women treated with cervical cone excision have not yet given birth to their first child. Our results underscore the need for a careful clinical approach to women with previous cervical conisation when they become pregnant. Our study encourages research aimed at identification of women at true risk of cervical carcinoma since today's overtreatment of all women with CIN2+ has side-effects.

We have illustrated a potential effect of an HPV16/18 vaccination programme on prevention of preterm deliveries. Our estimations suggest that an HPV 16/18 vaccination programme for prevention of cervical cancer also would have preventive effect on preterm delivery through reducing the need for cervical cone excisions. This may have implications for cost effectiveness evaluations and policy making with regards to the introduction of prophylactic HPV vaccination programmes. Future research may give more accurate values to the factors that influence the number of preterm deliveries that may be prevented by HPV16/18 vaccination.

Since few precancerous cervical lesions actually progress to cervical carcinoma, there is reason to improve identification of women at risk for such progression, and thereby prevent unnecessary cervical cone excisions. It has been suggested that detection of oncogene activity through the detection of HPV mRNA transcripts rather than the presence of HPV DNA, may be a better indicator to identify women at risk of developing high-grade cervical lesions and cervical cancer. Our results showed that the concordance between HPV DNA testing and HPV mRNA testing was poor, and the mRNA-based test was the least sensitive test with regards to the detection of HPV. However, the mRNA-based test was the only test method that correlated with histology grade. DNA and mRNA testing may be applied together to take advantage of the higher sensitivity and specificity of these respective tests. In such a situation,

mRNA testing may act as a biomarker for progression of disease, but further data is needed to consolidate this. Future studies should focus on developing screening tools that have higher specificity for developing cervical carcinoma. This to help distinguish the women with high-risk precursor lesions that will progress to invasive carcinoma from the vast majority of precursor lesions that spontaneously regress.

We have performed a large Norwegian study of HPV genotype distribution in women with CIN2+. Our study provides a basis for future trend analyses of HPV distribution in Norway. Our results suggest that HPV16 and HPV33 increase the risk of CIN3+ compared to other high-risk HPV genotypes. However, the proportion of CIN3+ attributable to HPV16 is much higher than for HPV33, due to the much higher prevalence of HPV16. Our results do not support an association of multiple infections with increased severity of cervical neoplasia. Future studies should involve follow-up studies of a large number of women with incident HPV infection to give reliable estimates of each genotype's specific prognosis. Knowledge of the natural history of each HPV genotype may be helpful to identify women with cervical precursor lesions that will spontaneously regress. Hence, unnecessary cervical cone excisions may be avoided and thereby preterm deliveries attributable to cervical cone excision.

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Performance of Human Papillomavirus DNA and mRNA Testing Strategies for Women with and without Cervical Neoplasia[∇]

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In the present study we investigated the cross-sectional positivity for DNA and E6/E7 mRNA from high-risk human papillomavirus (HPV) types in 643 women with high-grade cervical neoplasia (135 cases of cervical intraepithelial neoplasia grade 2 [CIN2], 495 cases of CIN3/adenocarcinoma in situ [ACIS], and 13 cases of invasive carcinoma) and in 736 women with normal cytology by using the Amplicor and PreTect HPV-Proofer assays. In addition, genotyping was performed using Linear Array for women with normal cytology and a positive HPV test and in all women with histologically confirmed CIN2+. In women with normal cytology, 8.3% (61/736) were Amplicor positive and 3.3% (24/736) were PreTect HPV-Proofer positive ($P < 0.001$). Concordant results between the Amplicor and PreTect HPV-Proofer tests were present in 90.3% (665/736). In women with CIN2+ lesions 96.4% (620/643) were positive by Amplicor, 98.4% (633/643) by linear array, and 64.1% (412/643) by PreTect HPV-Proofer. Concordant results for the three HPV assays were present in 63.8%. The genotype profile detected by linear array and PreTect HPV-Proofer showed substantial agreement for HPV types 16, 18, 33, and 45. HPV type 16 and/or 18 was detected in 58.8% (378/643) of the women with high-grade neoplasia. Detection of E6/E7 mRNA by PreTect HPV-Proofer increased with severity of the cervical lesion. Detection of HPV DNA, however, was not associated with histology grade. In conclusion, the detection of HPV varied according to the assay used, and the concordance between the tests was poor. Our results indicate that mRNA testing may be a biomarker for progression of cervical neoplasia, but the optimal genotype mix remains to be determined.

Infection with human papillomavirus (HPV) is considered the cause of the vast majority of premalignant and malignant epithelial lesions of the cervix (8, 21, 29, 31). However, most HPV infections are asymptomatic and transient, and more than 90% of new infections will resolve within 2 years (19). Progression to carcinoma is associated with a persistent infection with high-risk (HR) HPV types, integration of the HPV genome into the host chromosomes, and upregulation of E6 and E7 oncogenes, which can lead to abrogation of normal cell cycling events and tumor suppressor activity (7, 30, 31).

Large, randomized clinical trials have shown that HPV DNA testing has a higher sensitivity but lower specificity than cytology for detecting high-grade cervical lesions in primary screening (2, 5, 6, 12, 23, 24). As most HPV infections are transient, HPV DNA testing could result in follow-up of women with clinically insignificant infection, resulting in increased costs

and patient anxiety. This is why an informed approach to HPV testing is imperative, with clinical contexts and reasons for testing clearly defined and justified, respectively.

Most commercially available HPV tests detect the presence of HPV DNA; however, it is possible to detect HPV mRNA transcripts coding for E6/E7 and thereby the presence of oncogene activity. A nucleic acid sequence-based amplification method detecting E6/E7 transcripts from the five most common HR HPV types in cervical carcinoma (types 16, 18, 31, 33 and 45) is commercially available from two companies (the PreTect HPV-Proofer [Norchip AS, Klokkearstua, Norway] and the NucliSens EasyQ [bioMérieux S.A., France]). The prevailing consensus is that upregulated expression of E6/E7 is necessary for the initiation and progression of cervical neoplasia. Detection of HPV oncogene activity through the detection of mRNA transcripts may therefore be a better indicator of HPV infection associated with increased risk of progression to neoplasia than detection of HPV DNA (14, 17, 18).

The aims of our study were to investigate the cross-sectional positivity of HR HPV DNA and E6/E7 mRNA expression in women with and without cervical neoplasia by using two commercial assays. A third broad-spectrum commercial genotyping

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assay was included so that type-specific analysis could be performed (on women with high-grade disease and on HPV-positive women with normal cytology). We also wanted to study the association between testing positive by the different methods and the severity of the cervical lesion.

MATERIALS AND METHODS

Study population. Women were recruited from four hospitals and nine gynecologists in private practice in Health Region East, Norway. Enrollment took place from 1 January 2005 to 31 December 2006. Included in the study were (i) 764 women 30 years or older attending routinely administered clinical services and with normal Pap smear cytology, normal cytological results from the preceding 2 years, and no previous history of treatment for cervical neoplasia and (ii) 655 women (no age criterion imposed) with histologically confirmed cervical intraepithelial neoplasia grade 2 or 3 (CIN2+), adenocarcinoma in situ (ACIS), or invasive carcinoma. A total of 623 of these patients were treated with conization. The median age among women with normal cytology was 51 years (range, 31 to 82 years), and it was 37 years (range, 17 to 76 years) for women with CIN2+.

The Pap smears were evaluated without knowledge of the HPV results by different, experienced cytotechnicians at the Department of Pathology, Akershus University Hospital. The smears were classified according to the criteria of the Bethesda Classification 2001 (26).

The histological analyses were performed on colposcopically directed biopsies and/or cone specimens. All specimens were reevaluated blindly by one experienced pathologist (A. K. Lie) and diagnosed according to the WHO classification (1). The specimen with the most severe lesion was chosen for analysis. Histology revealed CIN2 in 21.0% (135/643), CIN3 in 73.7% (474/643), ACIS in 1.6% (10/643), ACIS together with CIN2/3 in 1.7% (11/643), and invasive carcinoma in 2.0% (13/643) of the cases.

Collection of specimens for HPV testing. Cervical specimens were collected with a Cytobrush Plus (Medscan Medical AB, Sweden). For the normal cytological group a conventional Pap smear was taken first and the brush was transferred to a PreServ Cyt vial (Cytoc Corporation) for HPV testing. For the CIN2+ group, samples were transferred directly to the PreServ Cyt medium at the time of conization or at the time of biopsy within 2 months before conization. Cells were stored in PreServ Cyt medium for up to 21 days at room temperature or at 4°C before HPV testing.

Total nucleic acid extraction. To allow one extraction for both mRNA and DNA, the manual DNA extraction protocol (AmpliLute; Roche/Qiagen) supplied with the Amplicor HPV test was replaced by the semiautomatic NucliSense miniMag (bioMerieux) or automatic easyMag (bioMerieux) total nucleic acid extraction protocol recommended by the PreTect HPV-Proofer test manufacturer. Briefly, 5 ml of each cell sample in PreServ Cyt medium was pelleted by centrifugation. In cases with visible blood, only 3 ml of the cell sample was used, and in cases with few visible cells 10 ml of the cell sample was used. For the miniMag procedure, 1 ml of lysis solution and 100 µl of elution buffer were used, and for the easyMag procedure 2 ml of lysis solution and 55 µl elution buffer were used. Isolated nucleic acid was kept cold and analyzed within 4 hours following extraction or stored at -80°C until analysis.

Validation of the nucleic acid extraction procedure. To compare the performance of easyMag extraction with AmpliLute extraction, 66 samples with high-grade lesions were extracted in parallel by both methods. The DNA concentrations in the extracts were determined using an in-house real-time beta-globin PCR for absolute quantification, using a dilution of human DNA with known concentrations as a standard (data not shown). Undiluted and diluted extracts were compared, as undiluted AmpliLute extracts were replaced by 1:10-diluted easyMag extracts in the modified Amplicor test.

HPV DNA testing. The Amplicor HPV test (Roche Diagnostics, Switzerland) detects the following HPV DNA genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The Amplicor test does not include genotyping, and a positive result of the test is interpreted as the presence of one or more of the above genotypes. As the AmpliLute manual extraction protocol was replaced by the automatic extraction protocol described above using a larger input of sample (5 ml versus 250 µl), 5 µl total nucleic acid was diluted with 45 µl PCR-grade water instead of using 50 µl undiluted AmpliLute-extracted DNA in each PCR mixture. All other steps in the analyses were performed according to the manufacturer's recommendations.

HPV DNA genotyping. HPV DNA genotyping was performed with the Linear Array HPV assay (Roche Diagnostics, Switzerland) in women with normal cytology and positive HPV test and in all women with histologically confirmed

TABLE 1. HPV genotypes detected with the Linear Array

Classification ^a	HPV genotypes detected
High risk	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Probably high risk.....	26, 53, 66, 68, 73, 82, IS39
Low risk	6, 11, 40, 42, 54, 61, 70, 72, 81, CP 6108
Not yet classified.....	55, 62, 64, 67, 69, 71, 83, 84

^a The classifications are based on IARC recommendations (21).

CIN2+. This assay detects 37 different genotypes, including HR, probably HR, low risk, and HPV types not yet classified (Table 1) (21). This was done retrospectively using the same extracts used for the Amplicor HPV test and PreTect HPV-Proofer test. As for the Amplicor test the input for the PCR was 5 µl extract added to 45 µl PCR-grade water. All other steps including analysis were as recommended by the manufacturer.

HPV mRNA testing. The PreTect HPV-Proofer (Norchip, Norway) detects HPV 16, 18, 31, 33, and 45 E6/E7 full-length mRNA transcripts. Briefly, 5 µl undiluted isolated nucleic acid was analyzed according to the manufacturer's instructions using the Lambda FL 600 fluorescence reader (Bio-Tek Instruments, Inc.) and the PreTect analysis software (Norchip; Norway). Samples that were HPV mRNA negative, internal control negative, or internal control indeterminate (signal between 1.4 and 1.7), as well as samples that were HPV indeterminate, were reextracted and reanalyzed using up to 10 ml PreServ Cyt sample as recommended by the manufacturer (PreTect HPV-Proofer user guide version 1105 720001 and earlier versions; Norchip). Samples that tested indeterminate twice were considered negative if the internal control was positive.

Statistical analyses. We compared the percentages of test positives according to the assay used. Statistical analyses were performed using two-by-two contingency tables with two-sided *P* values calculated with a Pearson chi-square test. Fisher's exact test and McNemar's test were used for comparisons of paired proportions. *P* values of <0.05 were considered statistically significant. We also calculated percent agreement between the different HPV assays, and values for Cohen's κ statistic were used as indicators of concordance; κ values of <0.20 indicated poor agreement, 0.21 to 0.40 fair agreement, 0.41 to 0.60 moderate agreement, 0.61 to 0.80 substantial agreement, and >0.80 indicated nearly perfect agreement. Kappa values were calculated for agreement between assays on detection of HPV 16, 18, 31, 33, and 45. Data analyses were performed by using SPSS software (version 16.0). The relative sensitivity and specificity were calculated based on valid test results on 736 women with histologically confirmed CIN2+ and 643 women with normal cytology.

Ethics. The Regional Committee for Ethics in Medical Research, East Region, Norway (676-04239), Norwegian Social and Health Directorate (05/163), and Norwegian Data Inspectorate (07/00975-2/SVE) approved the study. Written informed consent was obtained from all study participants.

RESULTS

Validation of nucleic acid extraction procedure. Based on the beta-globin real-time PCR performed on 66 samples with high-grade lesions, the mean DNA concentration in undiluted AmpliLute extracts was found to be 25 ± 30 ng/µl (mean \pm standard deviation). The mean DNA concentration in 1:10-diluted easyMag extracts was found to be 10 ± 9 ng/µl. Subsequent analysis of the same samples with the Amplicor test revealed 100% agreement regarding beta-globin gene detection (66 out of 66 samples were beta-globin positive) and 86.4% (95% confidence interval, 76.1 to 92.7) agreement regarding HPV detection (57 out of 66 samples revealing the same result). A total of 38 HPV-positive samples were detected combining both extractions. Five of these were only positive when extracted using the AmpliLute procedure, and four were only positive when extracted using the easyMag procedure. In conclusion, the agreement regarding detection of the internal control was 100% and for HPV DNA it was substantial (Cohen's κ , 0.74). Mean DNA concentrations used as input to the Amplicor test were similar, but DNA concen-

TABLE 2. Outcomes of HPV testing with PreTect HPV-Proofer, AmpliCor, and Linear Array in the normal cytology group

Comparison test and result	Result with PreTect HPV-Proofer		
	% Negative (n)	% Positive (n)	Total % (n)
AmpliCor			
Negative	89.4 (658)	2.3 (17)	91.7 (675)
Positive	7.3 (54)	1.0 (7)	8.3 (61)
Total	96.7 (712)	3.3 (24)	100.0 (736)
Linear Array			
Not tested	89.4 (658)	0 (0)	89.4 (658)
Negative	2.0 (15)	2.2 (16)	4.2 (31)
Positive	5.3 (39)	1.1 (8)	6.4 (47)
Total	96.7 (712)	3.3 (24)	100.0 (736)

trations in the easyMag extracts appeared to be more uniform than the AmpliLute extracts. Based on these results we concluded that the total nucleic acid automatic extraction method could replace the more laborious manual AmpliLute extraction method.

HPV detection in women with normal cytology. In women with normal cytology, 3.7% (28/764) of the cases were excluded because both the internal control for DNA and/or RNA quality and HPV were negative, leaving 736 with valid test results. A total of 10.6% (78/736) tested positive for HR HPV (DNA and/or mRNA). The AmpliCor test was positive in 8.3% (61/736) and the PreTect HPV-Proofer test was positive in 3.3% (24/736) (Table 2). Concordant results between AmpliCor and PreTect HPV-Proofer were found in 90.3% (665/736). The HPV-positive cases ($n = 78$) were genotyped using Linear Array, and 47 cases tested positive (60%). HPV was more frequently detected using the AmpliCor test or Linear Array than with PreTect HPV-Proofer (Pearson chi-square test, $P < 0.001$) (Table 2). By Linear Array multiple infections with two or more genotypes were detected in 1.4% (10/736) of all women and in 12.8% (10/78) of women with positive samples, but by PreTect HPV-Proofer no women were determined to have multiple infections.

HPV detection in women with high-grade cervical neoplasia. In women with CIN2+, 1.8% (12/655) of the cases were excluded because the internal control for DNA and/or RNA quality and HPV were negative, leaving 643 with valid test results. A total of 97.0% (624/643) tested positive for HR HPV (HPV DNA and/or HPV mRNA). AmpliCor was positive in 96.4% (620/643), Linear Array was positive in 98.4% (633/643), and PreTect HPV-Proofer was positive in 64.1% (412/643) (Table 3). In women with CIN2+ HPV was detected in 99.4% (639/643) when all HPV types detected by linear array were considered. Agreement between AmpliCor and PreTect HPV-Proofer was found in 66.7% (429/643) (Table 3) and between Linear Array and PreTect HPV-Proofer in 64.1% of the samples (412/643) (Table 3). Concordant results for the three HPV assays were present in 63.8% (410/643).

In total, Linear Array detected the presence of 34 different HPV genotypes in women with CIN2+, and the distribution of the HR HPV genotypes is shown in Fig. 1. HPV 16 was the most common HPV type, detected in 51.3% (330/643) of the women, followed by HPV 31, 33, 52, 18, 51, 58, and 45. HPV 16 and/or 18 was detected in 58.0% (373/643). Probable HR

TABLE 3. Outcome of HPV testing by PreTect HPV-Proofer, AmpliCor, and Linear Array in the CIN2+ group

Comparison test and result	Result with PreTect HPV-Proofer		
	% Negative (n)	% Positive (n)	Total % (n)
AmpliCor			
Negative	3.1 (20)	0.5 (3)	3.6 (23)
Positive	32.8 (211)	63.6 (409)	96.4 (620)
Total	35.9 (231)	64.1 (412)	100.0 (643)
Linear Array			
Negative	0.8 (5)	0.8 (5)	1.6 (10)
Positive	35.1 (226)	63.3 (407)	98.4 (633)
Total	35.9 (231)	64.1 (412)	100.0 (643)

HPV genotypes were detected in 13.5% (87/643), low-risk HPV genotypes in 18.0% (116/643), and genotypes that have not yet been classified in 13.2% (85/643) of the women. Linear Array detected multiple infections in 52.6% of the cases (338/643). HPV 6/11 was detected in 2.0% (13/643), together with other HPV types in most of the cases (1.7% [11/643]).

The distribution of HPV genotypes detected by the PreTect HPV-Proofer is shown in Table 4. HPV 16 was the most prevalent genotype, found in 42.3% (272/643) of the women, followed by HPV 33 (13.2%), HPV 45 (6.1%), HPV 18 (5.3%), and HPV 31 (2.3%). HPV 16 and/or 18 were detected in 47.1% (303/643) of the women. Multiple infections with two or more genotypes were detected in 5% (33/643) of the specimens by PreTect HPV-Proofer. Of the 33 specimens with multiple infections detected by HPV-Proofer, Linear Array results showed at least one of the same genotypes.

For women with CIN2+ the HR HPV genotype profile detected by Linear Array compared to PreTect HPV-Proofer is shown in Table 4. Agreement between the two tests was poor to moderate for HPV 31 (κ value, 0.18) and substantial for HPV 18, 16, 33, and 45 (κ values, 0.68 to 0.81). In women who were AmpliCor positive and PreTect HPV-Proofer negative ($n = 211$), genotyping with Linear Array revealed an HR HPV genotype not included in the mRNA test in 41.2% (87/211). There was 96.7% concordance between AmpliCor and Linear Array test results.

HPV test results according to severity of cervical disease. AmpliCor was positive in 95.6% cases of CIN2 (129/135), in 97.0% cases of CIN3/ACIS (480/495), and in 84.6% of cases of invasive carcinoma cases (11/13) (Table 5). Two invasive carcinomas were AmpliCor negative. mRNA testing revealed oncogene expression from HPV 45 in one of these cases, and results were negative in the other. PreTect HPV-Proofer was positive in 50.4% of the women with CIN2 (68/135), in 67.5% with CIN3/ACIS (334/495), and in 76.9% of the women with invasive carcinomas (10/13). Three invasive carcinomas tested negative with PreTect HPV-Proofer, and genotyping with Linear Array revealed HPV 11, 33, 81, and 56. The HPV11-positive invasive carcinoma was classified as a condylomatous type of squamous cell carcinoma, a newly described type in the WHO 2004 classification. The mRNA test was significantly more often positive in the CIN3+ lesions compared to CIN2 lesions (Pearson chi-square test, $P < 0.0001$). Detection of HPV DNA, however, was not associated with histology grade. The relative cross-sectional sensitivity and specificity were calcu-

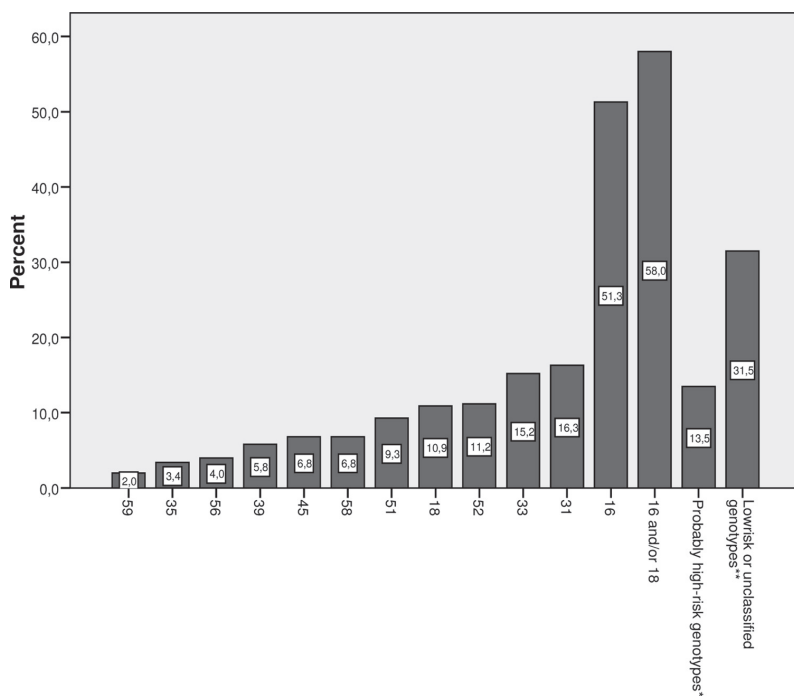


FIG. 1. Distribution of genotypes in positive tests among 643 women with CIN2+ detected by linear array. *, probably high-risk HPV genotype (25, IS 39, 53,66, 68, 73, and 82); **, HPV low-risk genotype (6, 11, 40, 42, 54, 61, 70, 72, 81, and CP 6108) or unclassified genotype (55, 62, 64, 67, 69, 71, 83, and 84).

lated (Table 6) and revealed the highest sensitivity for HPV DNA testing and the highest specificity for HPV mRNA testing.

DISCUSSION

The aims of our study were to compare relatively new commercially available assays for detection of HPV in Norwegian women with and without high-grade cervical neoplasia as the baseline for longitudinal analyses. HR HPV was detected in 10.6% of women above the age of 30 with normal cytology and 8.3% tested positive with Amplicor, which is in agreement with other European studies using HC II or consensus PCR (3, 9, 10, 17). Among the specimens from women with normal cytology,

a significantly higher number were positive by Amplicor than by PreTect HPV-Proofer ($P < 0.001$). The reason for this may be that more genotypes are included in the DNA test (13 versus 5 genotypes) and/or that the chemistry behind the mRNA test renders it more specific for the detection of clinically significant infection. Those with HPV E6/E7 mRNA-negative detection in HPV DNA-positive samples can be interpreted as HPV carriers without active viral transcription. However, it may be that transcriptional activity occurs but at levels insufficient for PreTect HPV-Proofer detection. Surprisingly, with PreTect HPV-Proofer samples tested positive in 2.3% of the Amplicor-negative cases. This could be have been caused by a false-positive mRNA test (oncogene expression

TABLE 4. Distribution of HPV genotypes detected by PreTect HPV-Proofer and Linear Array in the CIN2+ group ($n = 643$)

HPV Genotype(s)	PreTect HPV-Proofer		Linear Array		% with positive results in both tests ^a	P value	κ value
	% Positive (n)	% Negative (n)	% Positive (n)	% Negative (n)			
16	42.3 (272)	57.7 (371)	51.3 (330)	48.7 (313)	79.7 (267)	<0.001	0.79
18	5.3 (34)	94.7 (609)	10.9 (70)	89.1 (573)	48.6 (34)	<0.001	0.63
31	2.3 (15)	97.7 (628)	16.3 (105)	83.7 (538)	12.1 (13)	<0.001	0.18
33	13.2 (85)	86.8 (558)	15.2 (98)	84.8 (545)	71.0 (76)	<0.001	0.80
45	6.1 (39)	93.9 (604)	6.8 (44)	93.2 (599)	69.4 (34)	<0.001	0.81
16/18	47.1 (303)	52.9 (340)	58.0 (373)	42.0 (270)	78.8 (298)	<0.001	0.75

^a Percent (number) of HPV-positive women who tested positive on both PreTect HPV-Proofer and Linear Array.

TABLE 5. Relationship between morphology and HPV testing

Morphology (n)	PreTect HPV-Proofer		Amplicor		P value, Proofer vs Amplicor	Linear Array		P value, Proofer vs Linear Array
	% Positive (n)	% Negative (n)	% Positive (n)	% Negative (n)		% Positive (n)	% Negative (n)	
Normal ^a (736)	3.3 (24)	96.7 (712)	8.3 (61)	91.7 (675)	<0.0001	60.3 (47)	39.7 (31)	0.001
CIN2 (135)	50.4 (68)	49.6 (67)	95.6 (129)	4.4 (6)	0.091	98.5 (133)	1.5 (2)	0.992
CIN3/ACIS (495)	67.5 (334)	32.5 (161)	97 (480)	3 (15)	<0.0001	98.8 (489)	1.2 (6)	0.072
Carcinoma (13)	76.9 (10)	23.1 (3)	84.6 (11)	15.4 (2)	0.326	84.6 (11)	15.4 (2)	0.4
CIN2+ (643)	64.1 (412)	35.9 (231)	96.4 (620)	3.6 (23)	<0.0001	98.4 (633)	1.6 (10)	0.35

^a For women with normal cytology, genotyping with Linear Array was performed only in cases with a positive HPV test (positive by Amplicor and/or PreTect HPV-Proofer).

not associated with cervical neoplasia), lack of specificity of PreTect HPV-Proofer, or false-negative DNA tests due to a breakpoint in the L1 region during HPV integration. We intend to follow these women with repeat cytology and HPV testing after 12 months; if HPV infection is persistent and/or cytology is positive by colposcopy, biopsy will be performed.

In our study PreTect HPV-Proofer was positive in 3.3% of the women with normal cytology, which is higher than reported from another, larger cross-sectional Norwegian study where PreTect HPV-Proofer tested positive in 1.7% (68/3970) of women above the age of 30 with normal cytology (17). Castle et al. tested women in a routine screening program with the Aptima HPV assay (which can detect E6/E7 mRNA from 14 carcinogenic HPV types) and found that 8% (10/125) of women with normal cytology tested positive (4). It could be argued therefore (notwithstanding the analytical sensitivities of the two mRNA assays) that the smaller type range of the PreTect HPV-Proofer has contributed to the lower detection rate.

Due to the lower detection of HPV mRNA in women with normal cytology, it may constitute a better first-line screen compared to HPV DNA testing, provided the sensitivity for significant disease is not compromised and clinically significant infections are not missed. Longitudinal follow-up (including that associated with mRNA-negative/DNA-positive women) from this study should elucidate the prospective performance of the tests.

HR HPV was detected in 97.0% of women with histologically confirmed CIN2+. A significantly higher number of women with CIN2+ were HPV DNA positive rather than HPV mRNA positive. Concordant results for the three HPV tests were found in 63.8%. There are several explanations for the different outcomes of the tests. The different assays are not uniform with regard to the analytical sensitivity, use of template, and the spectrum of detectable genotypes. PreTect HPV-Proofer detects transcripts and oncogene activity from

out of the 13 HR HPV types included in the Amplicor test. The Linear Array, which detects 37 different genotypes, has lower analytical sensitivity than the Amplicor test. The concordance between Amplicor and the Linear Array in the CIN2+ group was 96.7%, which is almost the same as in the study of Steven et al. (97.8%) (27).

We detected an HR HPV genotype not included in the mRNA test in 41.2% of the women with CIN2+ (i.e., positive Amplicor and negative PreTect HPV-Proofer). HPV DNA testing will not discriminate between active and latent or transient infections, while mRNA testing may be more likely to. It is estimated that only 12 to 31% of CIN3 lesions will progress to invasive carcinomas if they are left untreated (15, 16, 22), so it could be that the HPV mRNA-negative/DNA-positive CIN2+ cases were those infections associated with regressing lesions. However, this will be impossible to confirm, since Norwegian women with CIN2+ lesions are routinely treated with conization.

In our study 96.4% of women with CIN2+ tested positive with Amplicor, which is in accordance with the large POBASCAM and ARTISTIC trials, in which HPV DNA testing was performed with PCR or hybrid capture 2 (2, 9). The Amplicor test was negative in 23 women with CIN2+, and among these, 3 patients tested positive with PreTect HPV-Proofer. As discussed earlier, the reason for this may be false-negative DNA tests associated with viral integration.

There is a lack of data on mRNA testing in clinical contexts. Cross-sectional Norwegian studies have shown that mRNA transcripts from HPV types 16, 18, 31, 33, or 45 can be detected in 77% of women with histologically verified CIN2+ and in 89% of invasive squamous cell carcinomas, compared to 94.5% and 92%, respectively, by HPV DNA testing (11, 14, 17). These studies support our results that HPV detection in preinvasive lesions will differ depending on whether you use mRNA methods with fewer genotypes or HPV DNA detection methods with a broad spectrum of genotypes. The Aptima HPV assay, a Gen-Probe test detecting E6/E7 mRNA for 14 carcinogenic HPV types, showed a prevalence of 92.4% in women with CIN2+ (4). Adding extra (probably) oncogenic HPV types in mRNA HPV tests may negatively influence the specificity of the test for high-grade lesions (13, 25). The performance of the PreTect HPV-Proofer assay is clearly influenced by the choice and number of genotypes included in the assay. It remains to be documented whether mRNA assays need to be intrinsically quantitative to be effective. In determining the optimal genotype mix for an mRNA test, indeed any HPV test is a contentious area. There will have to be a

TABLE 6. Sensitivities and specificities for the three tests

Test	% Sensitivity ^a	% Specificity ^b
Amplicor	96.4	91.7
Proofer	64.1	96.7
Linear Array	98.4	NA ^c

^a Based on 643 women with histologically confirmed CIN2+.

^b Based on 736 women with normal cytology.

^c NA, not available. Specificity could not be measured using the Linear Array as only some (n = 78 HPV positive) of the 736 women with normal cytology were tested via this technique.

compromise between including more rare HPV types to maximize sensitivity and detecting large numbers of what could be clinically irrelevant infections. Defining the appropriate analytical sensitivity for clinical utility is equally challenging.

According to the known prevalence of HPV types in invasive cervical carcinomas, more than 80% of the potential cases can be detected by the PreTect HPV-Proofer assay. The IARC pooled analysis of 3,085 invasive cervical carcinomas revealed that the five most common HPV genotypes were, in descending order of frequency, HPV 16, 18, 45, 31, and 33 (20). These genotypes were detected in 82.9% of the cases, which corresponds well with a Norwegian study of 204 women diagnosed with squamous cell carcinomas (11). In that previous study the five most common HPV genotypes were 16, 18, 31, 33, and 45.

DNA and mRNA testing may be employed together for screening to take advantage of the higher sensitivity and specificity, respectively, of the tests, and patients are then referred for a biopsy if both tests are positive. If only HPV DNA is positive, the patient may be retested for HPV DNA at a later date and then referred for colposcopy if persistently positive. mRNA testing alone for screening appears to be too insensitive, at least for the currently evaluated PreTect HPV-Proofer assay. Moreover, as HPV vaccination becomes more common and the prevalence of HPV vaccine types is reduced, there will be a requirement to reconsider/recalibrate HPV assays in line with the shifting dynamics of HPV type-specific prevalence and associated disease.

Accurate geographical data on HR HPV genotype distributions have implications not only for follow-up protocols in cervical cancer screening programs but also for assessing the expected impact of an HPV 16/18 vaccine program on CIN2+. In our study, 58.8% of the women with CIN2+ tested positive for HPV 16 and/or 18 as detected by either Linear Array or PreTect HPV-Proofer. This result corresponds with a recent meta-analysis which showed that HPV 16 and/or 18 was detected in 52% of women with high-grade precursor lesions (25a).

So far only one study has investigated the predictive values of HPV DNA versus mRNA testing in triage (28). This study revealed that PreTect HPV-Proofer has the highest specificity and the lowest sensitivity, which seems to be in accordance with our findings. At this stage we cannot calculate positive or negative predictive values from our study, due to the absence of histology results from the normal cytology group.

In conclusion, the detection of HPV varied according to the assay used, and the concordance between the tests was low. Our results indicate that mRNA testing may be a biomarker for progression of cervical neoplasia, but further data are needed to confirm this. mRNA testing for the five HR HPV types described may be a more specific approach and appropriate for risk evaluation. It is not clear whether the increased specificity of mRNA testing via the PreTect HPV-Proofer is driven by truly detecting transcripts or by detecting a more limited range of HPV types. Consensus on the number and types of genotypes that should be included in a diagnostic test to achieve the best sensitivity and specificity has not been reached and will likely evolve as interventions such as HPV vaccination become more common.

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We have no conflicts of interest for this study.

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LIST OF ERRATA

(Updated December 15, 2010)

1. Page 6. The headline “Implications of findings and future research” was missing in “Table of contents” and is now included.
2. Page 9. The word “now” was missing in line three in the second paragraph and is now included.
3. Page 17. In line six in the second paragraph, “high-risk cervical neoplasia” is now replaced by “high-grade cervical neoplasia”
4. Page 19. In line eight, the word “dived” is corrected to “divide”.
I thank Rudi Henriksen for this correction.
5. Page 28. In line one in the fourth paragraph, the word “Gradacil” is corrected to “Gardacil”.
I thank Rudi Henriksen for this correction.
6. Page 40. In line two in the third paragraph, the abbreviation “LCL” is now corrected to “CLC”.

