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NEW DIAGNOSTIC AND PROGNOSTIC METHODS TO IMPROVE THE EFFECTIVENESS OF CERVICAL CANCER SCREENING

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ABSTRACT

This thesis has made use of real-life clinical practice to provide guidance for secondary prevention of cervical cancer. We combined epidemiological, virological and biostatistical investigations using HPV DNA and HPV E6/E7 mRNA tests in women who were identified with abnormal findings through the organised cervical cancer-screening programme. The majority of cytological abnormalities detected through the Swedish cervical cancer-screening programme are minor, i.e. atypical squamous cells of undetermined significance (ASC-US, 3.6 %) or low-grade intraepithelial lesions (LSIL, 2.1 %). Since only a minority of women with minor abnormal cytology harbour underlying cervical disease, or rarely invasive cervical carcinoma (ICC), management of ASC-US/LSIL remains a major challenge to system efficiency. The realisation that infection with high-risk human papillomavirus (HR-HPV) causes cervical cancer and its precursor lesions has led to development of new molecular tests for detection of HR-HPV.

Study 1 compared the effectiveness of HR-HPV DNA testing, using Hybrid Capture 2 (HC2), with repeat cytology using Pap smear for detection of underlying high-grade cervical intraepithelial neoplasia (CIN2-3) in an evaluation of 177 women with minor cytological abnormalities, identified through the population-based cervical cancer screening programme. The HPV DNA test was positive in 66% of the women. Sensitivity for detection of CIN2-3 was 82% (95% CI; 80-97) using HR-HPV DNA testing and 61% (95% CI; 45-74) using Pap smear. These results indicated that HR-HPV DNA testing found more underlying disease than follow-up with repeat Pap smear and suggest that HR-HPV DNA-positive women should be referred for further examination including colposcopy and directed biopsies.

This study was published in 2005 and since then a large number of other studies have addressed this issue. There is now consistent evidence indicating that HPV triage with HC2 is more accurate than repeat cytology to triage women with equivocal (ASC-US) cytology. HR-HPV DNA testing has also been considered useful as a follow-up test in women treated for high-grade CIN to predict success or failure of treatment. HPV testing identifies residual disease more rapidly, with higher sensitivity and with similar specificity compared with follow-up cytology or histological assessment of section margins. Since expression of the HR-HPV E6/E7 oncogenes, resulting from deregulation of p53 and retinoblastoma protein, is necessary for malignant transformation and persistence in cervical tissue, a diagnostic test that identifies E6/E7 mRNA in 14 HR-HPV types was developed (APTIMA HPV Assay).

In study 2 we compared the performance of APTIMA with HR-HPV DNA testing and cytology in 143 women treated for CIN with conisation. These women were followed over a median time of 3.2 years and high-grade residual/recurrent disease was identified in 7 (4.9%) of them. Presence of HR-HPV DNA at the first follow-up visit predicted all (100%; 95% CI; 64.6-100) high-grade disease during follow-up, while the APTIMA failed to identify 3 women with high-grade disease (sensitivity 57.1% 95% CI 25.0-84.2). We concluded that APTIMA sensitivity was too low to be used for follow-up surveillance after conisation.

HPV DNA triage of ASC-US cytology is an established technique, but less effective in women with LSIL due to high prevalence of HPV. HPV DNA testing has high sensitivity, but is also positive in many women without disease (low specificity). Thus,

a molecular marker, such as HPV mRNA, which allows more specific identification of a transforming infection, holds promise.

In study 3 we compared the triage effectiveness of APTIMA testing with for HPV16 DNA testing, HPV16/18 DNA testing and repeat cytology, in a cohort of 205 HR HPV-positive women with minor cytological abnormalities. Nine of 25 (36%) women with ASC-US and 64 of 180 (36%) with LSIL developed CIN2+ over a 4-year follow-up period. APTIMA had the highest sensitivity to predict CIN2+ and CIN3+ among patients with ASC-US (77.8% and 100%) and LSIL (78.1 and 75.8%), although specificity was insufficient (<50%). HPV16 DNA testing, HPV16/18 DNA testing and repeat cytology were all less sensitive, but more specific than APTIMA. Our results support the use of APTIMA in triage of women with ASC-US, but not with LSIL. All evaluated tests showed accuracy estimates that indicated poor LSIL triage capability and risk of disease remained even when triage tests were negative. Additional biomarkers need to be evaluated to stratify women with LSIL.

Finally, in study 4 we addressed long-term risk of developing cervical pre-cancer among 314 women with ASC-US or LSIL in relation to age, HPV status and HPV DNA genotype. Median follow-up was 3.8 years. Data for these women were linked to the Swedish National Quality Register for Cervical Cancer Prevention (NKCx) to identify cases of histologically confirmed CIN2+. We showed that HPV status was the most important factor in determining risk for developing pre-cancer. Risk was low among HPV-negative women during the first 4.5 years, suggesting that they could safely return to the regular screening programme. The highest risk of pre-cancer was observed among women positive for HPV16/18, suggesting a need for more aggressive follow-up.

The purpose of our findings is to facilitate risk stratification in women with minor cytological abnormalities and in women treated for CIN, as well as to provide guidance for clinical management. In Sweden the second major peak in incidence of cervical cancer is among women aged 60-85. Older women are clearly a vulnerable risk group for cervical cancer. One approach to reduce CC incidence in Sweden is to extend the screening programme up to at least the WHO-recommended age of 65 years and to complement or replace cytology screening with HPV testing in older women and in those who never participate in screening programmes. Women’s health would greatly benefit from more clinically effective and cost-effective screening strategies to identify more women at risk of developing cervical cancer, thereby reducing costs for society.

Key words: HR HPV DNA, ASC-US, LSIL, HPV mRNA, risk stratification

LIST OF PUBLICATIONS

I. Sonia Andersson, Lena Dillner, Kristina Elfgren, Miriam Mints, MARIA PERSSON, Eva Rylander
A comparison of the human papillomavirus test and Papanicolaou smear as a second screening method for women with minor cytological abnormalities
Acta Obstetrica et Gynecologica Scandinavica 2005; 84: 996-1000

II. MARIA PERSSON, Sophia Brismar Wendel, Linda Ljungblad, Bo Johansson , Elisabete Weiderpass and Sonia Andersson
High-risk human papillomavirus E6/E7 mRNA and L1 DNA as markers of residual/recurrent cervical intraepithelial neoplasia
Oncology Reports 28: 346-352, 2012

III. MARIA PERSSON, K. Miriam Elfström, Sophia Brismar Wendel, Elisabete Weiderpass and Sonia Andersson
Triage of HR-HPV positive women with minor cytological abnormalities: A comparison of mRNA testing, HPV DNA testing and repeat cytology using a 4-year follow-up of a population-based study
PLoS ONE 9(2):e90023.doi:10.1371/journal.pone.0090023, published February 26, 2014

IV. MARIA PERSSON, K. Miriam Elfström, Sven-Eric Olsson, Joakim Dillner and Sonia Andersson
A seven-year follow-up of human papillomavirus DNA positive and negative minor cytological abnormalities
In manuscript, to be submitted in International Journal of Cancer

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LIST OF ABBREVIATIONS

AIS	Adenocarcinoma in situ
ASC-H	Atypical squamous cells - HSIL cannot be ruled out
ASC-US	Atypical squamous cells – uncertain significance
CC	Conventional cytology
CI	Confidence interval
CIN	Cervical Intraepithelial Neoplasia
CIP	Cumulative incidence proportion
CIS	Cancer in situ
FIGO	International Federation of Gynaecology and Obstetrics
HPV	Human papillomavirus
HR HPV	High-risk HPV
HSIL	High-grade squamous intraepithelial lesion
IARC	International Agency for Research on Cancer
ICC	Invasive cervical cancer
LBC	Liquid based cytology
LCR	Long control region
LEEP	Loop electrosurgical excision procedure
LR HPV	Low-risk HPV
LSIL	Low-grade squamous intraepithelial lesion
mRNA	Messenger ribonucleic acid
NPV	Negative predictive value
PCR	Polymerase chain reaction
pHR HPV	Probably high-risk HPV
PPV	Positive predictive value
SCC	Squamous cervical cancer
SCJ	Squamocolumnar junction
STI	Sexually transmitted infection
TBS	The Bethesda system
TZ	Transformation zone
VLP	Virus-like particle

1 INTRODUCTION

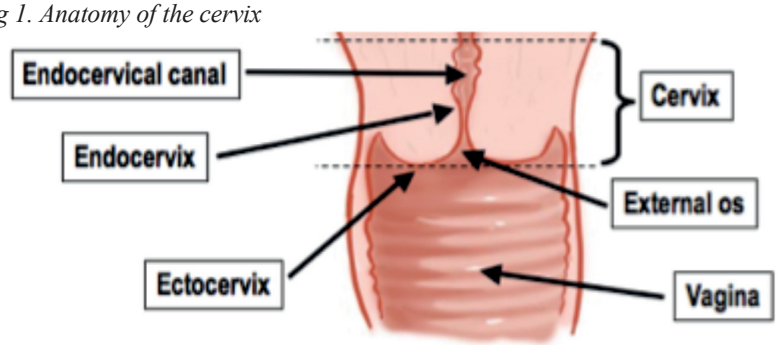
Since HPV infection is the requisite common denominator underlying cervical cancer, new approaches aimed at prevention have evolved in recent years through improved screening methods and HPV vaccination. In fact, it has become the leading preventable cancer and although research continues to make significant strides, several challenges remain before fully effective cancer control can be achieved. Pap smear screening has successfully reduced morbidity and mortality from cervical cancer over the past 50 years. Unfortunately, the incidence of squamous cervical cancer (SCC) is no longer decreasing, while the incidence of adenocarcinoma (AC) is now increasing. Pap smear testing has been found to be inefficient due to high rates of false negative findings and the need for repeat testing. The introduction of liquid based cytology (LBC) improved laboratory efficiency in processing cytology slides, but the problems of low sensitivity, subjective interpretations and sampling error still remain. Adjunctive HPV DNA testing has opened up new opportunities. Studies have consistently demonstrated advantages such as greater sensitivity—albeit lower specificity—than cytology, higher reproducibility, less need for staff training, automation with high-volume testing and the opportunity to use self-collected cervical samples. However, many questions still remain before successful implementation can become feasible. HPV is the most common sexually transmitted infection (STI) worldwide and is so prevalent that it is almost unavoidable. However, the majority of infections are transient and cervical cancer is the rare end stage of persistent infection with certain high-risk types. Despite our tremendous knowledge about HPV and interactions with host cells, tissues and immune systems, we still cannot predict whether a specific infection will regress or persist. A range of promising new biomarkers has emerged from the research pipeline, one of which is mRNA from the HPV E6 and E7 oncogenes, which provides high specificity to distinguish between benign productive infection and those where neoplastic progression has been initiated or already resulted in cancer.
This thesis aims to evaluate testing for the presence of HR HPV DNA, specific viral genotypes and HR HPV mRNA in women with minor cytological abnormalities and women treated for dysplasia, for the ultimate goal of improving the effectiveness of secondary cervical cancer prevention and thereby perhaps contribute a small piece of knowledge to this vast puzzle.

2 BACKGROUND

2.1 CERVIX

The cervix is the inferior part of the uterus and is divided into two portions. The lower portion (portio) extends into the vagina and can be visualized through speculum examination. The upper or supravaginal portion extends from the vaginal attachment to the lower uterine segment. The cervix is about 3 cm long and 2 cm in diameter. The cervical canal is approximately 3 cm long and extends from the external os to the internal cervical os. The ectocervix is covered by stratified squamous epithelium, while a single layer of columnar cells lines the endocervical canal. The area where these two cell types meet is known as the squamocolumnar junction (SCJ). In this area, a gradual transformation process takes place where columnar cells transform into squamous cells in a process known as metaplasia. The new squamous epithelium may obstruct mucus secretion, thereby causing formation of Nabothian cysts. Underneath the epithelium is a layer of connective tissue and beneath that is smooth muscle. Factors that induce metaplasia are still poorly understood, but may include environmental conditions, mechanical irritation, chronic inflammation, pH changes or changes in sex hormone balance.
The original squamocolumnar junction (SCJ) between squamous and columnar epithelium, called the congenital junction, remains unchanged until puberty. Post puberty, in addition to the congenital junction, the adult or functional junction forms and is termed the new SCJ. The TZ occupies the area between the original and the new SCJ and becomes larger with age as it expands towards the cervical opening. There are three types of TZ. In the type 1 TZ, the SCJ is fully visible on the ectocervix, in type 2 TZ, the SCJ is only fully visible if the cervical os is open and the endocervix can be inspected, while in type 3 TZ, the SCJ is not visible. The TZ plays a crucial role in the pathogenesis of cervical cancer (1) (2).

Fig 1. Anatomy of the cervix



2.2 HPV INFECTIONS AND RELATED DISEASES

The worldwide prevalence of HPV infection in women with normal cytology is around 11–12%, with the highest rates in sub-Saharan Africa (24%), Eastern Europe (21%) and Latin America (16%). There are large regional variations with particularly high prevalence in Eastern Africa and the Caribbean, where rates exceed 30%. Maximum

HPV prevalence is observed in women under age 25 and declines in older age groups (>45 years), though in many populations an unexplained secondary peak occurs in perimenopausal women. Globally, the five most prevalent types are HPV16 (3.2%), HPV18 (1.4%), HPV52 (0.9%), HPV31 (0.8%) and HPV58 (0.7%). The prevalence of all other HPV types is 0.6% or less. The overall prevalence of high-risk HPV (HR HPV) among women with normal cytology has been estimated at around 0.4%-2.6%. In a Swedish study of 44 146 adolescents, who were offered free Chlamydia trachomatis testing, samples were subjected to HPV genotyping (2008) in order to evaluate HPV prevalence before implementation of the HPV vaccination programme. The results showed that HPV positivity peaked at 54.4% among 21-year-old women and at 15.0% among 23-year-old men, although urine samples uniformly show lower HPV prevalence than genital swab samples. In general, HPV positivity was 37.8% in women and 11.2% in men. The most prevalent types among men and women were HPV16 (10.0%), and HPV51 (6.0%), HPV31, HPV18, HPV66 and HPV52, in descending order (3). HPV type distribution although, varies among different populations and among different sample assays (4).

Prevalence increases in direct proportion to the severity of lesions in women with cervical dysplasia by cytology, with 12.6% prevalence seen in normal cytology, and about 90% in women with cervical intraepithelial neoplasia grade 3 (CIN3) and invasive cervical cancer (ICC). Retrospective investigations have shown that nearly 100% of all cervical cancer patients are HPV positive. The proportion of HPV-positive women in whom HPV16 is detected also increases with lesion severity. The three most commonly found HPV types in women with invasive cancer are HPV16, 18 and 45, which were found in 20%, 8% and 5%, respectively, of HPV-positive women with normal cytology and in 63%, 16% and 5%, respectively, of women with cancer (5-7). A major novelty found in a recent review by Bzhalava et al. was the broad range of non-HR HPV types commonly detected in both low-grade and high-grade cervical abnormalities, and the extraordinarily high prevalence of multiple infections associated with these lesions. The proportional protective impact of HPV16/18 vaccination on cervical lesions can be predicted to rise from 17% in ASC-US, through 49% in HSIL and up to 70% in ICC, given that all HPV16/18 infections are causally related to the lesions in which they are found, even when other HPV types are present (Table 1).

HPV prevalence data from other body sites are less readily available. Detection of genital HPV infections in men depends on sampling techniques. HPV is most commonly detected in the shaft, glans, and scrotum; less often in the urethra. Prevalence of genital HPV in men generally correlates with prevalence of genital HPV infections in women within the same population, but does not vary by age as it does in women. HPV is also frequently detected in the perianal region and anal canal in both sexes. The highest prevalence of anal HPV is found among individuals positive for human immunodeficiency virus (HIV) and in men who have sex with men (MSM). Incidence of anal cancer among HIV-positive MSM in the US is similar to the rate of cervical cancer among women in sub-Saharan Africa(8).
Detection of oral HPV infection also varies substantially depending on sampling technique. In a meta-analysis the pooled prevalence of oral HPV infection was 4.5% among 4581 cancer-free individuals, with equal prevalence in men and women (9).

HPV prevalence in the anogenital tract and in the oral cavity is associated with anal and oral intercourse (8).

Table 1. Results from a meta-analysis showing women tested for HPV and HPV16, percent positive by cervical disease grade (5).

Grade of cervical disease	% HPV positive	% HPV 16+/ HPV+
Normal cytology	12	20
ASC-US	52	23
LSIL	76	25
HSIL	85	48
CIN1	73	28
CIN2	86	40
CIN3	93	58
ICC	89	63

Around 2 million (16%) new cancer cases that occurred in 2008 were attributable to infections(10). Infection with HR HPV is a major cause of infection-related cancer. Strong evidence for HPV causality has been cited by the International Agency for Research on Cancer (IARC) for cancers of the cervix, penis, vulva, vagina, anus and oropharynx (including base of tongue and tonsils). In 2008, 610 000 new cases of cancer attributable to HPV were diagnosed, representing 4.8% of the worldwide burden of cancer. Cervical cancer is the third most common female cancer, with an estimated 530,000 new cases in 2008. Approximately 86%, occurred in underdeveloped regions. The other five HPV-related types of cancer accounted for the remaining 80 000 cases. There is a strong association between cervical cancer incidence and level of development. Incidence and mortality rates tend to be at least four times higher in less developed countries, as indicated by the much lower 5-year survival rate (20%, compared with 65% in developed countries) (6). Unlike other important cancer-causing infections (Helicobacter pylori and hepatitis B and C virus), HPV is almost exclusively sexually transmitted and is not sensitive to general improvements in medical care or standard of living. Consequently, only vaccination and organised cervical cancer screening programmes can prevent or combat HPV epidemics and their cancer sequelae in any given population.
The 12 most common HPV types associated with cervical cancer worldwide, by decreasing prevalence, are HPV16 (57%), 18 (16%), 58, 33, 45, 31, 52, and 35, with small regional variations. HPV16 is by far the most likely to persist and cause CIN3 and cervical cancer.

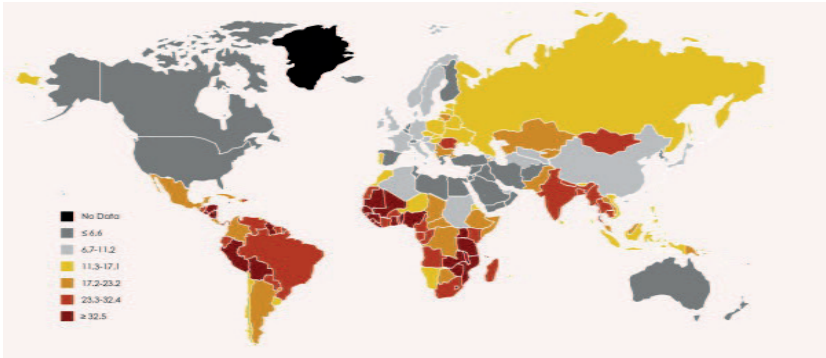


Figure 2 Cervical cancer, global map showing estimated age-standardised (world standard) incidence per 100,000 in 2008 (all ages). Based on GLOBOCAN 2008(6).

2.3 CERVICAL CANCER SCREENING IN SWEDEN

In 1968 Wilson and Jungner established a list of general criteria for screening that have stood the test of time; cervical cancer screening satisfies these criteria well (11).
The criteria are as follows:

1. The condition should be an important health problem.
2. There should be accepted treatment for patients with recognised disease.
3. Facilities for diagnosis and treatment should be available.
4. There should be a recognisable latent or early symptomatic stage.
5. There should be a suitable test or examination.
6. The test should be acceptable to the population.
7. The natural history of the condition, including development from latent to declared disease, should be adequately understood.
8. There should be an agreed policy on what patients to treat.
9. The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole.
10. Case-finding should be a continuous process and not a “once and for all” project.

The aim of cervical screening is to reduce the incidence of invasive cervical cancer (ICC) through detection and treatment of cancer precursors, as well as through early detection of invasive disease to thereby improve prognosis and reduce mortality (12). Swedish population-based screening with Pap smear has been used to detect precancerous lesions since the early 1960s (1964) and by 1977 all counties participated. Current screening guidelines from the National Board of Health and Welfare (1998) recommend a Pap smear every three years for women aged 23-50 and every five years for women aged 51-60. Most counties (70%) call women for screening based on elapsed time since last recorded cytology, including opportunistic tests (tests on demand), and most counties re-call non-responders the following year or earlier.
The screening programme has had a major impact on both morbidity and mortality from squamous cervical cancer (SCC), reducing the incidence of SCC and mortality by 35-70% (13-16). From 2007 to 2011, approximately 446 new cases of cervical cancer were detected annually (1.9% of all cancer cases in Sweden), among which 139 deaths

occurred. The age- standardized rate was 7.0/100 000 person years. (17) and relative 5-year survival was 73% (relative 1-year survival 89%). Nearly 700 000 Pap smears are performed annually in Sweden, of which 8% show some form of cellular atypia; 3.6 % were ASC-US, <1% koilocytosis, 2.1 % LSIL (CIN1), 0.1% ASC-H, 0.8% HSIL (CIN2) and 0.4% HSIL (CIN3)(18). Despite an increase in the number of cases of adenocarcinoma in situ (AIS) found by screening, the incidence of adenocarcinoma (AC), has increased (15), calling the protective effect of the screening programme (Pap smear) against AC into question (19).

Bray et al. recently presented an overview of cervical cancer and other HPV-related diseases in Central and Eastern Europe, where the incidence of cervical cancer is high in many countries due to lack of effective screening. They found a clear birth cohort effect in which risk of cervical cancer is increasing in successive generations of women born after 1940-1950, a general phenomenon reflecting changes in sexual behaviour with increased risk of persistent HPV infection (20).

In 2012, participation in the Swedish screening programme among women aged 23-50 was 78% and among women aged 51-60, 84%. Variation between counties was large, ranging from 66% in Uppsala county to 93% in Dalarna. In 2012, 681 411 cervical cytology samples were taken in Sweden, 71% of which were through the screening programme (18). The National Register for Cervical Cancer Prevention (NKCx) includes a copy of the same file used to report the diagnoses from all cytological and histopathological laboratories in Sweden, for which reason the data are 100% complete. The NKCx presents annual reports of these data. “Cytburken”, an information system on gynaecological cytology and histopathology results, provides information on patient history directly to healthcare providers.

Andre et al. used this nationwide population register to identify all cases of invasive cervical cancer (ICC) diagnosed in Sweden between 1999 and 2001. They verified the histopathological diagnoses and checked the Pap smear screening histories. They demonstrated that non-adherence to screening was the major reason for cervical cancer morbidity. Approximately 64% of all cervical cancers and 83% of advanced cases (FIGO stage II or higher) were diagnosed in women who were not screened. Two-thirds of the cancer cases were younger than 66 years at diagnosis, implying that they have had the opportunity to participate in screening. The results from the audit support benefit from screening of women younger than 30 but as only one case of ICC occurred below the lower screening age 23, this, supports the view that screening of women younger than 23 years is unnecessary. Women found to have abnormal Pap smears when screened within the recommended interval comprised 11.5% of all cases of ICC, implying that risk of ICC could partly be reduced through improved management of abnormal Pap smears. Women with abnormal Pap smears who did not have a follow-up biopsy were at even greater risk; 7% of cancer cases were diagnosed in women with an abnormal Pap smear who were not followed up with histopathology (12). However, about 92% of women with cervical cancer discovered through screening were cured, including those who underwent screening for the first time. Early diagnosis significantly improves the chance of successful treatment, which is a powerful argument for having a Pap smear when called for cervical screening.

A nationwide population based cohort study of all cervical cancer cases diagnosed during 1999-2001 (n=1230) and prospectively followed for 8.5 years found that, in addition to preventing cervical cancer, screening detected ICC also had a better prognosis than cancers detected on the basis of symptoms. Andre et al. also showed

that the improved cure rate was largely attributable to detection at an earlier clinical FIGO stage. Symptomatic cancers are generally associated with a later stage (21). This effect was not attributable to lead time bias. Lead-time bias is “when detection at an early stage adds time to follow-up but does not alter the course of the disease”. Lead-time is the length of time between detection of disease by screening and the detection resulting from the usual clinical presentation and stage at diagnosis.
Analysis of where the greatest medical benefits of quality improvement can be realised has identified four important areas: 1. Increased participation; 2. Increased protection for women over age 60; 3. Improved prevention through better management of AIS; 4. More sensitive screening tests (HPV test)(18).

2.3.1 Screening by conventional cytology (Pap smear)

The method was first described by Dr Papanicolaou in 1941 and ever since this test has been referred to as the Pap smear. Since precursor lesions of cervical cancer usually arise in the transformation zone (TZ), it is important that cell material is adequately sampled from this zone. The presence of metaplastic squamous cells and endocervical cells indicates that the TZ has been properly sampled (22). European guidelines recommend three sampling methods (22): 1. the combination of a wooden spatula (Ayre or Aylesbury) and an endocervical brush (e.g. Cytobrush), 2. a cervical broom (Cervex-Brush, Rovers) or 3. an extended tip spatula alone (Aylesbury). The best sampling device is the combination of Cytobrush with spatula, particularly with extended tip (23). The cells are smeared on a glass slide and immediately fixed to prevent air-drying, which distorts cellular detail. Ethyl alcohol 95% is used as fixative. The slides are stained according to the Papanicolaou staining method, which includes five separate dyes applied in a three-step process.
Cytology testing has limited sensitivity for histologically confirmed CIN2+ (70-80%), with a high degree of variation among cytopathologists due to low-moderate reproducibility (24) (25) (26) (27) (28). Specificity for pronounced cytological abnormalities is high (92%-96%) and positive predictive value (PPV) is about 42%. Cervical cancer screening has been successful despite the low sensitivity of a single Pap smear because the repeated testing of screening programmes improves sensitivity.

2.3.2 Liquid-based cytology (LBC)

Liquid-based cytology (LBC) was developed to improve the quality of conventional Pap smears. It is a new technique for transferring cellular material and improving sample preparations on microscope slides. The cells are collected just as in conventional cytology, but only plastic sampling devices may be used. The cells are then immersed and rinsed in a vial containing 20 ml of collection fluid (PreservCyt Solution) and samples are processed according to the methodology used. Two major types of LBC are commercially available, ThinPrep (Cytec Corp. Marlborough, MA, USA) and SurePath (BD, Franklin Lakes, NJ, USA). The ThinPrep 2000 is a semi-automatic processor that prepares one slide at a time. In the laboratory the vial is placed into the ThinPrep 2000 Processor, where cells are separated using a dispersion technique that breaks up blood, mucus, non-diagnostic debris and mixes the cell sample. The cells are then collected on a ThinPrep Pap Test Filter specifically designed to collect diagnostic cells in a thin layer, after which they are transferred to a glass slide

in a 20 mm-diameter circle and deposited into a fixative solution (29). The slides are then stained as in a conventional smear. The ThinPrep method was US FDA-approved (Food and Drug Administration) in 1996. The advantages of the method include greater likelihood of representative smears, fewer obscuring factors such as blood, mucus and inflammatory cells, and cellular material deposited in a thin layer that facilitates microscopic interpretation, thereby increasing laboratory efficacy and importantly, also facilitating supplementary analysis (e.g. HPV reflex testing).
Previous studies have demonstrated that LBC increases the proportion of abnormal cytology findings. A Swedish study by Zhu et al. (30) comparing sensitivity for detection of high-grade lesions showed that the ThinPrep method was somewhat more accurate (66%) than conventional cytology (CC) (47%). Another Swedish randomised controlled trial (RCT) comparing the performance of LBC with CC in a population-based screening setting showed a 40% increase in sensitivity for detection of high-grade lesions with LBC (31), but the question is still controversial since subsequent studies failed to support improved diagnostic accuracy. A meta-analysis by Arbyn et al. from 2008 (32) found LBC is neither more sensitive nor more specific for detection of high-grade CIN compared with conventional Pap testing. A large 2009 RCT from the Netherlands (33) involving 89 784 women concluded that neither sensitivity nor positive predictive value (PPV) for detection of cervical cancer precursors improved with LBC compared with conventional Pap testing. Another large RCT from Italy, Ronco et al., involving 45 000 women, came to the same conclusion, but also showed a large reduction in unsatisfactory smears. In a Swedish study by Fröberg et al. 2013, no significant differences in screening performance between LBC+HPV triage and conventional cytology were observed. Both methods showed similar detection rates for high-grade CIN and PPVs for detection of these lesions, indicating similar sensitivity and specificity for both cytological methods (34). Despite its higher cost, LBC has largely replaced CC in several countries and the Swedish Society for Obstetrics and Gynaecology now recommends LBC (35).
However, cytological interpretation is subjective and requires quality control and assurance to achieve and maintain accurate clinical performance. Since it is also labour-intensive, automated high-throughput screening has been difficult to achieve. Despite its low cost, cytology may not be the most cost-effective screening option for the reasons mentioned above (25). Although LBC has its advantages, it is more expensive and neither more sensitive nor more specific than conventional cytology, for which reason newer methods need to be evaluated. A 2013 Danish study (36) showed that implementation of image-assisted reading of LBC samples, regardless of the brand used, increased the proportion of abnormal findings by about 30% in all age groups (range from 19% to 41%). A laboratory equipped with conventional technology showed no trend towards an increase in abnormal findings during the study period.

2.3.3 Cytology and Histology Classification systems

Different classification systems have been used to evaluate cytological smears. Uniform grading of cellular abnormalities is essential for registration and comparisons over time and between different settings and countries. The Bethesda System (TBS) was developed to serve as a uniform system of terminology that would provide clear guidance for clinical management (37). This system was most revised in 2001.

European guidelines state that laboratories should use a nationally agreed terminology for cytology that is translatable into TBS (38). (Table 1)
The Swedish Society of Pathology and Clinical Cytology has formulated a uniform classification system that is essentially based on the CIN (Richart) System (39, 40) and which can easily be translated into TBS. This classification system is termed "Sverige-remissen". According to the Swedish Society for Clinical Cytology, koilocytosis without nuclear atypia should be reported as non-pathological and not as LSIL; however, this would be a rare finding. Furthermore, AIS and adenocarcinoma are classified together and not subdivided as in TBS, because both are unusual diagnoses.

Table 2. Conversion table for different cytological classification systems (41).

WHO	CIN	TBS 2001
Normal		Negative for epithelial abnormality
Atypia		ASC-US, ASC-H
Atypical glandular cells		Atypical glandular cells
Mild dysplasia	Condyloma CINI	LSIL
Moderate dysplasia	CIN II	HSIL
Severe dysplasia	CINIII	
CIS		
AIS	CGIN	AIS
Invasive carcinoma		

European guidelines for cervical histopathology strongly recommend CIN classification for histological diagnosis (42). CIN grading reflects the biology of the underlying lesion. CIN1/koilocytosis (correlating to LSIL) is likely to be reversible, associated with productive HPV infection and has low potential to progress to cancer (43). Cytological, virological and molecular profiles of CIN1 appear to be similar to those of epithelium without CIN (44). CIN2 and CIN3/carcinoma in situ (CIS) (correlating to HSIL) are more likely to persist or progress if left untreated and are also more likely to be associated with HPV integrated into the host genome (43), indicating a considerable risk of developing cancer. There is consensus on recommending treatment for CIN2 and CIN3 (45), which is why they are often included under the term high-grade CIN. However, it is clinically relevant to distinguish CIN2 from CIN3 (46). CIN2 is an intermediate condition, which contains over-called CIN1 and under-called CIN3, while CIN3 is a more robust and reproducible diagnosis than CIN2 and is therefore more useful as a gold standard for outcome (46). Any distinction between individual grades of CIN is poorly reproducible, but improves with increasing grade. Intraepithelial squamous lesions are characterised by abnormal cellular proliferation and maturation, together with nuclear atypia.
Intraepithelial lesions are classified into three categories following Richart´s description of cervical intraepithelial neoplasia (CIN) terminology (47). In CIN1 (flat condyloma, koilocytosis, mild dysplasia), atypical cells are present in the lower third of the epithelium. These lesions frequently show marked HPV cytopathic effects including

perinuclear halos, multinucleation with nuclear membrane irregularities, and hyperchromasia (e.g. “koilocytosis”). In CIN2 (moderate dysplasia), neoplastic basaloid cells and mitotic figures occupy the lower two thirds of the epithelium. In CIN3 (severe dysplasia; carcinoma in situ) the full thickness of the epithelium is engaged where the cells have high nuclear/cytoplasmic ratios with scant cytoplasm and dense, hyperchromatic nuclei with coarse clumped chromatin and irregular nuclear outlines (48). CIN terminology is used for reporting both histological and cytological diagnoses.

When attempting to distinguish reactive squamous proliferations from HPV-induced lesions pathologists frequently make errors. The most common error in the category of mild dysplasia is “overcall” of non-specific inflammatory or reactive lesions as productive HPV infections. In the ASCUS-LSIL Triage Study (ALTS), 45% of biopsies initially classified as CIN1 were downgraded to non-CIN when reviewed by a panel of expert gynaecological pathologists (49). Distinction between CIN2 and both CIN1 and CIN3 in biopsy specimens is complicated by the fact that the thickness of the epithelial changes often varies greatly within any given cervical biopsy specimen, while the angle at which the epithelium has been cut during histological sectioning may also have an effect. Immature metaplasia, atrophy, and reparative processes are lesions unassociated with any risk for progression to carcinoma and may be misinterpreted as CIN2 and CIN3. In such cases p16 staining and repeat cytology after oestrogen administration, as well as additional HPV testing may be helpful.

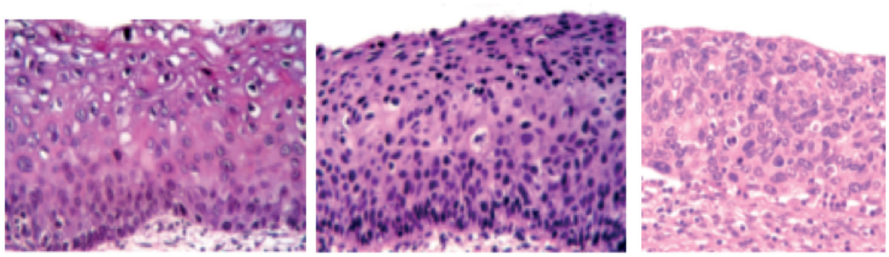


Fig 3. CIN1-3 From Tavassoli & Devilee (2003)

However, in regard to reproducibility, LSIL is one of the most robust cytological interpretations. Conditions that mimic HPV-associated koilocytosis include intracytoplasmic glycogen, especially in women taking oral contraceptives or hormone-replacement therapy, and “pseudo koilocytosis”, which may be identified in some older women with atrophic findings. HPV-negative LSIL women in the ALTS study who demographically resembled a lower risk population were more likely to be older and report fewer sex partners than women with HPV-positive LSIL, supporting the view that some HPV-negative LSIL represents false-positive cytology. Newer staining methods and biological markers will help to distinguish between these diagnoses.

2.4 COLPOSCOPY

The purpose of colposcopic examination is to identify diseased tissue by localising and diagnosing suspicious areas of the ectocervix and vaginal fornices (sites where dysplasia may occur) to allow targeted sampling (biopsies) from these areas for histopathological confirmation. The procedure provides illuminated magnification (6-40x) of the cervix and the vagina. Various solutions (normal saline, 3%-5% acetic acid

and Lugol's iodine) are then applied in a given order to the cervical epithelium, which turns precancerous lesions "acetowhite". A green filter can also be used to examine the sub-epithelial vascular pattern (Sellors & Sankaranarayanan, 2003). Abnormalities preferentially develop within the TZ and are graded according to morphological features such as acetowhiteness, margins, blood vessels and iodine uptake. Microinvasive and invasive squamous cancers are densely acetowhite with markedly atypical blood vessels. The surface configuration gradually changes from small protuberances to raised edges, irregular surface contours and bleeding blood vessels. Dr. Hans Hinselmann from Germany first described this method in 1925.
The International Federation of Cervical Pathology and Colposocpy (IFCP) recommended new terminology for colposcopy in 2011. The new nomenclature covers terminology related to the cervix and the vagina. Various types of cervical excision methods and specimen dimensions are also included. The terms "satisfactory colposcopy and unsatisfactory colposcopy" have been replaced. The colposcopic examination should be assessed for three variables: Adequate or inadequate, including reason why, squamocolumnar junction visibility and transformation zone type (Table3).

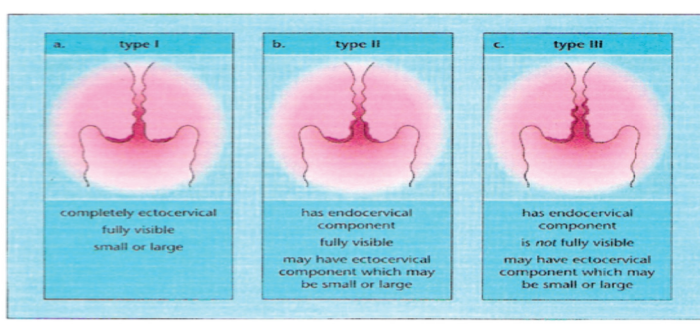
2.4.1 Terminology and the Transformation Zone (TZ)

The TZ is the area between the original squamous epithelium and columnar epithelium within which varying degrees of maturity may be identified. Three types of TZs have been described and are classified according to these findings:

- Size of the ectocervical component
- Position of upper limit
- Visibility of the upper limit

- Transformation zone Type 1 Completely ectocervical and fully visible
- Type 2 Fully visible with an endocervical component
- Type 3 Endocervical component present, but not fully visible (50).

Fig 4, TZ types



The following points may provide clinical guidance:

- •Describe features
- • Surface contour and edges
- • Speed of uptake of acetic acid/whiteness
- • Features of acetic acid/whiteness
- • Iodine staining
- •Transformation zone type

- •Extent of lesion
- •Colposcopic opinion
- •Management plan

2.4.2 Colposcopic findings and biases in assessment

Studies have shown poor correlation between colposcopic impressions and histological diagnosis and therefore scoring systems have been developed to improve clinical prediction. The Reid Colposcopic Index (RCI) is the most well known (51). Strander et al. (52) developed a new scoring system, which in addition to the parameters evaluated in the RCI also includes lesion size. Studies have shown that the sensitivity of colposcopic biopsies is dependent on lesion size (53). Kärrberg et al. also used the Swedish scoring system to evaluate pregnant women and concluded that it seems to be a useful tool that may reduce the need for diagnostic biopsies (54).
Colposcopically directed punch biopsy from abnormal cervical areas is of great importance because a small piece of cervical tissue, often <5 mm in diameter, may be taken to confirm the clinical impression, since colposcopy alone misses approximately one-third of high-grade CIN. Biopsies may sometimes be randomly taken from the TZ when no lesion is observed (55). Such biopsies are often combined with histological sampling from the endocervical canal, especially if the "new" squamocolumnar junction cannot be examined (inadequate colposcopic examination) or when an endocervical lesion is suspected. Colposcopy can also be used to assess the vagina, vulva and perianal skin.
"Assessed sensitivity and specificity in colposcopy and directed biopsies are susceptible to bias". According to IARC handbook on cervical cancer screening, the colposcopic impression confounds the reference standard of diagnosis (histology) since it dictates where the histological specimen is obtained (48, 56). Verification bias exists when diagnostic tests influence whether or not directed biopsies (the gold standard) are used to verify test results. In clinical practice this is more likely to occur when colposcopy is negative and no biopsies are taken. In this scenario, sensitivity estimates may be too high and specificity too low. Underwood et al. looked at possible reasons for missed high-grade CIN. Such reasons may include limitation of colposcopy to identify the specific abnormality, skill level of colposcopist, or failure to sample an identified abnormal area. Improved image quality is associated with improved detection of CIN2+, but seemingly normal cervical tissue, even with enhanced imaging, has been shown to contain CIN2+ disease in 25% of women referred for colposcopy. The conclusion therefore was that colposcopy itself, rather than punch biopsy, is the limiting factor for detecting CIN2+ (57). Newer technologies, such as electrical impedance spectroscopy and computer-assisted diagnosis, have been shown to improve colposcopic performance (58, 59).

2.4.3 Accuracy of colposcopy

Underwood et. al stated that there is increasing concern over the accuracy of colposcopically directed punch biopsies to diagnose high-grade CIN since recent reports have indicated lower sensitivity than previous reports. A Norwegian study reported that 24% of women with negative colposcopy-directed biopsies were found to have CIN2+ when follow-up biopsy was performed (57, 60). For example, the meta-

analysis of colposcopic accuracy by Mitchell (61) found that sensitivity to predict CIN2+ was 96% and specificity 48%. Pretorius et al. (2004) demonstrated a 57% sensitivity to detect CIN2+ and that sensitivity increased to 95% by adding random biopsies at the squamocolumnar junction. Adding endocervical curettage also increased sensitivity by 5.5% in women with adequate colposcopy. Pretorius concluded that random biopsies should be considered if cytology was high-grade. (55, 62).
The ALTS trial demonstrated that sensitivity depends more on number of biopsies taken than on training of the colposcopist, and that taking random biopsies increases detection of CIN2+, but data are limited since only women with ASC-US or LSIL were included (53, 63, 64). Pretorius also stated that regardless of skill, performing more biopsies increases the sensitivity of colposcopy (65).
A meta-analysis by Underwood, which included 32 papers published between 1969 and 2011, demonstrated that pooled sensitivity for a single punch biopsy was 90% and when one or more punch biopsies were performed, the sensitivity increased to 93%. When multiple biopsies are routinely taken, sensitivity approaches 100%. Specificity to diagnose CIN2+ was 24.6%.
The TOMBOLA trial identified a proportion of false-negatives associated with punch biopsy, but concluded that the impact on clinical outcome was insignificant because the next screening round would pick up missed cases (66). Missed disease may simply represent an over-diagnosis of small regressive foci of CIN2/3.
Another valuable outcome from the TOMBOLA trial was the establishment of a policy of targeted punch biopsies with subsequent treatment for CIN 2+, as well as a policy of cytological surveillance for CIN 1 or less to provide the best balance between benefit and harms for management of women with low-grade abnormal cytology who were referred for colposcopy. Immediate large loop excision results in over-treatment and should not be recommended (67). Since management decisions on whether to treat or monitor abnormal cervical cytology are often determined by the findings on punch biopsy, it is important for colposcopists to be aware of the limitations of the method. Underwood concluded that the observed high sensitivity and low specificity for high-grade CIN in colposcopy-directed biopsy might result from verification bias. Sensitivity appears to be high, but is probably a “spurious” finding because most studies limited excision to women with a positive punch biopsy (57).

Section	Pattern
General assessment	**Adequate or inadequate for the reason** (e.g., Cervix obscured by inflammation, bleeding, scar) **Squamocolumnar junction visibility**: completely visible, partially visible, not visible **Transformation zone types 1,2,3**
Normal colposcopic findings	**Original squamous epithelium**; mature, atrophic **Columnar epithelium**; ectopy/Ectropion **Metaplastic squamous epithelium**; Nabothian cysts; crypt (gland) openings Deciduosis in pregnancy
Abnormal colposcopic findings	**General principles** Location of the lesion: Inside or outside the transformation zone; location of the lesion by clock position Size of the lesion; number of cervical quadrants the lesion covers Size of the lesion as percentage of cervix **Grad 1** (minor): Fine mosaic; fine punctation; thin acetowhite epithelium; irregular geographic border **Grade 2** (major): Sharp border; inner border sign; ridge sign; dense acetowhite epithelium; coarse mosaic; coarse punctuation; rapid appearance of acetowhitening; cuffed crypt (gland) openings **Nonspecific**: Leukoplakia (keratosis, hyperkeratosis) erosion Lugol's staining (Schiller's test): stained or nonstained
Suspicious for invasion	Atypical vessels **Additional signs**: fragile vessels, irregular surface, exophytic lesion, necrosis, ulceration (necrotic), tumor or gross neoplasm
Miscellaneous findings	Congenital transformation zone, condyloma, polyp (ectocervical or endocervical), inflammation, stenosis, congenital anomaly, posttreatment consequence, endometriosis

Table 3. Provided by the International Federation of Cervical Pathology and Colposcopy, 2011.

2.5 REFRAMING CERVICAL CANCER PREVENTION

Cervical cancer is a rare complication of persistent infection with high-risk types of HPV. There is no consensus regarding the definition of "persistence" other than presence of HPV DNA on repeated testing of cervical specimens. The lifetime probability of encountering HPV at some point is as high as 80%-90%. However, most infections clear spontaneously without clinical signs or symptoms. In all, 4%-10% of middle-aged women are estimated to be persistent carriers and thus represent a true high-risk group for cervical cancer and probably also for other HPV-related cancers. The underlying mechanisms that determine whether infections resolve or become persistent are still unclear. Because lag time between age at peak incidence of HPV

infection and age at peak incidence of cancer is two to four decades, the initiating infection and its precursor lesions become a perfect target for screening and early detection.
The majority of vaginal cancer and their precursor lesions are associated with high-risk HPV types, with a reported range of 51.4%-81% (68-71). Fuste et al. concluded that HPV-positive tumors of the vagina tend to affect women with history of cervical neoplasia (72) and Alonso et al. demonstrated that HPV-positive early stage (FIGO I and II) vaginal squamous cell carcinoma had a better prognosis than early HPV-negative tumors (69). An estimated 40%-50% of cancers of the vulva have also been associated with HPV. In men, HPV DNA is found in cancer of the penis (40%-50%) and in both sexes, HPV DNA is detected in anal cancers (88%-94%). In head and neck cancers, the prevalence of HPV varies, but has been found in 35%-50% of oropharyngeal cancers. HPV16 is the most common type in all non-cervical cancers. According to a meta-analysis by De Vuyst et a., HPV 16 was found more frequently (>75%) and HPV18 less frequently (<10%) in HPV-positive vulvar, vaginal and anal carcinomas than in cervical carcinoma. HPV6 and HPV11 are common among genital warts and respiratory papillomatosis (RRP) but De Vuyst also reported that HPV6 and HPV11 were common in VIN1 and AIN1, but not in VAIN1 (vulvar, anal and vaginal intraepithelial neoplasia)(73). Current vaccines have been shown to protect against type-specific precancerous lesions and against genital warts in both females and males. HPV vaccination of men has also shown high efficacy against HPV-related anal precancerous lesions and anal cancer.
The tremendous amount of new knowledge has “prompted a paradigm change” of cervical cancer prevention to include prevention of HPV infections and related diseases (74).

2.6 NATURAL HISTORY

Of all HPV infections, cervical infections are the best understood, but we still lack insight into the final steps of carcinogenesis. At all ages, new infections are benign unless they persist. Persistence of an infection is the known necessary event for development of cervical pre-cancer and cancer. There is conflicting evidence as to whether any single type of HR HPV (especially HPV16) persists longer than others in the absence of development of CIN3+.
Studies have demonstrated that up to half of infections clear within 6 months and the great majority (90%) clear within a few years after acquisition.

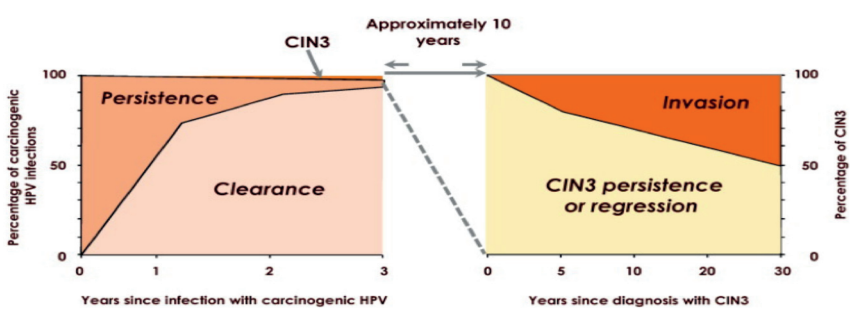


Fig 5. From: Updating the natural history of human papillomavirus and anogenital cancers(75)

According to Moscicki, the major steps in cervical carcinogenesis are:

1. Infection of the metaplastic epithelium of the cervical transformation zone (TZ) with one or more high-risk HPV types
1. Viral persistence
2. Clonal progression of the persistently infected epithelium to cervical pre-cancer
3. Invasion
4. The time between infection and clonal development of CIN3 cannot be precisely determined, because it depends on the intensity of surveillance and diagnostic limits of colposcopic biopsies, but the time required for detectable CIN3+ is likely 7-10 years.
5. The time from initial development of a small CIN3 lesion to invasive cancer may be up to 10 years(75).

2.6.1 Risk of CIN3+ following a positive HPV test.

Large cohort studies have estimated the absolute risks associated with different HPV genotypes. A Swedish study by Sundström et al. demonstrated that the presence of HPV16/18 in the first smear was associated with an 8.5-fold increased risk of CIS and an 18.6-fold increased risk of SCC, compared with women who are HPV-negative. Infection with other HR HPV types in the first smear was also associated with significantly increased risk. Persistence of HPV16 infection conferred a relative risk of 18.5 for CIS and 19.5 for SCC (76), which as expected represents a substantial increase in risk due to repeated HPV positivity.
Another Swedish study by Dahlström et al. investigating the risk of adenocarcinoma in situ (AIS) and invasive adenocarcinoma (AC) found that HPV 16-positivity in a normal smear was associated with an increased risk of both future AIS (OR: 12, 95% CI: 3-47) and AC (OR: 16, 95% CI: 4-67), when comparing with women who were HPV16-negative. Moreover, HPV 18-positive smears were associated with even higher risks for AIS (OR: 26, 95% CI: 3-192) and AC (OR: 28, 95% CI: 4-206)(19). A Danish cohort study by Kjaer et al. (77) showed that HPV16 had the greatest tendency to persist and was associated with the highest probability for progression when persistent, followed by infection with HPV18, HPV31, and HPV33, while HPV-negative women remained at very low risk. Women who tested positive for HR HPV at baseline and 2 years later were found at 12 years to have a 19.3% absolute risk of CIN3+. For a persistent HPV16 infection, the risk of CIN3+ within 12 years was 47.4%. However, rates of HPV16 persistence after an incident infection are similar to other high-risk HPV types (75, 77).

2.6.2 Risk of CIN3+ following a negative HPV test

Several large cohort studies have confirmed that risk of CIN3+ is very low in the years following one or two negative HPV tests, regardless of age. Dillner et al. studied long-term cumulative incidence of CIN3+ in six European countries and observed that after six years cumulative incidence rate was considerably lower among women who were HPV-negative at baseline (0.27%) than among women who only had negative cytology at baseline (0.97%) (78). The longest follow-up time was 16 years, where cumulative risk of cervical cancer among HPV-negative women was 0.26%(79).

Since HPV-negative women include both women who have cleared their infections and women who have never been positive (the two sub-groups cannot be distinguished) and because a negative HPV test is associated with a very low risk of ICC, the conclusion that can be drawn is that once cleared, infections neither reappear nor cause large numbers of CIN3+ cases (75). Young women are vulnerable to reinfection with a different or the same HPV types after documented clearance (80). Median time to the first negative test after incident infection (following a previously negative HPV test) was 9.4 months. In all, 90.6% of infections became undetectable within 2 years and of those, 19.4% were redetected within 1 year. The bulk of cancers in a population can be attributable to infections that were acquired at a young age and do not clear (81).

2.6.3 Non-viral co-factors

The most well-established co-factors (risk factors) for ICC among HPV-infected women are smoking, long-term hormonal contraceptive use, multiparity and human immunodeficiency virus (HIV) infection. It is unknown whether the mechanism of action for these co-factors is to increase risk of viral persistence or risk for progression, given persistent infection, with the exception of HIV, where results indicate that infection increases risk of persistence regardless of age. However, these known behavioural co-factors are less etiologically important than HPV genotype.
One study by Hwang L.Y. et al. found that smoking and oral contraceptives enhances squamous metaplasia, which is believed to support viral persistence (82). Studies have also found that age at first intercourse is a risk factor for cancer development. The prevalence of HPV peaks at a young age, when the process of metaplasia is also active. Risk of ICC was 2.4-fold higher among women reporting their first intercourse or first pregnancy before age 16, compared with those over 21 (83). Early first intercourse may also be a marker of high-risk behaviour, through which more HPV infections are acquired.
Lifetime number of sexual partners also correlates with higher exposure to HPV infections and a history of more than five partners doubles the risk(84). Risk of ICC also increases with the number of full-term pregnancies. More than seven full-term pregnancies have been shown to quadruple risk, while two full-term pregnancies double risk. Data from a recent Danish cohort study also support the notion that childbirth increases risk of subsequent CIN3+ in addition to the already increased risk for CIN3+ among women with persistent HPV infections. No associations were found with number of pregnancies, use of intrauterine devices, or sexual behaviour (85). Mechanisms that have been proposed to explain the increased risk of pre-cancer or ICC among multiparous women include elevated hormone levels and impaired immune responses. The TZ also remains on the ectocervix for a longer time, which may make these women more vulnerable to exposure to HPV and other cofactors. However, the Danish study could also be interpreted to mean that increased risk is related to delivery rather than to pregnancy per se, perhaps because of local tissue damage or cellular oxidative stress with DNA damage(85).
Sex hormones appear to be involved in cervical carcinogenesis. It has been suggested that oestrogen stimulates HPV gene expression, influences cervical immune response and stimulates cell proliferation in the TZ (85). Oral contraceptive (OC) use involves low-intensity hormone stimulation, but is often more long-term than pregnancy and childbirth. Data are conflicting regarding risk of pre-cancer and ICC, where some

prospective studies have shown no association (86, 87) while others indicate increased risk associated with long-term use of OCs (88).
However, the strongest risk factor resulting in persistent infection is likely to be lack of an adequate immune response, as reflected by high risk of HPV-related diseases among HIV-infected individuals, as well as among patients receiving immunosuppressive treatment.
The role of Chlamydia trachomatis as a co-factor is also controversial. A study by Safaeian et al. (89) suggested that the association between Chlamydia and HPV may partly be due to an increased susceptibility to HPV, since they found no association or risk of pre-cancer in this regard, as was found in earlier studies. However, a recent large European study with 9 years of follow-up also indicated that Chlamydia and Herpes Virus 2 may possibly contribute to cervical carcinogenesis. It further identified HPV16 E6 seropositivity as the strongest marker to predict ICC well before disease development (90). In addition, a recent Finnish study concluded that concomitant infection with HPV18/45 and C. trachomatis was associated with very high risk of CIN3 (91).
A European cohort study (92) also found that smoking is a major risk factor for developing cervical pre-cancer and ICC, which is consistent with previous findings. Smoking status, duration and intensity relate to disease and are associated with an up to a two-fold increased risk of CIN3/CIS and ICC, while length of time since quitting reduced risk by up to a factor of two. The strong beneficial effect of quitting smoking is an important finding that can support public health policies for smoking cessation.
In a large pooled analysis by Castellsagué, the only differences in cofactors observed relate to adenocarcinoma, where smoking and Chlamydia infection were unrelated, in contrast to SCC (93).

2.6.4 Re-infection, re-activation or clearance

This is a complex issue, but there is evidence that transient infections are cleared by innate immune responses and are less likely to result in a memory immune response, thereby leaving women vulnerable to re-infection. Moscicki et al. found that sexual behaviours were highly associated with redetection of infections, suggesting they may be due to re-exposure. The ambiguity may arise because antibodies are not the likely mechanism of protection against natural infections and cell-mediated immunity is more difficult to measure. Redetection may also be due to autoinoculation from other mucosal sites (75).

CIN2 is thought to be an intermediate stage between CIN1 (HPV infection) and CIN3 (direct cancer precursor), but this has recently been questioned, since many studies have shown that reproducibility of the CIN2 diagnosis is quite poor (94). Two review pathologists agreed with 84% and 81% of initial diagnoses of CIN3, compared with only 13% and 31% of the CIN2 diagnosis. However, several studies, especially in young women, have found rather poor reproducibility of the CIN3 diagnosis as well (95). Regression rates of CIN2 among young women under age 25 are high, up to 75% (96). In older women, regression rates are lower at around 30-50% over a 2-year period (97). For all ages, the regression rate for CIN2 appears to be lower than for CIN1, which is about 90%, but higher than for CIN3, which is about 20%-30%. The discrepancy in making diagnoses may also be due to misclassification of lesions. An

interesting study examined the protein expression from supernatants from fresh biopsies and found that several proteins could be used to differentiate CIN2 from CIN3 lesions, where Cytokeratin 2 was found to be the best discriminator with 90% correct classification (98). A frequently cited review from Östör reported that the likelihood of regression of CIN1 is 60%, persistence 30%, progression to CIN3 10%, and progression to invasive disease 1%. The corresponding numbers for CIN 2 are 40%, 40%, 20%, and 5%, respectively. The likelihood of CIN 3 regression is 33% and progression to invasive disease, greater than 12%. McCredie et al. reported results from an unethical clinical study where treatment for CIN3 was withheld from 1229 women in Auckland, New Zealand (1965 to1974). In women managed only by punch or wedge biopsy, cumulative incidence of invasive cancer of the cervix or vaginal vault was 30% (95% CI 23–42) at 30 years, and 50% (95% CI 37–65) in the subset of 92 women who had persistent disease within 24 months. The risk of cancer at 30 years was only 0.7% among women who were initially treated with conventional therapy (99).

2.6.5 Heterosexual transmission and autoinoculation

Transmission can be calculated in many different ways, like transmission probability per partnership, viral quantity, and nature of sexual encounters or per coital act. Transmission between heterosexual couples is very common, although rates vary widely among studies. The cumulative transmission probability over a 6-month period (i.e. the probability that an infected partner transmits HPV to a susceptible partner) ranged from 5%-28% for male-to-female and 19%-81% for female-to-male. Most studies have observed a higher rate of female-to-male versus male-to-female transmission. Specimens from women collected soon after vaginal intercourse (up to 48h) may result in false HPV positivity due to contamination from the sex partner and not to a true infection. The highest transmission rate was observed when one partner had a persistent infection (extended exposure) and probably a higher viral load (100). Sexual transmission to the oral tract via oral sex and open-mouthed kissing is associated with cancers of the oropharynx, tonsil and base of tongue (75, 101). Studies indicate that the anus may serve as a reservoir for HPV infection of the cervix but also the opposite. Moscicki reports a higher relative risk (RR) of acquiring an anal infection after a cervical infection with the same HPV genotype (20.5) than vice versa (8.8). It is more likely that the cervical infection serves as the source as vaginal discharge is frequently found on the perineum and can easily be transmitted to the anus (toilet paper). Anal sex can also be underreported in studies. HPV of the same genotype on the fingers as on the genitals most likely represents deposition of HPV DNA rather than a true infection but nonsexual routes of transmission (inoculation through fingers) cannot be ruled out (75).

Studies have shown that anal infections in women and in men who have sex with men are quite common but clearance is also common, unless the individual is infected with HIV. HIV strongly influences development of anal intraepithelial neoplasia (AIN). Women with other HPV-associated lesions, including CIN3+ and vulvar cancer, have higher rates of anal cancer (8).

2.7 HPV BIOLOGY AND LIFE CYCLE

2.7.1 HPV Classification

Human papillomaviruses (HPV) have evolved over millions of years and survived in a wide range of animal species and humans. HPVs comprise a large and diverse group of viruses with 174 fully characterised types and new HPV types are continually being found (102). There are five major HPV genera, based on DNA sequencing, each with different epithelial tropism and disease associations: Alpha papillomavirus, Beta papillomavirus, Gamma papillomavirus, Mu papillomavirus and Nu papillomavirus. HPVs infect epithelial cells of genital mucosa (alpha papilloma viruses only), oral mucosa and skin (all five genera). HPV types belonging to different genera have less than 60% similarity, based on the nucleotide sequence of the capsid protein L1. Different viral species within a genus share 60%-70% similarity. A novel HPV type has less than 90% similarity to any other HPV type; i.e., a new genotype differs by more than 10% in the DNA sequence of the L1 open reading frame (ORF) from the closest known HPV type. When DNA only differs by 2%-10%, the two viruses are considered subtypes of the same HPV type. Variants by definition differ from one another by a maximum of 2% in the L1 gene (103). A novel HPV type is assigned a number after the whole genome has been cloned and deposited at the International HPV Reference Center, which was established in Heidelberg in 1985 and subsequently transferred to Karolinska Institutet in 2012 (www.hpvcenter.se)(7).

HPV causes a wide range of diseases from benign lesions to invasive tumours. The various diseases appear to be associated with different strategies of transmission and propagation within the epithelium, as well as different interactions with the immune system. Various HPV types have adapted to specific epithelial niches with different types having different disease associations (102).

Alpha papilloma viruses are divided into cutaneous and mucosal types. Mucosal Alpha types are the most studied and clinically important and can be subdivided into high-risk and low-risk groups. High-risk Alpha types have been clearly linked with development of SCC and AC of the cervix. Approximately 40 different HPV types are known to infect the genital mucosal epithelium and a subset of 10–15 HPV types are associated with lesions that can progress to cancer. In 2009, an IARC working group (International Agency for Research on Cancer) classified 12 mucosal HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56 58 and 59) as carcinogenic to humans (Group 1) (104). They are referred to as high-risk (HR) HPV types. These 12 types cluster together in the same evolutionary branch or “high-risk clade”. Eleven additional types in the high-risk clade were classified as possibly carcinogenic (Group 2B) based on their phylogenetic relatedness to Group 1 types, with the exception of HPV68, which was upgraded to probably carcinogenic (Group 2A). Types that are closely related evolutionarily (e.g., HPV16 and 31) may exhibit different degrees of cancer risk, thought to be related to different protein functions and patterns of gene expression. Low-risk HPV types share many similarities with HR types, but are not found in ICC. HPV6 and 11, also belonging to the Alpha papillomavirus genus, cause benign genital condylomas. HPV3 and 10 are cutaneous Alpha types that cause flat warts (105). High-risk HPVs do not cause cancer in the vast majority of individuals they infect.

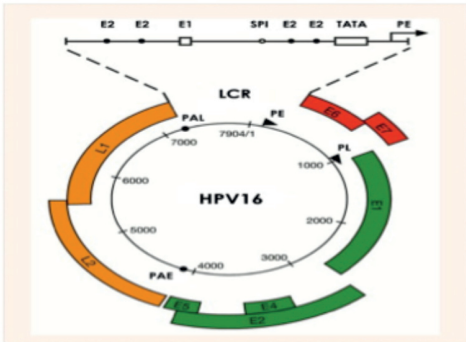


Fig 6. Alpha Papillomavirus Disease Association and Genome Organisation of HPV16 (102)

2.7.2 Genome organisation

HPVs are small, circular double-stranded DNA viruses containing almost 8000 base pairs. The viral icosahedral capsid is composed of two structural proteins, the major capsid protein L1 and the minor L2. The viral genome is functionally divided into three domains based on location – the long control region (LCR) and eight genes termed either "early" or "late". They encode for a large number of viral proteins that are necessary for different stages of the viral life cycle, where E and L signify early and late events in the viral life cycle. The LCR is non-coding and shows the highest degree of variation in the viral genome. It contains binding sites for transcription factors and for viral E1 and E2 proteins, which control viral replication and gene expression. HPV16 has two promoter elements known as PE (early promoter) and PL (late promoter) that regulate the expression of differentially spliced mRNAs during epithelial differentiation (102). The early region consists of six open reading frames (ORF): E1, E2, E4, E5, E6 and E7. An ORF is a sequence of bases coding for a protein. These early genes are involved in regulation of replication and transcription, cell growth, maturation and virus release and malignant transformation. The late genes code for the structural proteins L1 and L2.

2.7.3 Start of infection

HPVs are intracellular parasites, dependent on the cell machinery of basal cells in stratified epithelium to replicate. Through a micro wound in the epithelium, HPV binds via its L1 major capsid protein to heparan sulfate proteoglycans (HSPG) on the basement membrane (BM) and undergoes a conformational change that exposes the N-terminus of the L2 minor capsid protein. This leads to furin cleavage and proteolysis of the L2 protein exposes a previously occluded surface of L1 that then binds to a surface receptor on the keratinocytes. This second receptor binding leads to virus internalization and uncoating of the virus in endosomes. The endosomal escape mechanism is L2-dependent and the L2-genome complex is transported to specific subnuclear domains, ND10 bodies where viral gene transcription is initiated. The L1 protein is retained in the endosome and undergoes lysosomal degradation. The infectious process is slow and takes 12-24 hours before transcription begins. The effectiveness of neutralizing antibodies after vaccination might partly be due to the

extended exposure of antibody neutralizing determinants on the BM and the cell surface (106).

2.7.4 Function of LR and HR HPV proteins

It is thought that infection is followed by an initial phase of genome amplification and then maintenance of the viral episome at a low copy number in the basal cell layers (200 copies/cell). The viral replication proteins E1 and E2 are thought to be essential for the initial amplification phase, but their exact role is not yet clear. E2 regulates viral transcription, binds to the LCR and can recruit viral E1 protein. The role of E6/E7 proteins in infections with LR HPV types is uncertain, but the functional differences between the low-risk and high-risk E6/E7 proteins contribute to their different pathogenesis and to their different patterns of gene expression.
The proliferation of basal and parabasal cells by HR HPV leads to lesion growth and is mediated by the E6 and E7 proteins. A key function of the E6/E7 proteins is to stimulate cell cycle re-entry in mid-epithelial layers to promote genome amplification. Deregulation of E6/E7 expression is critical in determining neoplastic grade, even in the absence of viral integration. Functional differences between HR and LR E7 proteins relate to their different affinities for binding to the retinoblastoma (Rb) protein family. HR E7 binds and degrades p105 and p107, which control cell cycle entry in the basal layers, as well as p130, which is involved in cell cycle re-entry in the upper layers. HR E7 also stimulates host gene instability through deregulation of the centrosome cycle in the basal cells. A key difference between LR E6 and HR E6 is that the latter has a PDZ-domain motif at the C terminus and is able to interact with PDZ targets, which are involved in regulation of cell polarity, proliferation and signalling. Other important and unique differences from LR E6 include the capacity of the HR E6 protein to: 1. upregulate telomerase activity to maintain telomere integrity during repeated cell divisions and 2. to mediate intracellular p53 degradation. As mentioned above, a key function of HR E6 and E7 expression is to allow infected cells in the upper epithelial layers to re-enter S-phase in order to increase viral copy numbers. Viral proteins E1 and E2 are also upregulated and increase in quantity. The proliferating epithelial cells express both markers of differentiation (keratins 1, 10, 4 and 13) and markers of cell cycle entry (MCM, Ki-67, PCNA, Cyclin E and Cyclin A). Cellular DNA replication occurs first, followed by replication of the viral genome.
E4 and E5 proteins modify the cellular environment and E5 is involved in koilocyte formation (102).

2.7.5 HR HPV life-cycle, regulation and deregulation

As mentioned above, a micro wound in the epithelium is required to allow virions to gain access to the basal lamina. Infection of the columnar cells may be facilitated by their proximity to the epithelial surface and the TZ. The proliferating cells of the metaplastic epithelium in the TZ are accessible and particularly susceptible to infection. As these cells divide, they produce daughter cells that are pushed outwards towards the epithelial surface, which triggers various events in the virus life cycle.
In lesions caused by high-risk HPV types, cells in the lower layers express E6 and E7, which stimulate cell division. Genome amplification occurs when the proteins necessary for this event become elevated in the mid layers of the epithelium. The cells

will then express viral E4 protein and enter cell cycle phase S or G2. The S phase is when DNA replication occurs and the G2 phase is a gap between DNA synthesis and mitosis, during which a process is underway to ensure that everything is ready for mitosis to begin. When the cell cycle is finished in the upper epithelial layers, a subset of E4-positive cells starts to produce viral L1 and L2 proteins. These proteins form the shell of the virus and packaging of the viral genomes can now occur. An interesting hypothesis is that lesion formation begins with infection of basal stem cells and the longevity of the stem cells is the key factor in the formation of a persistent lesion (102).

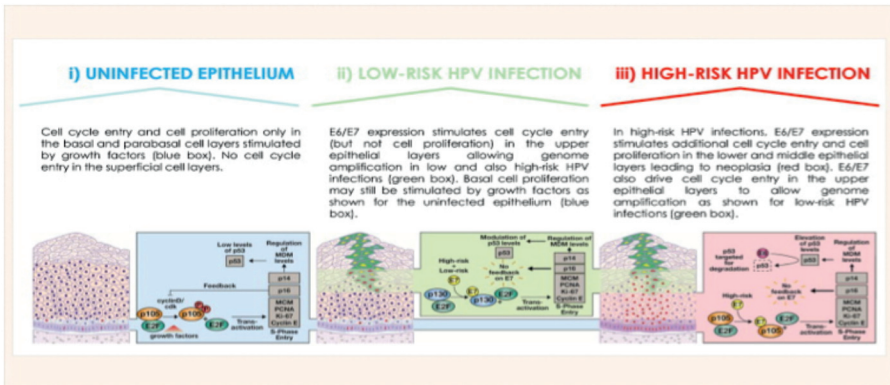


Fig 7. Regulation of Cell Cycle Entry and Proliferation in HR HPV Infected Epithelium. Locations of cells driven into cell cycle are marked by red nuclei in the epithelium. A yellow nucleus represents the appearance of L1 (102).

In HR HPV infections, the E7 protein indirectly inactivates the retinoblastoma tumour suppressor protein without the need for phosphorylation, as in uninfected epithelium, by displacing E2F from pRb (p105) and from p130 (pRb family member) thereby allowing transactivation of the genes necessary for S-phase progression. The absence of effective inhibition of cell cycle progression by p16ink4a leads to its accumulation in the cell and also to elevation of MCM, Ki-67 and PCNA levels in the infected epithelial layers. p16ink4a normally forms a negative feedback loop that suppresses growth factors (cyclinD/cdk) that control cell cycle entry and cell division activity, the overexpression of itself and other E2F-activated genes (MCM, Ki-67, PCNA). This leads to elevated p14arf levels which compromise the normal p53 degrading function of MDM. Consequently there is an increase in p53. P53 mediates cell cycle arrest, but this function is now countered by high levels of HR E6 proteins in the proliferating cells, which associate with E6AP. The result is ubiquination and proteasomal degradation of p53 (102). Detection of HPV in tissue biopsy or in exfoliated cervical cells may indicate productive infection (CIN1), abortive infection (CIN3), and presence of recently deposited virus particles that have not caused infection or latent infection. To distinguish between these possibilities, markers of viral gene expression may be useful to confirm active disease.
Methylation of the viral genome can sometimes suppress gene expression and lead to silent infection in which viral genomes are retained in the basal cell layer without apparent disease.

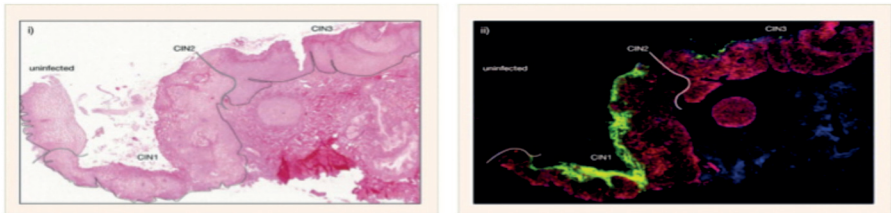


Fig 8. The image on the left is a histology section showing uninfected epithelium, CIN1, CIN2 and CIN3. The image on the right shows the same piece of tissue stained with two biomarkers (MCM and HPV16 E4). The cellular marker MCM (red) is expressed at low levels in the basal and parabasal layers in uninfected tissue. As a surrogate marker of E7 expression, MCM protein is elevated to different extents in neoplasia. In HPV-induced lesions viral E4 protein (green) becomes abundant as MCM levels decline during differentiation. Detection of E4 using type-specific antibodies confirms HPV16 as the causative HPV type in this lesion (102).

Expression levels of E6/E7 increase from CIN1 to CIN3, contributing to the accumulation of cellular genetic changes and ultimately leading to invasive disease. Viral deregulation is thought to facilitate integration of the viral episome into the host cell chromosome, where it encodes for viral oncogenes. Deregulation of early gene expression may follow from hormonal changes or epigenetic modifications, such as viral DNA methylation. The HPV16 LCR contains hormone response elements that can be stimulated by oestrogens, and there is abundant evidence that oestrogens and HPV co-operate in the development of cervical cancer. Different methylation patterns are linked to different degrees of disease severity and relate to changes in viral gene expression. Integration of the viral genome into the host cell genome occurs in many high-grade lesions, although cancer may arise from cells exclusively containing episomes. Host genetic susceptibility plays an important role. The host cell genome contains a number of fragile sites where viral integration is more likely to occur, although integration is generally a random event that may sometimes result in disruption of viral genes that regulate transcription from the LCR. E2 is of particular importance because it normally regulates E6/E7 expression. The majority of cervical cancers contain one or more copies of HPV randomly integrated into the host cell chromosome. Viral integration sites frequently lie within the regulatory E1 or E2 gene regions. Loss of E6/E7 regulation may facilitate persistent high-level expression of these genes. It is believed that once this occurs expression may increase even further. About 70% of HPV16-associated cervical cancers contain integrated HPV16 sequences in the host genome, while about 30% arise from cells containing only episomes. In the case of HPV18, the viral genome is almost always integrated (102).

2.7.6 Immune response

Cell-mediated immune response clears most infections, but HPV16 persists longer than other high-risk types, which may contribute to its higher risk for cancer. Models have provided evidence that cell-mediated immune responses and lesion regression are modulated by antigen-specific CD4+ T-cell dependent mechanisms. However, the hallmark of HPV infection is the effective evasion of innate immune recognition. The

viral productive life cycle is totally intraepithelial, which means there are no viraemia, no viral-induced cytolysis and no associated inflammation.
HPV down-regulates the innate immune signalling pathways and also inhibits Langerhans cell activation and recruitment of dendritic cells. In high-risk HPV types the mechanisms of immune evasion have essentially been established. Immune regression results from cross presentation of HPV antigens by Langerhans cells, followed by T-cell infiltration and shut-off of viral gene expression. However, apparently normal cells can still contain silent viral episomes and may not be effectively cleared from the basal cell layers. It has been suggested that the virus may be reactivated following immune suppression, hormonal changes, or as a result of aging. Immune response failure and inability to recognise viral antigens has been reported among cervical cancer patients (102).

2.8 TESTS FOR THE DETECTION OF HPV

HPVs cannot be cultured in conventional cell cultures, and classical virological diagnostic techniques (electron microscopy, immunohistochemistry) are suitable for routine detection due to lack of sensitivity and specificity. Serological assays for detection of anti-HPV antibodies have limited analytical accuracy and, so far, no clinical utility. All HPV tests currently in use are based on detection of HPV nucleic acids in clinical specimens. A recent review by Poljak et al. identified at least 125 commercially available HPV tests and, additionally, at least 84 variants of the original tests (107). They predicted that the number of commercial HPV tests will continue to increase in the future due to wide clinical applications which offer promising marketing opportunities for manufacturers. The design of new HPV tests will be guided by the categorisation of different HPV types into different risk categories. Since this varies over time and certain HPV types have been moved from one risk category to another, test design will need to accommodate such changes.
The key issue for HR HPV DNA testing is to detect HR HPV infections associated with high-grade cervical disease and to differentiate them from transient HR HPV infections. There needs to be a balance between clinical sensitivity and specificity for detection of CIN2+. Compared with cytology testing, HPV testing has been shown to have higher sensitivity and therefore higher negative predictive value (NPV). A negative HPV test is therefore reassuring that no clinically significant lesion is present. Other advantages of HPV testing include high reproducibility, no inter-observer variation, dichotomous results (positive or negative), high throughput and automation. HPV tests to evaluate HPV vaccine development require different analytical parameters (higher sensitivity) than clinical tests. Validated cut-off thresholds adapted for clinical use are therefore important. The Hybrid Capture 2 (HC2) HPV DNA Test (QIAGEN Inc., Gaithersburg, MD; USA (previously Digene Corp.)) was the first test to gain approval from the United States (US) Food and Drug Administration (FDA), which occurred in 1999. Currently five tests are approved by the FDA for clinical use. Four of them are DNA-based: HC2, Cervista HPV HR (Hologic, WI, USA), Cervista HPV 16/18 (Hologic Inc., Bedford, MA, USA) and Cobas 4800 (Roche Molecular Diagnostics, Pleasanton, CA, USA). One test is an RNA-based assay (APTIMA HPV assay (formerly GenProbe Inc., San Diego, CA, USA)(FDA-approved in 2011)(108). Cervista HPV16/18 is a test that screens for a panel of HR HPV types, and for HPV16/18 if the panel is positive (109). In April 2011 the FDA also approved the

Cobas 4800 HPV test, which allows detection of HPV16/18 while concurrently testing for 12 other high-risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). The Cobas 4800 was evaluated in the ATHENA (Addressing THE Need for Advanced HPV Diagnostics) study and is currently used by the Virology Department at Karolinska University Hospital in Stockholm (110). Below I will focus on the HPV tests that we used for our studies, namely the HC2 HPV DNA Test (HC2), the Linear Array® HPV Genotyping Test (LA) (Roche Molecular Systems Inc., Alameda, CA, USA) and the APTIMA HPV Test.

2.8.1 Hybrid Capture 2 Assay (HC2)

The Hybrid Capture® 2 HPV DNA test was originally developed by Digene Corporation (Gaithersburg, MD), but is currently marketed by Qiagen (MD, USA) and is the most commonly used HPV test worldwide. The B-probe of HC2 targets 12 HR HPV types (IARC-2009) plus HPV68. The US FDA approved HC2 in 2003 for triage in cases of atypical squamous cells of undetermined significance (ASC-US) and as an add-on test to cytology screening for women age 30 and older. HC2 has been evaluated in many RCTs and cohort studies, which have demonstrated the clinical value of HPV testing in general. It has therefore been recommended that new HPV tests do not need to be evaluated in extensive longitudinal clinical trials, but should demonstrate that they possess clinical characteristics equivalent (non-inferior) to HC2, before they can be used for screening purposes (107). The HC2 sampler kit includes a special cervical brush and a vial containing specimen transport medium (STM). Exfoliated cells are first treated with an alkali-denaturing reagent to release host and any existing HPV DNA molecules into the solution. The processed samples are hybridized with two mixtures of unlabelled single-stranded full-genomic-length RNA probes. One is complementary to the DNA sequence of 13 high-risk HPV types (high-risk probe cocktail B) and the other to five low-risk HPV (LR HPV) types: HPV-6, HPV-11, HPV-42, HPV-43 and HPV-44 (low-risk probe cocktail A).
Positive specimens are detected by binding the hybridization complexes onto the surface of a microplate coated with monoclonal antibodies specific to RNA–DNA hybrids. Immobilized hybrids are detected (or captured, hence the name “hybrid capture”), by the addition of a second alkaline phosphatase-conjugated antibody to the RNA–DNA hybrids and subsequently followed by addition of a chemiluminescent substrate. Light emission is measured semi-quantitatively as relative light units (RLU) in a luminometer. The emitted light from the specimen is compared to the average value of intensity of emitted light from three positive controls containing 1.0 pg of HPV16 DNA per ml (about 5000 copies of the HPV genome). RLU greater than or equal to 1.0 are considered positive. For high throughput HC2 testing, the Rapid Capture System (Qiagen) with semi-automated pipetting and microplate handling has been available for the last few years. One technologist can use the Rapid Capture System to process up to 352 patient specimens in 8 hours with 3.5 hours of hands-free operation (111). HC2 testing cannot determine specific HPV types since viral detection is performed using a combined probe mix. The main problems are cross-reactivity with low-risk HPV types, especially HPV11, 53, 54, 55 and 66 and the fact that HC2 does not contain an internal cellular control, to help adjudicate false negatives. HC2 also has an additional 5% false-positive rate in samples that contain no HPV DNA according to highly sensitive polymerase chain reaction (PCR) tests (108).

One study comparing clinical sensitivity and specificity for detection of high-grade CIN in a population of women referred for colposcopy because of abnormal cytology, showed that HC2 had a sensitivity of 99.6%, a specificity of 28.4% and a positive predictive value (PPV) of 36.1% (112). They studied the effect of different cut-off thresholds on clinical accuracy and found that clinical specificity of HC2 increased with increasing cut-off threshold. The authors suggested that HC2 might benefit from adjusting the positivity cut-off value to 2.0 RLU/CO, to improve clinical specificity and PPV, while still retaining similar clinical sensitivity. Based on these results, the manufacturer changed the positivity interpretation criteria, but only for samples collected in Thin Prep PreservCyt solution (Hologic, Madison, WI, USA) and not for samples collected in Digene STM (111).

2.8.2 Polymerase chain reaction (PCR)

PCR is a technology to selectively amplify a target sequence of DNA. Key components include primers (short DNA fragments) containing sequences complementary to target regions and heat stable DNA polymerase (for which the method is named). As the PCR progresses, the DNA itself is used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. The method makes use of thermal cycling, consisting of repeated cycles of heating and cooling of the reaction for the purpose of DNA melting (involving physical separation of the two strands of the DNA double helix) and enzymatic replication of the DNA. PCR has extremely high molecular sensitivity, permitting detection of less than 10 copies of HPV DNA in a mixture. The primers are able to amplify sequences from several different HPV types because they target conserved DNA regions within the HPV genome and the amplicons can be genotyped by sequencing or hybridization with type-specific probes using Dot blot, Southern blot or line strip hybridization. Three consensus primer systems are now well established: PGMY09/11, GP5+/6+ and SPF10LiPA. They target segments of considerably different sizes: 450 base pairs (bp), 140 bp and 65 bp respectively. Shorter fragments tend to yield better sensitivity when testing severely degraded specimens, such as paraffin-embedded, archival tumour tissue (48).

2.8.3 Linear Array HPV genotyping test (LA)

HPV DNA genotyping tests allow individual determination of several HPV types. However, the clinical value of HPV DNA-based genotyping assays has not yet been fully established, but these tests are indispensable research tools for the study of natural history, transmission, pathogenesis and prevention of HPV infections. DNA sequencing is still considered to be the ‘gold standard’ for HPV genotyping, even though it is costly, time-consuming and difficult to apply in routine diagnostic settings.
These tests are based on the principle of reverse hybridization, where a fragment of the HPV genome is first PCR-amplified, and the resulting amplicons are denatured and subjected to detection using HPV type-specific probes immobilized on a strip, filter or microtiter well.
The Linear Array HPV Genotyping Test (LA) is one of the most commonly used HPV genotyping assays for the identification of (37 HPV types in toto) 36 alpha-HPV types: HPV6, HPV11, HPV16, HPV18, HPV26, HPV31, HPV33, HPV35, HPV39, HPV40,

HPV42, HPV44, HPV45, HPV51-54, HPV56, HPV58, HPV59, HPV61, HPV62, HPV64, HPV66-73, HPV81-84 and HPV89, and one subtype (subHPV82 or IS39). LA is based on the co-amplification of a 450 base pair (bp) region of the HPV L1 gene and on a 268 bp region of the human β-globin gene, using biotinylated primer sets PGM09/PGMY11 and PC04/GH20, respectively. The resulting amplicons are then denatured and hybridized with HPV-specific oligonucleotide probes immobilized as parallel lines on nylon or a nitrocellulose membrane strip. After hybridization, streptavidin-conjugated alkaline phosphatase or horseradish peroxidase is added, which binds to any previously formed biotinylated hybrid. Incubation with chromogenic substrates yields a coloured precipitate at the probe positions where hybridization occurs. The genotyping strip is then read and interpreted visually by comparing the pattern of HPV-positive probes with the test reference guide for each of the targeted HPV types. At the same time, a region within the human beta-globin gene is amplified as a control for cell adequacy, nucleic acid extraction, and PCR efficiency. In a study by Szarewski et al. which compared the clinical sensitivity and specificity of six different HPV assays for detection of high-grade CIN in a population of 953 women referred for colposcopy because of abnormal cytology, LA had a sensitivity of 98.2%, a specificity of 32.8% and a PPV of 37.7% for detection of CIN2+ lesions (112).

2.8.4 HPV mRNA as biomarker and test (APTIMA HPV Assay)

As cervical lesions progress to cervical cancer the viral HPV DNA frequently integrates into the host-cell genome and the viral genes E6 and E7 are continuously expressed. Transcripts from the E6 open reading frame are either unspliced (full length (FL) E6 transcripts) or spliced. FL E6 proteins bind most powerful to p53, which results in degradation of the p53. Full length E6 therefore has the strongest association to carcinogenesis and FL transcripts are always present in cervical cancer. The E6 and E7 proteins have been difficult to analyze due their unstable nature in solution but their transcripts can be analyzed. Tests that detect these transcripts have are more likely to detect clinically significant disease (113).
Several studies have shown that testing for HPV mRNA instead of HPV DNA can be useful, due to higher clinical specificity and similar sensitivity. Transcripts encoding for viral oncoproteins E6 and E7 are of greatest interest. Viral mRNA can be detected by reverse transcriptase PCR or by nucleic acid sequence-based amplification (NASBA). The APTIMA HPV Assay (Gen-Probe Inc., San Diego, CA) was approved by the US FDA in 2011 and is at present the only FDA-approved mRNA-based HPV test. The indications are a) triage of women 21 years and older with ASC-US to determine whether referral for colposcopy is needed and b) screening of women age 30 and older in combination with cytology. The APTIMA HPV Assay (Gen-Probe, San Diego, CA, USA) is a transcription-mediated amplification-based assay, which allows detection of E6/E7 mRNA transcripts of 14 HR HPV types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. (i.e. IARC 2009 12 HR HPV types plus HPV66 and HPV68). The test result is qualitative for the presence or absence of the targeted HPVs and does not allow exact determination of the HPV type present in a clinical specimen (114). There is also The APTIMA 16 18/45 Genotype Assay which detects E6/E7 viral mRNA of HPV16, 18, and 45 from women with APTIMA HPV Assay positive results. The test can differentiate HPV16 from HPV18 and/or HPV45, but does not differentiate between HPV18 and 45.

APTIMA is a single tube test and is based on three main steps:

1. Target capture of E6/E7 mRNAs using HPV-specific capture oligomers linked to magnetic microparticles (Fig1). The procedure works by first lysing the cells to release the target mRNA.
2. Amplification of target E7 mRNA using transcription-mediated amplification (TMA) by using two enzymes: RNA polymerase and reverse transcriptase. The reaction is performed at the same temperature unlike PCR and results in ten-billion-fold amplification within 15 to 30 minutes.
3. Detection of resulting amplicons by hybridization protection assay (HPA) using chemiluminescent labelled probes. A selection reagent inactivates the label on the unhybridized probes. Emitted light is measured in Relative Light Units (RLU) in a luminometer. The assay result is based on the interpretation of the analyte signal-to-cutoff (S/CO).

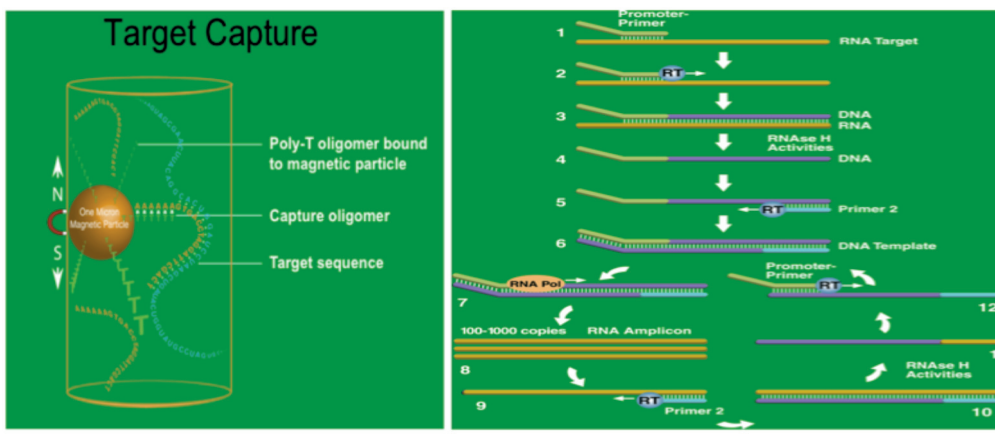


Fig 9 Aptima HPV Assay, Target Capture
The target sequence is hybridized to an intermediate capture oligomere with complementary sequences to specific regions of the HPVmRNA molecules and a string of deoxyadenosine residues. The complex is captured by poly-deoxythymidine oligomeres bound to the surface of magnetic particles. The particles are drawn to the side of the tube by magnets, concentrated and washed

Fig 10. Transcription-Mediated Amplification (TMA)
TMA uses two primers in the reaction. A primer contains a promoter sequence, which is a specific sequence that signals the enzyme to start transcribing at a particular site on the DNA/RNA template. RT; Reverse transcriptase creates a DNA copy of the target RNA. RNAse H degrades the resulting RNA: DNA duplex. A second primer then binds to the DNA copy and a new strand of DNA is synthesized. RNA polymerase recognizes the promoter sequence of the double-stranded DNA molecule and initiates transcription.

An internal control transcript is added to each reaction (via the Target Capture Reagent) to verify performance at each step of the assay: capture, amplification and detection (111). The internal control signal in each reaction is differentiated from the HPV signal by the magnitude of light emission from probes with different labels by the method,

Dual Kinetic Assay (DKA)). Internal Control-specific amplicon (and HPV 16 when use of APTIMA HPV16 18/45) are detected using a probe with a rapid emission of light (flasher), while amplicon specific to HPV (and 18 and 45 when use of APTIMA HPV16 18/45) are detected using probes with relatively slower kinetics of light emission (glower)(115). One positive calibrator (in vitro transcript in buffered solution) and one negative calibrator (buffered solution) are tested in triplicate at the beginning of each run. The purpose is to determine the validity of the run and to establish the assay cut-off values for the internal control and analyte signals.
The signal observed for each reaction is then compared with the cut-off values. Those reactions with an analyte signal to cut-off (S/CO) ratio >1.00 is considered positive for HPV. Samples with an analyte S/CO ratio <1.00 must have an internal control signal greater than or equal to the internal control cut-off value in order to be considered a valid negative result. (114). The analyte S/CO of the Aptima was recently changed (lowered) and now a S/CO ratio > 0.50 is considered positive. In our studies we considered S/CO >1 to be positive according to the manufacturer at the time of the studies (114). The assay software determines test results.
Analytical evaluation estimated the 95% detection limit for Aptima to be 38-488 mRNA copies/reaction in the DTS system (semi-automated) and 17-275 HPV mRNA copies in the TIGRIS DTS system (fully automated).
Analytical evaluation of APTIMA also showed that it did not cross-react with any low-risk HPV types, or with normal flora or opportunistic organisms that may be found in cervical samples (114). APTIMA was considered to be a robust test with low inter- and intra-run variability. Getman et al. demonstrated that Aptima detected not only HPV mRNA, but also HPV dsDNA, but the sensitivity for HPV16 mRNA was substantially higher than for HPV16 DNA (116). However, recently published manufacturer instructions state “False positive results may occur with this test. In vitro transcripts from low-risk HPV genotypes 26, 67, 70 and 82 exhibited cross-reactivity with the Aptima HPV Assay.”

2.8.5 Accuracy of APTIMA in triage and primary screening

In a meta-analysis including eight studies, the pooled sensitivity and specificity of APTIMA to triage ASC-US was 95.7% and 56.4%, respectively, for CIN2+ and 96.2% and 54.9%, respectively, for CIN3+. APTIMA and HC2 showed similar sensitivity for CIN2+ and CIN3+ lesions. However the specificity of APTIMA was significantly higher (ratio: 1.19 for detection of CIN2+ and ratio: 1.18 detection of CIN3+).
In LSIL triage, when considering CIN2+ as the outcome, pooled sensitivity and specificity were 91.0% and 42.5%, respectively, whereas for CIN3+, the figures were 96.7% and 38.7%, respectively. Here too the APTIMA was as sensitive as HC2, but substantially more specific for both outcomes (ratio: 1.37 and 1.35) (117). A study by Castle et al. seemingly corroborates this statement, though it is not included in the meta-analysis (118). The meta-analysis by Arbyn et al. concluded that Aptima is as sensitive as HC2 for detection of CIN2+ or CIN3+ in women with ASC-US or LSIL. The specificity of Aptima is on average 19% (95% CI= 8-29%) better in triage of ASC-US and 37% (95% CI=22-54%) better in triage of LSIL. They supported the use of Aptima in triage of ASC-US and suggested that the test might also be considered for triage of women with LSIL.

The largest trials supporting the use of ASC-US triage are a Canadian study of 1400 women (119), the PREDICTORS-1 study involving 950 UK women (112) and a French study involving 750 women (120).
In a primary screening setting, the FASE and CLEAR trials also demonstrate similar sensitivity, but higher specificity compared with HC2 (121). These were the studies that supported the FDA approval of Aptima for the indication of primary cervical cancer screening. The CLEAR study (Clinical Evaluation of Aptima mRNA) involved 11 000 US women and the FASE study 5000 French women. One part of the CLEAR study investigated Aptima HPV Assay as an adjunctive method for the triage of women with ASC-US over 21 years (939 women). They showed that Aptima HPV-positive women had a risk of CIN2+ 9 times higher than women with negative results and the risk for CIN3+ was nearly 13 times higher. Aptima sensitivity and specificity was 87 % and 63 % for CIN2+ (122). The overall prevalence of high-risk HPV mRNA was 42 % among women with ASC-US and 5.0% among women with a normal cytology test result over 30 years (123). A recent study compared clinical performance of the APTIMA for CIN2+ with the HR HPV GP5+/GP6+ PCR in a cross-sectional clinical equivalence analysis with > 900, cervical samples from population-based screening. The Aptima HPV assay showed a clinical sensitivity for CIN2+ of 94.2% and a clinical specificity for CIN2+ of 94.5%. These figures were 97.1% and 93.6%, respectively, for the GP5+/GP6+ PCR. The authors comment their findings are in line with those of other studies that compared the clinical performance of Aptima for ASC-US/LSIL or primary screening. In addition, high reproducibility was found for the Aptima HPV assay, as reflected by the intralaboratory reproducibility over time (kappa = 0.89) and interlaboratory agreement (kappa = 0.91) (124). They concluded that the Aptima meets the criteria of the international guidelines for HPV test requirements for cervical screening, but longitudinal data are needed to ensure that the long-term negative predictive value is similar to that of validated HPV DNA tests.

2.8.6 Other HPV mRNA tests

The other two commercially available mRNA tests based on NASBA technology are the PreTect HPV Proofer (NorChip, Klokkarstua, Norway) and the NucliSens EasyQ® HPV V1 test (Biomerieux, Marcy l'Etoile, France). They detect E6/E7 mRNA transcripts of the five most frequently identified HR HPV types in cervical cancer: HPV16, HPV18, HPV31, HPV33 and HPV45. Several studies have shown that they have lower clinical sensitivity for detection of CIN2+ than DNA-based tests, but significantly higher clinical specificity. The lower sensitivity has partly been explained by the detection of only five HR HPVs.

2.8.7 HPV test validation

HPV tests should be clinically validated before use in clinical practice, but only 10-15% of available tests on the market meet this recommendation. Furthermore, manufacturer's instructions for most HPV tests do not specify nucleic acid extraction methodology, which is a crucial step in molecular testing. Professional recommendations on how to evaluate a novel HPV test for safe use in primary cervical cancer screening have been published (125). New HPV assays should have an optimal balance between clinical sensitivity and specificity (111, 125). The new test should

show non-inferiority when compared with HC2 assay. The guidelines also incorporated an assessment of the technical robustness of new assays through the measurement of intra- and inter laboratory reproducibility. Stoler et al. proposed a minimum sensitivity of 92+3% for the detection of CIN3+, and a specificity of 85% to achieve an adequate positive predictive value (PPV) for CIN3+ for any new HPV DNA test (108, 126). These criteria aim to achieve a balance between excessive referral and risk of missing high-grade disease. When designing an HPV test the inclusion of HPV types that are rarely associated with cervical cancer must be carefully weighed against the loss of clinical specificity (e.g. HPV53 and HPV66). The World Health Organization has developed an international proficiency panel for HPV DNA detection and typing to establish international standards for all carcinogenic HPV types, to allow evaluation of analytical performance of HPV tests and compare data between different laboratories. Their manual also provides instructions on best practice and quality control procedures for laboratories performing HPV testing (107, 127).

2.8.8 HPV self-sampling

One advantage of HPV DNA testing is suitability for self-sampling. Self-sampling is likely to improve compliance and is particularly appealing in hard-to-reach populations where social or religious practices limit acceptance of vaginal examinations. A Canadian review found that women who were under/never screened and were offered HPV self-testing were twice as likely to participate in cervical cancer screening, but it was still unclear what HPV self-collection device is best for collecting reliable samples with minimal discomfort for women (128).
In addition, a UK study by Szarewski et al. (129) found that sending a self-sampling kit to women doubled response rate compared with repeat invitation to attend screening. Although the response rate for the self-sampling group was only around 10%, Wikström et al. found a response rate of around 40% to test kits sent by mail (130, 131). A recent meta-analysis of screening data from 36 studies on a total of 155 000 women (132) found that self-sampling for HPV detected, on average, 76% (95% CI 69–82) of CIN2+ and 84% (72–92) of CIN3+. The pooled absolute specificity to exclude CIN2+ was 86% (83–89) and to exclude CIN3+ 87% (84–90). The pooled sensitivity and specificity of HPV testing on self-samples was lower than HPV testing on samples taken by a clinician. The conclusion was that sampling by a clinician is preferable and should be recommended. Lower sensitivity of self-sampling can be explained by lower loads of HPV DNA in the vagina. At present, cytology triage cannot be carried out on self-samples and therefore an adequate molecular reflex test needs to be developed for triage of HPV-positive women. Hyper-methylation of some viral or human genes involved in carcinogenesis has shown promising accuracy profiles and may be applicable to self-sampling, but further validation is needed (133).

2.9 HPV TESTING IN SECONDARY PREVENTION

Detection of high-risk HPV DNA is considered to be useful for three clinical applications: (134, 135).

1. 1 As a triage test to select women with equivocal or mildly abnormal cytology, needing referral for further diagnostic evaluation (colposcopy)

2. As a follow-up test for women treated for high-grade CIN to predict cure or failure of treatment
3. As a primary screening test, to detect or rule out cervical precancer in the population over age 30.

2.9.1 Triage of women with minor cytological abnormalities

The management of women with minor cytological lesions has been subject to discussion (49, 136, 137). Until recently, follow-up recommendations for women with ASC-US or LSIL varied from conservative repeat cytology (138) to immediate referral for colposcopy and biopsy (139). The natural history of minor cytological lesions is difficult to predict on morphological grounds. These lesions often regress spontaneously without treatment (43, 140). Therefore, referring all women with minor cytological lesions for further gynaecological examination would entail increased risk for over-diagnosis and over-treatment, as well as risks for adverse obstetric outcomes following excision of CIN lesions (135). Over-referral would also cause unnecessary anxiety among women (141), substantially increased costs to the healthcare system and a shortage of colposcopic resources. ASC-US and LSIL are the most common cytological abnormalities and in ASC-US, one to two thirds of cases is not associated with HPV. The majority of women do not have clinically significant disease, but a substantial proportion of them do have histopathologically-confirmed high-grade CIN (142). From a population of women screened in the US, an estimated one third of CIN cases were discovered on follow-up of a previous smear showing ASC-US (143).
An appropriate triage method should therefore be able to identify women who have or will develop cervical cancer precursor lesions while at the same time reducing the risk of over-diagnosis (144, 145).Since evidence exists concerning the etiological role of high-risk HPV infections for the development of cervical cancer and its precursors (146) (147) (148), HPV testing has been proposed as a triage method to distinguish between women with minor cytological lesions who need referral for colposcopy, and women who can be referred back to the normal screening schedule (149).

2.9.1.1 ASC-US

A recent Cochrane review of diagnostic test accuracy of HPV testing (HC2) versus repeat cytology for triage of minor cytological cervical lesions (135) corroborates conclusions from previous meta-analyses, which all indicated that HC2 triage of women with ASC-US predicts presence of underlying high-grade CIN with greater accuracy than repeat Pap smear, considering ASC-US+ as cut-off (significantly higher sensitivity, similar specificity)(150). mRNA testing with the APTIMA (Gen-Probe Inc., San Diego, CA, USA) test has similar sensitivity, but higher specificity than HC2, which leads to the conclusion that APTIMA is also useful for triage of ASC-US. The pooled absolute sensitivity of APTIMA to find underlying CIN2+ was 95.7% and the specificity 56.4% in ASC-US triage.

2.9.1.2 LSIL

A productive HPV infection usually manifests as LSIL with low potential for neoplastic transformation (151). Therefore, HPV DNA testing is nearly always positive, which limits its capacity to distinguish between cases with or without severe underlying lesions. The prevalence of LSIL in women with a positive HC2 test, which

was reported in the studies included in the Cochrane review by Arbyn et al., ranged from 55% to 89%. Test positivity rates were always higher than in ASC-US. HPV positivity is clearly age-dependent with a decline after age 30. A study by Moss showed that 89% of women under age 35 with mild dyskaryosis on Pap smears tested positive by HC2, as were 69% of women between the ages of 35 and 49 and 51% of women aged 50 or over (152). The specificity of HC2 for the outcome CIN2+ in the ALTS study was 16% in women under 29 years, and 30% in women 29 years or older (153). However, the Cochrane conclusions concerning triage of LSIL differ from previous reviews, which stated that HC2 triage was associated with no significant gain in sensitivity, but a substantial and statistically significant loss in specificity compared with repeat cytology (154) and therefore could not be recommended. The current Cochrane review, which included more studies, confirmed the lower specificity of HC2 triage of LSIL, but demonstrated a significant gain in sensitivity, which could justify recommending HR HPV DNA testing for triage of LSIL. However, recommendations should be based on local cost-effectiveness analyses, local HPV prevalence, performance of the specific HPV test in use and compliance with follow-up. Swedish guidelines recommend HPV triage of all women with minor cytological abnormalities regardless of ASC-US or LSIL diagnosis. HPV-positive women are referred for colposcopy and HPV-negative women are recommended to undergo repeat cytology testing one year later, just to be certain (39, 155).

However, there is a need for more specific triage tests for women with LSIL (135). Virological triage could be made more specific by increasing viral load cut-off, adding a second triage test, or by excluding young women. A few studies have used a higher test threshold (HC2), resulting in gains in specificity, but simultaneous loss of sensitivity (156). Triage of ASC-US or LSIL with new molecular markers is the focus of ongoing research (E6/E7 transcripts of different HPV types, p16 immunostaining). Analyses have also identified a significant increase in specificity by age in triage of LSIL when the outcome was CIN3+. The increase in specificity reflects the drop in HPV test positivity rate with increasing age. The break-even point for lower costs in HPV triaging policy was found to be at an HPV prevalence of about 70%, corresponding with an age of >35 years for ASC-US and >40 years for CIN1 according to a Swedish randomised health services study by Dillner et al., (155).

Since the APTIMA test was more specific than HC2 without showing loss in sensitivity, the meta-analysis by Arbyn concluded that APTIMA could also be recommended for triage of women with LSIL (117). Pooled sensitivity and specificity for CIN2+ in triage of LSIL were 91.0% and 42.5%, respectively.

Table1. Absolute and relative pooled sensitivity and specificity comparing HC2 with HPV DNA or RNA testing for triage of women with ASC-US or LSIL to find underlying CIN2+, based on a meta-analysis by Arbyn (134).

		Absolute accuracy		Relative accuracy	
Triage group	**Test**	**Absolute sensitivity**	**Absolute specificity**	**Relative sensitivity**	**Relative specificity**
ASC-US	HC2	90	58		
	LA	94	46	1.0	0.9
	PreTect	81	79	0.8	1.8

	APTIMA	96	56	1.0	1.2
	HPV16	54	87	0.6	1.6
	HPV16/18	58	83	0.6	1.7
LSIL	HC2	95	28		
	LA	99	28	1.0	1.0
	PreTect	76	77	0.8	2.8
	APTIMA	91	42	0.96	1.4
	HPV16	51	81	0.6	2.5
	HPV16/18	58	75	0.6	2.3

Table 4. Absolute and relative pooled sensitivity and specificity comparing HC2 with HPV DNA or RNA testing for triage of women with ASC-US or LSIL to find underlying CIN2+, based on a meta-analysis by Arbyn (134).

2.9.2 Follow-up after treatment of high-grade CIN

Neither the section margins nor cytology used for evaluation of treatment efficacy has proven sufficiently accurate for detection of residual or recurrent disease. However, detection of HR HPV DNA has been recommended as a follow-up test for women treated for high-grade CIN in order to predict cure or failure of treatment (154). The short-term rate of residual or recurrent high-grade CIN, evaluated over a 2-year period, is estimated at an average of 8% (4-18%). The risk of recurrent CIN2+ is higher in women older than 50 years, consistent with the observation that viral persistence increases with age (157). A study by Castle et al. (158) indicated that women treated for CIN are at increased risk for cervical cancer, compared with the general population, for at least 10 years and perhaps up to 20 years after treatment. Currently available data suggest that HPV testing picks up residual disease quicker and with higher sensitivity and similar specificity compared with follow-up cytology or histological assessment of section margins (134, 159). Pooled sensitivity was 93% for HPV DNA and 72% for cytology. Pooled specificities for HPV DNA and cytology were 81% and 84%, respectively. HPV testing was significantly more sensitive (ratio of 1.25), but not less specific (ratio of 0.97%). Combined testing with cytology and HPV was not significantly more sensitive, but was significantly less specific than HPV DNA testing alone.

Kocken et al. reported on long-term follow-up risk of recurrent high-grade CIN after treatment (160). If the woman was HR HPV negative at 6 months after treatment, the 10-year risk of CIN3+ was 2.1%. If the woman cytology-negative, the risk was 2.8% and if both tests were negative, the risk was 1.4%. Three consecutive negative cytology results at 6, 12, and 24 months, or negative findings in both HPV DNA and cytology at 6 and 12 months were associated with low risk of residual CIN3+, 0.7% and 0.0%, respectively, which is comparable to the risk within the general population. On the other hand, a positive HR HPV test at 6 months increased risk of CIN3+ to 29%, a positive cytology test (ASC-US+) to 13%, averaging out to 23% when one of these two tests was positive. In a recent Swedish study of recurrence after LEEP conisation, no women without type-specific persistence of HPV had recurrent or residual disease. HPV genotyping appeared to be useful for improving specificity when HPV testing was used for in post-treatment follow-up (161). A population-based cohort study of long-

term incidence and mortality found that women previously treated for CIN3 are at increased risk of developing and dying from cervical or vaginal cancer and that risk accelerates above age 60, suggesting a need for lifelong surveillance of these women (162). More research is needed to identify biomarkers that accurately predict a woman's long-term risk of future cancer.

2.9.3 Primary screening for cervical cancer

A meta-analysis by Arbyn et al. (134) confirmed that HR HPV testing is substantially more sensitive than cytology for identifying underlying CIN2+ and CIN3+. One drawback is the lower specificity, especially among younger women where HPV infection is usually transient. In both European and North American cross-sectional studies, the pooled sensitivity of HC2 for CIN2+ was 96%, whereas the pooled specificity was 91%. HC2 sensitivity was on average 23-43% higher than cytology and specificity for excluding CIN2+ was significantly lower, ratio 0.97. Four large European randomised controlled trials (RCT) of primary screening consistently demonstrated second screening round data that showed a significantly lower cumulative incidence of CIN3+ and cervical cancer in women over age 30, who were HR HPV-negative versus cytology-negative at enrolment (163-166). Moreover, cohort studies presented data showing a low cumulative incidence of CIN3+ associated with a negative HR HPV test (134). For HPV-negative women without or with cytological abnormalities the risk was 0.2% and 1.2%, respectively. For women who were cytology-negative without or with HR HPV test, the risk was 0.2% and 6.1%, respectively. The baseline HPV-negative group had essentially the same rates of dysplasia as the patients who were both HPV-negative and cytology-negative at baseline (0.2% cumulative risk to develop CIN3+ over the next 5 years), suggesting that screening with HR HPV testing alone without cytology offers similar protection. Screening intervals for HPV-negative women can safely be extended to at least 5 years. However, in women who were HR HPV-positive, the risk at the second screening round was substantially higher: 6% and 17%, respectively, for women without or with ASC-US or higher-grade cytology at baseline. Cytology testing of HPV-positive cases clearly stratified the risk for CIN3+, indicating that cytology may be a useful method for effective triage in HPV-based screening (167-169).
Other evaluated triage methods include genotyping for HPV16 and HPV16/18, as well as HPV retesting to detect persistent infections. Kahn et al. reported the 10-year cumulative risk associated with HPV16, HPV18 or other HR HPV infections to be 17%, 14% and 3%, respectively (170). The ATHENA trial demonstrated similar sensitivity and PPV for detection of CIN3+ among HR HPV-positive women using either the Cobas 4800 test (allows detection of HPV16/18 while testing for 12 other high-risk HPV types) or cytology (171). Additional markers under consideration for triage of HPV-positive cases are testing for HPV mRNA, p16INK4a and p16INK4aKi67 double staining. However, HR HPV-positive women who are cytology-negative or HPV16/18-negative must be kept under surveillance and invited for repeat testing (134). The cumulative incidence rate of CIN3+ among women who were positive for HR HPV with negative cytology results increased continuously over time, reaching 10% at 6 years, whereas the rate among women with positive cytology results who were negative for HPV remained below 3%(172). A recent follow-up study of the four European randomised controlled trials investigated the efficacy of HPV-based

screening and found that HPV-based screening provides 60-70% greater protection against ICC than cytology (173).
One reason that HR HPV-based cervical cancer screening has not been recommended earlier is that despite the higher cross-sectional sensitivity of HR HPV testing for detecting CIN2+ and CIN3+, it could not be excluded that HPV testing simply picks up more regressive disease. However, a 13-year follow-up of the Swedescreen RCT of primary screening involving 12 500 women aged 32-38 concluded that the cumulative incidence of CIN2+ was the same for HPV screening and for cytology, but that HPV screening detects CIN2+ earlier rather than over-diagnosing this condition (174).
In summary, the evidence base at present indicates that HR HPV DNA testing is more effective for primary screening of women aged 30 or older than cytology and that screening intervals for HPV-negative women can safely be extended to at least 5 years. The possible advantages offered by HPV-based screening also require a well-organised programme with good compliance to screening and triage policies.
Four HR HPV DNA tests are currently considered clinically validated for the purpose of primary screening according the equivalence criteria of Meijer CJLM et al. (125). These are HC2, GP5+/6+, Cobas 4800 and the Abbott Real Time PCR HR HPV tests. The author states that in addition to accuracy, other characteristics must be considered such as high-throughput capacity, costs, applicability for self-sampling and the option of performing ancillary triage tests.

2.10 TREATMENT OF CERVICAL DISEASE

Most guidelines recommend treatment when CIN2+ is detected. Women with CIN1 are usually followed-up and treated depending on persistence and age. Excisional treatment is mandatory for women with inadequate colposcopy, suspicion of invasive disease or glandular abnormality. The goal of treatment is to eliminate high-grade cervical lesions and the cervical TZ, while minimising harm to the cervix. Low morbidity excisional methods are preferred to generate specimens for histopathology. Common procedures include loop electrosurgical excision procedure (LEEP), cold knife cone biopsy, electrofulgaration, laser conisation, laser ablation and cryotherapy. Large loop excision of the transformation zone is often abbreviated to LLETZ in the UK or LEEP (loop electrosurgical excisional procedure) in the US. Treatment success is reported to be 91-98% in non-randomised studies. Cold knife cone biopsy is no longer often used. Loop excision is easier and faster to learn than conisation with laser, but one advantage of laser therapy is the ability to customise the size of the cone. A Cochrane review from 2012 found no obvious superior technique for treating CIN in terms of failures or operative morbidity. No differences were found between laser conisation and loop excision regarding risk of residual disease, preoperative severe pain, significant thermal artefact, vaginal discharge or cervical stenosis at follow-up. Laser conisation was associated with significantly longer operating time and increased risk of perioperative severe bleeding compared with loop excision, but no difference in risk of secondary haemorrhage. Excisional treatments are estimated to be 90-95% effective (175).

2.10.1 Adverse pregnancy outcomes

A meta-analysis from 2008 concluded that all excisional procedures to treat CIN seemed to be associated with adverse obstetric morbidity, but only cold knife

conisation was associated with a significantly increased rate of severe outcomes (perinatal mortality RR 2.9 95% CI 1.4-5.8) (144). Concerning LLETZ, the possibility of increased risk could not be excluded. Removal of large amounts of cervical tissue is a risk factor for adverse obstetric outcomes, but most loop excisions in young women with fully visible TZ only need to be 1 cm deep, which should help protect against serious dysfunction. Moreover, women with CIN are known to have demographic, behavioural, and sexual characteristics that increase their risk of adverse obstetric outcomes, which may blur the results of the studies (144). An observational study from Dublin revealed that greater thickness and total volume of the excised transformation zone were associated with increased risk of preterm labour (<37 weeks of gestation) (176). A recent large meta-analysis from China including 37 000 cases also concluded that LEEP is associated with an increased risk of subsequent preterm delivery (<32/34, <28 weeks) and other adverse pregnancy outcomes. (177).

2.10.2 Development of novel treatments

The immune system plays an important role in controlling development of HPV-associated cancer. Two E6/E7 vaccines have shown some clinical promise in high-grade VIN patients. Clinical response correlated with development of HPV-specific T-cell response and local immune factors. Treatments that can shift the balance of immune effectors locally may be effective (imiquimod) by themselves or may be combined with new drugs targeting molecular pathways mediated by HPV in cancer (8). Small molecule inhibitors targeting the DNA-binding activities of HPV E1/E2 or the anti-apoptotic consequences of E6/E7 oncogenes are in preclinical development. Proteosome and histone deacetylase inhibitors, which can enhance apoptosis in HPV-positive tumour cells, are also being tested in early clinical trials. Combinations with vaccination are also now being tested (8).

2.11 NEW BIOMARKERS FOR CERVICAL CANCER SCREENING

Because of superior sensitivity and reproducibility, HPV DNA testing for detection of high-grade CIN is under consideration for use in primary screening for cervical cancer. The main objection has been that HPV DNA testing cannot separate transient from persistent infections and therefore it could not be excluded that HPV testing may just picks up more regressive disease. New approaches with improved specificity are needed to triage HPV-positive cases. New technologies include a) genotyping for specific HR HPV types, b) p16INK4a staining to identify proliferating cells and c) methylation of host and viral genes. Repeated HPV genotyping may also be an option.

2.11.1 Genotyping for HPV16 or HPV18

Several studies have confirmed the higher absolute risk of CIN3+ among HPV16- and HPV18-positive women and these observations have led to the development of newer commercially available diagnostic tests. In the ATHENA trial (Addressing the Need for Advanced HPV Diagnostics) over 40 000 HPV positive women with ASC-US or worse cytology were evaluated using HPV16/18 genotyping for triage. Strategies that referred only HPV16- or HPV18-positive women had a sensitivity for detection of CIN3+ similar to other strategies which referred only women with ASC-US or worse cytology

(50.4%, 59.5%, and 52.8%, respectively), suggesting that triage of HR HPV-positive screening tests is equally feasible by cytology or by HPV16/18 genotyping (171). Another population-based screening study in the Netherlands evaluated similar triage strategies and concluded that triage of HR HPV-positive women with cytology was preferable based on relative balance of benefits (high negative predictive value) and harms (modest colposcopy referral rate)(167). Studies in both Denmark (77) and Sweden (178) have reported that the absolute risks for progression to high-grade cervical disease in women with HPV31 and HPV33 are similar to those for HPV16/18-positive women.

2.11.2 P16^{INK4a}

Over expression of the cyclin-dependent kinase inhibitor p16^{INK4a} is a surrogate marker for transforming activity of the E7 viral protein that is essential for initiation and maintenance of the neoplastic process. High-level expression of HPV E7 protein can up-regulate p16, and increasing levels of p16 correlate with increasing grades of CIN. Several studies have evaluated the clinical utility of p16^{INK4a} immunochemical staining as a diagnostic adjunct both in cervical histology and cytology specimens. It has been shown that diagnostic accuracy, reliability and quality in histopathology of cervical lesions can be improved. Studies evaluating potential use of p16^{INK4a} as a progression marker for low-grade CIN have consistently revealed that the majority of p16^{INK4a}-negative lesions regressed, whereas most p16^{INK4a}-positive CIN1 lesions progressed to high-grade CIN (179). A meta-analysis of eight studies in which both p16 and HC2 were performed for triage of low-grade disease concluded that p16 had a similar sensitivity compared with HC2 for detection of CIN2+ in ASC-US, but a lower sensitivity and higher specificity in triage of LSIL (180). However, p16^{INK4a} single-staining immunocytochemistry requires a morphologic interpretation of immunoreactive cells and it can be very difficult to distinguish a p16^{INK4a}-positive cell showing an intraepithelial lesion from a cervical cell that occasionally over expresses p16^{INK4a} to arrest its cell cycle. This is seen in squamous metaplastic cells or rare endocervical cells. Therefore a combination of antibodies detecting p16^{INK4a} and Ki67, a marker of cell proliferation, has been thought to identify true HPV transformation of cervical cells. P16^{INK4a}/Ki-67 in dual staining has been evaluated in triage of ASC-US and LSIL and provides high sensitivity for detection of CIN2+ as well as improved specificity compared with single morphology interpretations (179). Triage studies of HPV-positive cases with p16 or cytology have shown similar results, but dual staining increased sensitivity while maintaining high specificity. Promising techniques for the future may involve a combination of dual staining and computer-assisted image analysis.

2.11.3 Markers for aberrant S-phase induction

Topoisomerase 2 alpha (TOP2A) and minichromosone maintenance protein 2 (MCM2) are targets in an assay provided by Becton Dickinson (ProExCTM), which includes a cocktail of antibodies against these proteins. The MCM2 reflects an active cell proliferation state, but differentiation between benign or HPV-induced neoplasia is hardly possible. Top2A is a nuclear enzyme, which regulates DNA topology during chromosome replication. Deregulated production of both proteins has been associated

with disease and disease progression, but the major drawback is that normal proliferating cells also express MCM2 and Top2A to some extent, which limits its use (179).

2.11.4 Biomarkers for productive HPV infections: E4 and L1

Viral proteins that are expressed during the late phase of the replication cycle, such as the L1 capsid protein and the E4 protein, are only expressed in terminally differentiated squamous epithelial cells. They are capable of replicating the HPV particles. Therefore these late gene products have been suggested as markers for CIN1 in histology or LSIL in cytology samples. The inclusion of a viral protein marker can help to discriminate between viral and non-viral CIN1 and to subdivide lesions that fall into the ambiguous CIN2 category. Further work is required to evaluate the utility of disease staging in histology. However, markers that detect productive HPV infection are of limited value as screening tools since a positive test result will not exclude the presence of HSIL. HPV L1 is a major target of the immune response in HPV-infected squamous intraepithelial lesions and is only produced during productive HPV infection at the end of the natural viral life cycle (181). Studies have reported that the majority of HR-HPV-related LSIL expresses HPV L1, whereas HR-HPV-related HSIL fails to synthesise L1 (182). Therefore, loss of L1 expression could be used as a prognostic marker for development of preinvasive lesions. A 2013 study by Norman et al. showed that HPV L1 capsid protein detection can predict the clinical outcome of early dysplastic lesions, allowing differentiation between transient HPV infections and risk of progression to cancer, and can be used as an objective standard to optimise clinical management of squamous intraepithelial lesions (183).

2.11.5 Methylation of host cell genes and viral genes

Epigenetic changes are heritable changes in gene activity that are not caused by changes in the DNA sequence. DNA methylation (DNAme) and histone modification are two examples that alter how genes are expressed. Methylation is the transfer of methyl groups (CH3) to G–C-rich areas of a genome through the action of dimethyl transferases. Methylation affects gene regulation either through blocking of transcription factor access to promoter and enhancer regions (an enhancer is a short region of DNA that can bind proteins which enhance transcription of genes), or by altering conformation of nucleosomes (a nucleosome is the basic unit of DNA packing and consists of a segment of DNA wound in sequence around a histone protein core) favourable or unfavourable to transcription. This could lead to chromosomal instability, cell immortality and malignant transformation (108). The methylation status of both host and viral targets seems to be a promising biomarker. Mounting evidence suggests that methylation of CADM1 (host) and L1 and L2 genes (virus) is associated with a worse prognosis, but more studies are needed. Aberrant methylation can be detected in cervical smears up to seven years prior to diagnose of ICC and analysis is applicable to self-collected lavage samples.

2.11.6 E6 and other protein markers

Detecting overexpression of proteins E6 or E7 in the cervical epithelium allows differentiation between transient HPV infections and pre-cancer or cancerous changes. Currently two HPV-E6 tests are undergoing validation. Another E6 test has been developed by OncoHealth Corporation (San Jose, CA, USA) and is based on the use of antibodies against HPV E6-E7 proteins. It can be run using samples collected for liquid-based cytology and the preliminary results are promising.

2.12 HPV VACCINES

Virus-like particles (VLPs) are empty non-infectious viral shells consisting of recombinant L1 capsid proteins without containing any viral DNA. Systemic immunization with VLPs is able to evoke an immune response and offers protection via antibodies to L1, but precisely what epitopes are recognized remains unknown. The generated antibody concentration is 50 times higher than following natural infection, and then wanes to levels many times lower than peak concentration, where they remain stable for several years. The protective effect is likely made possible by high antigen concentrations and the delivery routes that grant access to lymph nodes and the spleen. IgG1 responses predominate, but high levels of IgA, IgG3 and IgG4 are also detected. The two available vaccines have demonstrated seroconversion in virtually 100% of vaccinees. No protective level of antibody concentration against infection has yet been defined and therefore the minimum protective antibody titre remains unknown. Affinity and avidity are likely to be important factors, but very low antibody titres may still be protective. L2 protein vaccines are poorly immunogenic compared with the L1 VLPs, but concatenated multitype L2 proteins can boost immunogenicity and show cross-protection against HPV 16, 18, 45 and 58 pseudovirions (8).
Antibodies against L1 appear 6–8 months following natural infection in about 50–70% of infected individuals. Other viral proteins like E1, E2 and E6 do not elicit antibody responses. Initially, the IgM response occurs, then decays and is followed by the IgG1 response, which is more persistent. The protective role of antibodies following a natural infection is still unclear (8).

2.12.1 Therapeutic and prophylactic vaccines

Lesion regression is often associated with cell-mediated immune responses to E2 and E6. When CD8+ T cell gain access to dysplastic lesions, they are likely to regress. Two clinical trials tested HPV therapeutic vaccines in patients with vulvar epithelial hyperplasia who showed complete responses in approximately 50% of cases over a 52-week follow-up period. The expected rate among unvaccinated women was 5%. However, more studies are needed (8).
After a long period of development, two licensed prophylactic HPV vaccines are now available on the market. Gardasil®, (Merck & Co., Whitehouse Station, NJ, USA) which is a quadrivalent vaccine containing VLPs of types 6, 11, 16 and 18, and Cervarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium), a bivalent vaccine containing VLPs of types 16 and 18. Two phase III studies, FUTURE I and FUTURE II, evaluated Gardasil® and two studies, PATRICIA and the Costa Rica HPV Vaccine Trial (CVT), evaluated Cervarix®. All four trials studied young women (mean age 20,

range 15-26) and were large (5 500-18 500 vaccinees) blinded, randomized controlled trials (RCT). Both vaccines demonstrated high and similar efficacy against HPV-related genital disease in women naïve to the corresponding HPV types. However, the vaccines had no therapeutic effect on established infections or on CIN. Both vaccines demonstrated cross-protection for infections and lesions related to non-vaccine types: HPV 31 for Gardasil (70%) and HPV31, HPV33, HPV 52, HPV45, and HPV51 for Cervarix. Gardasil has also been shown to protect mid-adult women (age 24-45) from incident infections and CIN caused by the vaccine types and to protect men (age 16-26) from incident infection, genital warts and AIN (anal intraepithelial neoplasia). One concern – removing HPV16 and HPV18 might leave an empty ecological niche for other HPV types to fill in, but so far there have been no such indications. Both vaccines demonstrated excellent safety in these clinical trials. Mild to moderate injection-site symptoms, headache and fatigue were the most common adverse events. Symptoms were transient and did not increase with the number of doses. Safety evaluations after vaccine introduction have also shown good results. The vaccines were introduced in 2006 and in recent years they have become more available due to reduced market prices. Australia was one of the first countries to reach high coverage with Gardasil; evaluations have shown great impact on genital warts in the vaccinated age groups, as well as decreased rates among men, suggesting herd immunity (8). In Sweden, HPV vaccination of fifth-grade girls has been included in the national vaccination programme since 2010. In Stockholm County, free catch-up vaccination is offered to girls up to age 18 and subsidized up to age 26. Vaccinated women are informed of the importance of continued participation in the national cervical cancer-screening programme since current vaccines do not offer 100% protection against ICC. The included HPV types are responsible for only 70% of ICC cases. A new nine-valent vaccine targeting additional HPV types (31, 33, 45, 52, and 58) has been developed and is expected to prevent almost 90% of ICC (184). Phase III trials are currently underway. A recent Swedish study demonstrated a considerable reduction in the risk of acquiring condyloma after only 2 doses of vaccine, a finding that requires further investigation since this may reduce costs and increase vaccination programme feasibility (185).

2.12.2 Cost-effectiveness of including males

Including males in the vaccination programme would have a direct benefit on protection against HPV-related cancer and genital warts in men. Indirect benefits would include protection of non-vaccinated females and males through increased herd immunity (a form of immunity that occurs when the vaccination of a significant portion of a population or herd provides protection for individuals who have not developed immunity) and some protection for men who have sex with men. However, any cost-effectiveness analysis should take into account that the HPV-related burden of disease in males is lower than in females and that males benefit from female vaccination via herd immunity. If vaccine coverage in females is less than about 50% it can be cost-effective to include males in the HPV vaccination programme but increase of female coverage is probably more effective (8). Intense debate concerning male vaccination is currently underway. The Australian government approved male vaccination (Gardasil) in 2012 and commenced in 2013. Results from a mathematical model of HPV

transmission concluded that this would lead to the near elimination of genital warts in both female and male heterosexual populations (186).

2.13 HPV, HIV AND IMMUOSUPPRESSION

The majority of women infected with HIV will be co-infected with HPV. Studies indicate that the rate of cervical cancer among HIV-positive women is 2 to 22 times greater than in HIV-negative women. It has also been reported that immunocompromised individuals are resistant to treatment of HPV-related diseases and prone to accelerated development of HPV-associated cancers. HIV-positive women are also more likely to have multiple HPV infections. Possible explanations include alteration in cell-mediated immunity, increased susceptibility and possible reactivation of latent HPV infections. Prevention of HPV-associated diseases in HIV-positive individuals includes HPV vaccination, although the efficacy of HPV vaccination in HIV-positive children requires further research (8).

2.14 TRANSLATIONAL RESEARCH AND BIOBANKING

Successful cancer research depends on the availability of biological specimens derived from individuals who have been followed-up for several years and who finally developed the outcome of interest. This can be achieved by using biobanks that are linked to population-based cancer registries. These registries generate high-quality, standardised and complete data, but the main advantage is the opportunity to obtain extremely long follow-up data on health outcomes without having to wait. A variety of information such as living conditions, lifestyle and risk factors can be accessed, and different kinds of bio-specimens from both patients and volunteers can be stored after collection. “Translational research” is a crucial part of prevention research and includes searching for new biomarkers for early diagnosis or for use in new screening tests. The Swedish biobank, including Pap smears from the screening programme, has served as a useful resource for research concerning HPV-based cervical screening (187). In the past, each research study or laboratory had to develop its own biospecimen storage system, which was a major endeavour. In many cases the specimens were of limited use due to inappropriate handling or storage. Naturally, such factors could hamper comparability between different studies, making them less effective. Given the increasing complexity of biobanks, nowadays researchers use specialised services to handle acquisition, quality control, storage, processing and distribution of biospecimens for laboratories. International biobanking networks have been developed to foster common international standards. Examples of such major biobanking networks are the Biobanking & Molecular Resource Infrastructure (BBMRI) and the Public Population Projects in Genomics (P3G). The SCCB was first established in 2012 as a pilot project of the clinical cytology centre at Karolinska Hospital in Stockholm. The Swedish BBMRI supported the financing, development and implementation of this first national prospective repository for LBC obtained from women participating in organised cervical cancer screening. Standardised methods for handling and long-term storage were evaluated and developed and to date at least seven other counties across Sweden have become involved. Information from the biobank will be linked to the national cancer registry and will become an invaluable resource for future research. An aspiration volume of 4 ml was chosen to obtain the largest amount of cells from the

LBC sample, but the final storage volume is only a 300-ml aliquot of cells. The samples will be stored in “96-well format vials” and systematically labelled. Evaluation of storage conditions demonstrated that cells remain intact in fluid at temperatures ranging from + 4°C to - 35°C, but were frozen at - 80°C. Therefore, - 25°C was found to be the optimal temperature for long-term storage, since all cells remained intact in the fluid medium. This is an advantage since it allows new slides to be made for cytological diagnosis, immunohistochemistry, and in situ-hybridizations. At the same time, the low temperature used for storage is advantageous for preserving high-quality DNA and RNA for long-term use. Patient information is provided in the screening invitation letters. The Open Access principle prevails, which does not discriminate against or favour any requesting organisation and which will make samples available for health-related research (188).

A randomised health services study (RHS) entails a randomised design with observations in routine health care, regardless of whether randomisation is at the individual, population or process level (189). Unlike the randomised controlled trial (RCT), which is recognised as the most valid study design, implementations of new methods and policies in routine health care have not been defined by formal design, which has impaired the ability to evaluate and improve health care. Therefore the RHS design should be considered when incorporating new routines into clinical practice in order to generate valid, large-scale data on effectiveness and safety of medical practices in routine health services (155).

3 AIMS

3.1 GENERAL AIM

The general aim of this thesis is to evaluate new diagnostic methods and to identify long-term risk factors for development of cervical cancer among women either with minor cytological abnormalities or who have been treated for cervical high-grade disease.

3.2 SPECIFIC AIMS

3.2.1 Paper I

The aim of this study was to compare the sensitivity of HR HPV DNA testing to detect high-grade cervical disease with conventional Pap smear among women with minor cytological abnormalities.

3.2.2 Paper II

To assess the utility of HR HPV mRNA testing after treatment of high-grade cervical intraepithelial neoplasia and to compare the prognostic value of HPV mRNA with HR HPV DNA and cytology.

3.2.3 Paper III

To assess the usefulness of HR HPV mRNA to triage HR HPV DNA-positive women with minor cytological abnormalities and to compare this method with testing for HPV 16 DNA, HPV16/18 DNA and repeat cytology.

3.2.4 Paper IV

To assess long-term risk of high-grade cervical disease among women with minor cytological abnormalities as stratified by HPV-status, cytology and age, to serve as a guide for disease management.

4 MATERIALS AND METHODS

4.1 STUDY SUBJECTS AND STUDY DESIGN

4.1.1 Paper I

Women with minor cytological abnormalities (ASC-US and LSIL) detected through population-based screening were referred for colposcopy 4-6 months later to the department of gynaecology at three different university hospitals in Stockholm. We consecutively enrolled 177 of these women. All women underwent pelvic examination, repeat cytology (Pap smear), HPV DNA testing and colposcopy either with directed biopsies from acetowhite areas, or with biopsy near the squamocolumnar junction at 12 o'clock if no lesions were observed. We compared the results from HPV DNA tests and Pap smears with the histopathological diagnoses. Diagnosis was based on the most severe grade of atypia found in each biopsy. A local pathologist evaluated the samples and classified them according to the cervical intraepithelial neoplasia (CIN) classification (World Health Organization).

4.1.2 Paper II

The present study considered women who were treated for CIN by conisation between September 1999 and June 2009 and all of the 149 women who returned for at least one follow-up visit were invited to participate. In all, 143 women were treated by loop electrosurgical procedure (LEEP) using a C-LETZ electrode (Utah Medical Products Inc., Midvale, UT, USA) (190). Six of the study women did not undergo LEEP; instead, 2 were treated by cryotherapy and 4 by cold knife or laser conisation. Table 1 presents the histopathological diagnoses and margin status of the cone specimens. Since no information on histopathology or margin status was available for the two women (1%) treated with cryotherapy, the pre-treatment histopathological diagnoses (CIN1 and CIN2) were used. Six (4%) cone specimens contained no CIN and therefore they were excluded from further analysis, leaving 143 women (96%) eligible for the study.

Table I. Conisation results for 149 invited women and for 143 included women.

Characteristics	**N (%)**
Histology results in cone specimens of invited women (n=149)	
CIN3+/AIS	78 (52.3)
CIN2	32 (21.5)
CIN1	33 (22.1)
No CIN[b]	6 (4.0)
Margin status of women included in	

final analyses (n=143)	
Free margins	89 (62.2)
Positive margins	52 (36.4)
No information about margins[c]	2 (1.4)

CIN, cervical intraepithelial neoplasia; AIS, adenocarcinoma in situ; [b] Excluded from analyses; [c] treated by cryotherapy.

Inclusion follow-up and subsequent visits.
The inclusion visit consisted of a complete work-up, including pelvic exam, cytology testing, HPV DNA, and HPV E6/E7 mRNA testing. When indicated, colposcopy-directed biopsies were also taken. Women were divided into two groups based on when post-surgical HPV DNA and mRNA analysis were performed: 'early' (<12 months) and 'late' groups (>12 after conisation). All 143 women came for at least one additional follow-up visit after the initial inclusion visit (subsequent/final visit), during which Pap smear and, when clinically indicated, colposcopy-directed biopsies were performed. Cytological results from these subsequent/final visits were available for 137 (96% of 143) women and histological results for 30 (21% of 143). Average follow-up time was 1333 days (3.6 years), median 1182 days (3.2 years), with a range of 71-5622 days (2.4 months -15.4 years).

4.1.3 Paper III

This study included LBC samples from 219 high-risk HPV (HR HPV)-positive women with minor cytological abnormalities detected within the population-based screening programme. Of these, 190 women had low-grade squamous intraepithelial lesions (LSIL) (87%) and 29 had atypical squamous cells of undetermined significance (ASC-US) (13%) (34, 191, 192).
Briefly, women with ASC-US or LSIL were referred for further investigation, including colposcopy, directed biopsies, and/or repeat cytology according to screening programme guidelines. Histological samples were evaluated and classified as within normal limits, CIN1, CIN2+ or CIN3+ based on the most severe lesion present (47). (In the absence of histological results, the most severe cytological result was used.) Cytological results were classified according to the CIN classification of the Swedish Society for Clinical Cytology (47)[, but were re-classified using the Bethesda system for the purposes of this study, although excluding koilocytosis without nuclear atypia from the LSIL diagnosis (37). Women were followed for 4 years after the ASC-US/LSIL index cytology, during which time all histological and cytological results were obtained through medical and laboratory records, as well as from the Stockholm Oncology Centre in cases where information was insufficient. All women were offered annual follow-up and were treated by conisation if high-grade disease was diagnosed or if low-grade disease persisted for more than two years. HR HPV mRNA testing was performed on residual material from the LBC samples in April 2010. Mean age of study participants was 32.0 (range: 23-60 years (standard deviation (SD) 8.5 years). Half the women were aged 30 or younger and there was no statistically significant difference in age between the ASC-US and LSIL groups (p=0.60). Mean age in the ASC-US and LSIL groups was 32.8 years (SD 9.0) and 31.9 years (SD 8.4), respectively. The age distribution of study participants was as follows: 25.1% were 23-

24, 23.7% were 25-29, 15.1% were 30-34, 16.0% were 35-39, 10.5% were 40-44, and 9.6% were 45-60 years of age.

4.1.4 Paper IV

A total of 326 women were identified with minor cytological abnormalities detected through the population-based cervical screening programme in Stockholm, Sweden, between September 2005 and December 2008. To be included in this longitudinal analysis and contribute to follow-up time, women had to have at least one follow-up test (cytology or histology) taken after study-entry. One woman who only had index cytology without follow-up testing was excluded. Furthermore, 11 women who were diagnosed with CIN2+ lesions on the same day as the ASC-US/LSIL index cytology were also excluded, yielding a final study population of 314 women. Of the remaining 314 women with minor cytological abnormalities, 76 (24.2%) had a cytological diagnosis of ASC-US and 238 (75.8%) had LSIL. Baseline characteristics (age, HPV status and cytological diagnosis) were cross-checked against the National Quality Register for Cervical Cancer Prevention (NKCx). The women were linked to the population-based NKCx using personal identification numbers in order to identify individuals who develop histologically confirmed CIN2+ during follow-up through December 2012. We estimated the cumulative incidence proportion of CIN2+ by baseline characteristics (age, cytological diagnosis and HPV status) using one minus the Kaplan-Meier curves. Univariate and multivariate Cox regression models were used to show the association between index visit data and outcome (CIN2+ in histopathology). We also assessed the association between index data and outcome of CIN2+. Women were followed according to accepted clinical practice guidelines, which stipulate that women with minor cytological abnormalities be referred for repeat cytology (Pap smear) and colposcopy with directed biopsies if indicated. HPV detection and genotyping were carried out as reflex testing using the liquid based cytology (LBC) samples. Women were treated when investigation revealed cervical intraepithelial neoplasia grade 2 or worse (CIN2+) or for persistence of minor lesions.

4.2 CYTOLOGICAL SAMPLES

Sweden uses the old US cytological nomenclature (193) in which CINI is a cytological diagnosis as well as a histopathological diagnosis (155). For the purposes of these studies the cytological results were translated into the Bethesda nomenclature system (37). The Bethesda classification was modified according to Swedish recommendations that define samples with koilocytosis without nuclear atypia as non-pathologic findings. However, this is a rare event. Therefore, LSIL only includes cases of mild dysplasia (CIN1). Local cytotechnologists and/or pathologists read the samples.

Papers I, II, III and IV considered tests incorporating conventional cytology, where cells from the ecto- and endocervix were collected using an Ayres spatula and cervical brush, smeared onto a glass slide, immediately fixed in 95% ethanol and air-dried for cytological examination (22). The slides were stained according to the Papanicolaou method (Papanicolau GN, 1941) and read by local cytotechnologists and/or pathologists. In addition, the cervical material on the endocervical brush was used for HPV DNA detection and genotyping for the purposes of Papers I and II.

Papers III and IV studied LBC samples. Cells were collected from the ecto and endocervix just as for conventional smear, but using a plastic spatula. The cells were rinsed into a vial containing PreservCyt solution (ThinPrep®, Hologic, Marlborough, Ma, USA), which is used to prepare slides for cytological analysis. Local cytotechnologists and/or pathologists read the samples.

4.3 HPV TESTING

Hybrid Capture 2 (HC2, Qiagen, previously DiGene Inc, Gaithersburg, MD) was approved by the US Food and Drug administration (FDA) in 2003. It is the most commonly used HPV DNA test worldwide and is associated with the largest evidence base for HPV DNA applications (108). The Linear Array HPV genotyping Test (LA) is one of the most commonly used genotyping assays and has frequently been used for discordant analyses in research settings (111).
The only HPV mRNA assay currently approved by the FDA for clinical use is the Aptima HPV Assay (formerly GenProbe Inc., San Diego, CA, USA, which has now merged with Hologic), used for detection of E6 and E7 mRNA from 14 high-risk HPV types.

Hybrid Capture 2 (Paper I)
Cell samples were obtained from the ectocervix and endocervix with a cervical brush, spread onto a slide for Papanicolaou staining, after which the brush was inserted into a transport medium provided by the manufacturer of HC2. The specimens were tested according to the manufacturer's protocol at the Department of Virology, Karolinska University Hospital, Stockholm, Sweden. DNA from cervical material was denatured and hybridized with a cocktail of 13 RNA probes to oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Hybrids were captured with alkaline phosphatase conjugated antibodies specific to HPV DNA-RNA hybrids. A dioxetane-based chemiluminescent substrate was added, and the resultant relative light units (RLUs) were measured in a luminometer. Specimens with RLUs equal to or above the mean RLUs of triplicate positive control specimens containing 1 pg of HPV DNA/µl (about 5000 copies of the HPV genome) were designated as positive.

Linear Array (Paper II, III, IV)
In Paper II, cells from the cervix were suspended in PreservCyt fixative. A 1 ml suspension was centrifuged and the cell pellet lysed according to instructions supplied in the Total Nucleic Acid Isolation kit (Roche, Basel Switzerland). In Papers III and IV, 2 ml of the cell suspension remaining from the LBC samples were similarly centrifuged, lysed and used for HPV DNA detection and genotyping. DNA was extracted using the MagNA Pure LC Robot and analysed with the Linear Array HPV Detection and Genotyping Test (LA) according to manufacturer's instructions (Roche, Basel, Switzerland). The LA test combines PCR amplification and reverse line-blot hybridization for identification of 37 HPV genotypes. It is based on co-amplification of a 450 bp region of the HPV L1 gene and a 268 bp region of the human b-globin gene, using biotinylated primer sets PGM09/PGMY11.The resulting amplicons hybridize with matching type-specific DNA probes coating a nylon strip and are detected by colorimetric determination. The result is a pattern of blue lines that is visually read by comparing patterns with a reference guide. The HPV types were categorized into 12

HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), 6 probable high-risk (pHR) HPV types (26, 53, 66, 68, 73, 82) and 19 low risk or undetermined risk (LR) HPV types (6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39, CP6108) according to Munoz et al. (194).

HPVmRNA testing by APTIMA HPV Assay (Paper II,III,IV)
In Papers II and III, LBC samples previously used for HPV DNA analysis were retrieved from the archives and used for HPV E6/E7 mRNA analysis using the Aptima HPV Assay (formerly GenProbe Inc., San Diego, CA, USA, has now merged with Hologic). All analyses were performed in April 2010. One ml of Thin Prep liquid specimen was transferred to an Aptima Specimen Transfer tube containing 2.9 ml of buffered detergent solution (Specimen Transport Medium) that lyses the cells and releases mRNA. Four hundred microlitres of the diluted liquid specimen were tested by Aptima HPV Assay according to manufacturer's instructions. Aptima is a qualitative nucleic acid amplification test that detects the HPV E6/E7 mRNA of 14 HR HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). The test involves three main steps: capture, amplification and detection of target mRNA. The assay provides results that are positive or negative for the presence or absence of these high-risk HPV types, but does not determine the specific HPV type in the sample. An internal control transcript was added to verify performance at each step of the assay. One positive and one negative calibrator were used at the beginning of the run to determine the validity of the run and to establish the assay cut-off values for the internal control and analyte signal to the cut-off values. The signal observed for each reaction was compared with the cut-off values. An analyte signal to cut-off (S/CO) ratio equal to or greater than 1.00 was considered positive. The analyses were performed at the Department of Virology, Karolinska Hospital.

4.4 COLPOSCOPY AND BIOPSIES

In Paper I, all women were examined with colposcopy, using a Zeiss OMPI colposcope for magnification. The ectocervix and distal part of the endocervix were stained with 5% acetic acid. Punch biopsies were obtained from acetowhite areas. If no acetowhite areas were observed, a biopsy was taken close to the squamocolumnar junction, at 12 o'clock. The biopsies were fixed in buffered 4% formalin, embedded in paraffin and then sectioned and stained with haematoxylin/eosin for histological diagnosis. The samples were evaluated by a local pathologist and classified according to the cervical intraepithelial neoplasia (CIN) classification (WHO) system. Diagnosis was based on the most severe grade of atypia found in each biopsy and was compared with results of HPV testing and Pap smear. In Paper II, all women were examined with colposcopy at the inclusion visit after treatment of CIN. Punch biopsies were obtained if indicated by an abnormal colposcopy examination during follow-up. The biopsies were handled and assessed in the same manner as described above by a local pathologist.
In Papers III and IV, all women with minor cytological abnormalities were routinely referred for further examination by a gynaecologist including colposcopy and punch biopsies if lesions were suspected. Women were followed annually according to clinical practice; colposcopy and directed biopsies were performed when indicated. In paper III, information about histology and cytology results was obtained from medical and laboratory records, and from the Stockholm Oncology Center (OC). In paper IV,

information about high-grade disease was obtained from the National Quality Register for Cervical Cancer Prevention (NKCx).

4.4.1.1 Cervical conisation (Paper II)

In paper II, women treated for high-grade CIN underwent loop excision electrosurgical procedure (LEEP) using a C-LETZ electrode (Utah Medical Products Inc., Midvale, UT, USA) (190). Six women did not undergo LEEP; two of them were treated by cryotherapy and four by cold knife or laser conisation. Following excision the cone was paraffin-embedded, sectioned and diagnosed according to WHO (ICD10, Geneva 1990).

4.5 NKCX

In Paper IV, all women were linked through their personal identification numbers to the population-based National Quality Register for Cervical Cancer Prevention (NKCx) in order to identify individuals who developed histologically confirmed, cervical intraepithelial neoplasia grade 2 or worse (CIN2+) during follow-up through December 2012. The NKCx contains a copy of the same file used to report cytological and histopathological diagnoses from all cytological and histopathological laboratories in Sweden. The completeness of the NKCx is therefore 100%. All smears, histopathological results and hospital visits in Sweden are registered using a Personal Identification Number (PIN) that is unique for each individual and either assigned at birth or at time of immigration to Sweden. Since the PIN is linked to a complete registry, the current study has nationwide information on all follow-up smears and histopathological results obtained from all women in the studied cohort. The National Cervical Screening Register (NCSR) was initially established ten years ago at the Department of Medical Epidemiology and Biostatistics (MEB), KI. In 2011 Stockholm County Council took over the register and it is now part of the NKCx. It includes screening information dating back to the 1960s, millions of records of cervical smears and histological sample results from all Swedish women. A national steering group of several different experts runs the register. Data delivery, coverage and multiple quality indicators are evaluated annually, which enables feedback.

4.6 STATISTICAL ANALYSES

Paper I

Chi2 statistics were calculated to test the significance of the data with evident ordering. All statistical tests were two-sided and were considered statistically significant at $p<0.05$. Logistic regression was used to assess the significance of differences in paired data, such as the differences in sensitivities of cytology and HPV testing in the same patient.

Paper II

In the absence of histological data, the most severe cytological finding, ASC-H/HSIL+, was considered to be equivalent to CIN2, LSIL to be equivalent to CIN1 and ASC-US to be normal. Therefore the outcome was a composite of cytological and histological data. Two disease thresholds defined treatment failure: low-grade disease (LSIL-CIN1) and high-grade disease (HSIL-CIN2+), including CIS and AIS detected during follow-

up visits. Accuracy parameters for prediction of treatment failure according to these two thresholds were calculated, including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Women without CIN in the cone specimen were excluded from accuracy calculations. Data were analyzed with the software STATISTICA 6.1 (Statsoft Inc., Tulsa, OK, USA).

Paper III

The most severe histological diagnosis recorded during the 4-year follow-up period was considered to be the outcome. Accuracy parameters for prediction of CIN2+ and CIN3+ were calculated for APTIMA, HPV16 DNA testing, HPV16/18 DNA testing, and repeat cytology at three different cut-off levels. The parameters that were calculated included: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), diagnostic odds ratio (DOR) and likelihood ratios (LR), as stratified by ASC-US and LSIL diagnoses at baseline. Relative sensitivities, specificities, PPV and NPV with 95% confidence intervals (CIs) were calculated for APTIMA and compared with HPV16 DNA testing, HPV16/18 DNA testing, and repeat cytology at three different cut-off levels: ASC-US+, LSIL+ and high-grade squamous intraepithelial lesions or worse (HSIL+). Receiver-operating characteristic (ROC) curves were plotted in the same figures to compare the different tests.
Analyses were performed using Stata 13 (Stata, College Station, TX, USA).

Paper IV

In this prospective cohort study, we included women with minor cytological abnormalities who had complete HPV testing at baseline and at least one follow-up cytological or histological test. Follow-up time was counted from the date of the index ASC-US/LSIL to the date of the first histologically confirmed CIN2+ lesion, or to the last registered cytology date if the individual did not develop a CIN2+ lesion.
First, we estimated the cumulative incidence proportion of CIN2+ by baseline characteristics (age, cytological diagnosis and HPV status) using one minus the Kaplan-Meier curves. The log rank test was used for comparisons. Second, univariate and multivariate Cox regression models were used to show the association between index visit data and outcome (CIN2+ in the histopathology). Hazard ratios (HR) and their 95% confidence intervals (95% CI) were obtained from the regression models, using time since baseline ASC-US/LSIL result as the underlying timescale. The proportional hazards assumption was checked and no evidence of non-proportionality was found. A p-value of <0.05 was considered statistically significant and all analyses were conducted using STATA version 13 (Stata Corp. Stata Statistical Software, USA).

5 RESULTS

5.1 PAPER 1

5.1.1 Index cytology and age distribution

A total of 177 women with minor cytological abnormalities were enrolled. 52 (29%) had atypical squamous cells of undetermined significance (ASC-US) and 125 (71%) had low-grade squamous intraepithelial lesions (LSIL). Mean age was 34 years (median: 31; range 23-60).

5.1.2 Outcome

Histopathological analysis of colposcopy-directed biopsies revealed 101 (57%) normal samples and 76 (43%) abnormals, including various degrees of cervical intraepithelial neoplasia (CIN). 38 (50%) women had high-grade lesions (CIN2 and CIN3) and 38 (50%) had CIN1. (Table 1)

5.1.3 Repeat Pap testing and HPV testing

The second Pap smear was normal in 93 (53%) women and abnormal in 84 (47%). Among the 93 women with a normal second smear, 15 (16%) had been shown to have high-grade dysplasia (CIN2-3) on histopathological analysis of the biopsy, and among the women with an abnormal second Pap smear, 23 (28%) showed CIN2-3 on histopathology. (Table 2.)
A total of 116 women (66%) were HR HPV-positive. 31 (27%) had high-grade dysplasia (CIN2-3) on histopathology, while among women with a negative HPV test, only 7 (11%) were shown to have CIN2-3 on histopathology.
48 HPV-positive women had a normal second Pap smear, 11 (23%) of whom had CIN2-3 on histopathology.68 HPV-positive women had an abnormal second Pap smear, 20 (29%) of whom were shown to have CIN2-3.

5.1.4 Pap smear and HPV test accuracy

	CIN2-3	No disease	Total
Pap smear positive	23	61	84
Pap smear negative	15	78	93
	38	139	177

The sensitivity of the Pap smear for detection of CIN2-3 was: 23/38=61% (95% CI 45-74). The specificity of the Pap smear for detection of CIN2-3 was: 78/139=56% (95% CI 48-64). The positive predictive value (PPV): 23/84=27% (95% CI 19-38) The negative predictive value (NPV): 78/93=84% (95%CI 75-90)

HR HPV sensitivity: 31/38=82% (95% CI 67-91). HR HPV specificity: 54/139=39% (95% CI 31-47). HR HPV PPV: 31/116=27% (95% CI 20-35). HR HPV NPV: 54/61=89% (95% CI 78-94)

	CIN2-3	No disease	Total
HR HPV positive	31	85	116
HR HPV negative	7	54	61
	38	139	177

HPV-positive findings without dysplasia. Among women 30 years or younger, 33 (42%) were HR HPV-positive but had no signs of CIN, compared with women older than 30 years, where 23 (23%) were HR HPV-positive without any signs of CIN.
In conclusion, our study demonstrates that 39% (false negatives 15/38= 39%) of the high-grade cervical lesions would not have been detected using a Pap smear as the sole second screening test. If an HPV test had been used instead, 18% (false negatives 7/38= 18%) of these lesions would have been missed. If these two methods were combined, only 11% (false negatives 4/38= 11%) of CIN 2+ cases would have been missed.
HR HPV-testing with HC2 is a more sensitive method for detection of high-grade lesions.

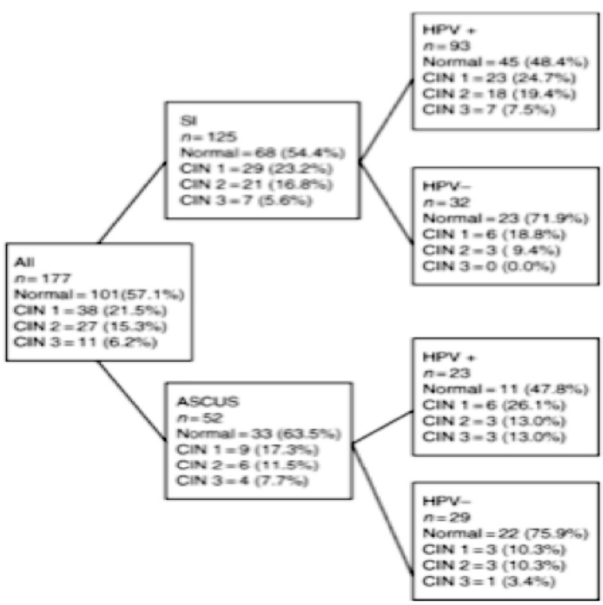


Table 1. Results of the histopathological analysis of biopsies and HPV in women with cytological atypia interpreted as LSIL and ASC-US. ASC-US, atypical squamous cells of undetermined significance; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesions.

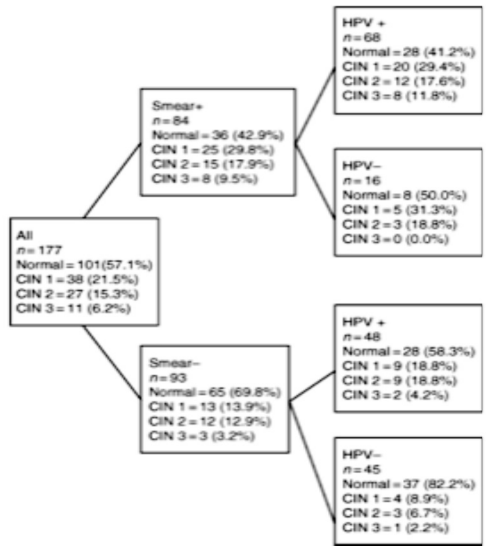


Table 2. Results of the histopathological analysis of biopsies and HPV in Pap smear-negative and Pap smear-positive women with a primary diagnosis of cytological atypia interpreted as LSIL and ASC-US. ASC-US, atypical squamous cells of undetermined significance; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesions.

5.2 PAPER 2

5.2.1 Cytological and histological diagnoses during follow-up.

Cytological results from subsequent follow-up visits were available for 137 (96%) women and histological results for 30 (21%). 20 (14%) women had three or more follow-up visits after conisation. In the absence of histological data, we created an outcome comprising both cytological and histological results. In the absence of biopsy data, a cytology finding of ASC-H/HSIL+ was equated with CIN2, LSIL with CIN1 and ASC-US and WNL (without neoplastic lesions =negative for intraepithelial abnormality) was equated with a normal outcome, even when cytology was ASC-US. When all follow-up visits were taken into account, a total of 32 (22.4%) treatment failures were detected, 25 (17.5%) of which were low-grade disease and 7 (4.9%) high-grade disease. Regarding high-grade disease outcomes, 5/7 (71%) of the diagnoses were based on histology (Table 2).

Diagnosis	Inclusion visit N (%)	Subsequent/last visit N (%)	All follow-up visits N (%)
WNL	114 (79.7)	124 (86.7)	110 (76.9)
Low-grade disease[a]	20 (14%)	12 (8.4%)	25 (17.5%)
High-grade disease[b]	5 (3.5)	4 (2.8)	7 (4.9)
Unsatisfactory[c]	4 (2.8)	3 (2.1)	1 (0.7)
Total	143	143	143

Table II. Combined cytological/histological outcome during follow-up WNL, without neoplastic lesions; CIN, cervical intraepithelial neoplasia; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesions. [a]CIN1/LSIL; [b]CIN2/HSIL; [c]histology was unsatisfactory for diagnosis

5.2.2 Age distribution and follow-up time

Mean age of treated women (n=143) was 31.3 years, median age 30 years and age span ranged from 21 to 56 years. Average follow-up time was 1333 days (3.6 years), median 1182, (3.2 years) and range 71-5622 days (2.4 months -15.4 years).

5.2.3 HR HPV DNA and HR HPV mRNA at inclusion visit

33 (23%) women were HR HPV-positive at the inclusion visit. The most frequently detected HPV types were HPV52 (4.2%), 33 and 56 (3.5% each). HPV16, 18, 51, 58 and 66 were equally common, 2.8% each. Six women had multiple infections (4 with double infection, 1 with triple and 1 with quadruple infection).
13 (9.0%) women were positive for HR HPV E6/E7 mRNA. Women who were HPV DNA-positive for types 33, 52, 56 (three cases each) and 18 (two cases), expressed mRNA more frequently than women who were HPV DNA-positive for types 16, 31, 45 and 59 (one case each). However, 26 discordant samples were found. Three HR HPV E6/E7 mRNA-positive women were HR HPV DNA-negative and had a cytological result of WNL. Among the 23 HR HPV mRNA-negative women, two were HR HPV DNA-positive for types not included in the Aptima HPV Assay (HPV type 82 and 53). These women had a low-grade disease outcome. Among the remaining 21 mRNA-negative, DNA-positive women 3 had high-grade disease and 4 had low-grade disease (Table III). Women were divided in two groups based on an early (<12 months after conisation) or late (> 12 months after conisation) inclusion visit. Table VI shows the distribution of HPV DNA and mRNA positives in the different groups (Table VI).

	Early group n=77 (%)	Late group N=66 (%)	Total n=143
HPV DNA pos	24 (31)	9 (14)	33 (23)
HPV mRNA	11(14)	2 (3)	13 (9)
Tot	35	11	46

Table III. Discordant results for 26 women.

Case no.	HPV DNA	HPV mRNA	Histological/cytological outcome
25	66	0	WNL
30	16, 51, 59	0	CIN2/HSIL
32	52	0	CIN1/LSIL
35	18	0	WNL
37	16	0	WNL
40	66	0	WNL
49	51, 73	0	WNL
57	33	0	CIN1/LSIL
59	18	0	WNL
71	66	0	WNL
79	33	0	CIN2/HSIL
81	82	0*	CIN1/LSIL
103	53	0*	CIN1/LSIL
104	31, 66	0	CIN1/LSIL
107	52	0	WNL
108	66	0	WNL
115	58	0	WNL
122	56	0	WNL
131	51	0	WNL
136	58	0	WNL
138	16	0	WNL
151	56	0	CIN1/LSIL
18	0	+	WNL
129	0	+	WNL
164	0	+	WNL

5.2.4 Accuracy parameters in prediction of treatment failure

5.2.4.1 High-grade disease

Presence of HR HPV DNA at the inclusion visit predicted 100% (95% CI 64.6-100) of residual/recurrent high-grade disease, with a specificity of 80.9% (95% CI 73.5-86.6) (Table V A). Cytology at the inclusion visit (ASC-US+) had a sensitivity of 85.7% (95% CI 48.7-97.4), and a specificity of 87.5% (95% CI 80.9-92.1) for prediction of high-grade outcome. HPV E6/E7 mRNA was a poor predictor of treatment failure in the present study. Sensitivity was 57.1% (95% CI 25.0-84.2), but specificity was 93.4% (95% CI 87.9-96.5) for prediction of high-grade outcome. The positive predictive value (PPV) was 30.8% (95% CI 12.7-57.6) and the negative predictive value (NPV) 97.7% (95% CI 93.4-99.2) (Table V B). Biopsy margin status and presence of CIN2+ in the cone specimen were also poor predictors of treatment failure with sensitivities of 57.1% (95% CI 25.0-84.2) and 71.4% (95% CI 35.9-91.8) and specificities of 64.2% (95% CI 55.8-71.8) and 22.8% (95% CI 16.5-30.5), respectively. (Table IV A)

5.2.4.2 Low-grade disease

Considering low-grade disease or worse as the threshold for treatment failure resulted in lower sensitivity and higher specificity values. (Table IV B)

Table V.A Accuracy of HR HPV DNA testing

HPV DNA test	Disease (CIN2+/HSIL)	Disease free	Total
Positive	7	26	33
Negative	0	110	110
Total	7	136	143

•HPV DNA Sensitivity 7/7=1.00 Specificity: 110/136=0.809
•PPV 7/33=0.212 NPV 110/110=1.00

Table V. B Accuracy of HR HPV mRNA testing

HPV mRNA test	Disease (CIN2/HSIL)	Disease free	Total
Positive	4	9	13
Negative	3	127	130
Total	7	136	143

HPV mRNA Sensitivity 4/7=0,571 Specificity 127/136=0,934
PPV 4/13=0,308 NPV 127/130=0,977

Table IV A Accuracy parameters in prediction of treatment failure of high-grade disease or worse.

Test	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
HR HPV DNA	100.0 (64.6-100)	80.9 (73.5-86.6)	21.2 (10.7-37.8)	100 (96.6-100)
Cytology at inclusion visit (ASC-US+)[a]	85.7 (48.7-97.4)	87.5 (80.9-92.1)	26.1 (12.6-46.5)	99.2 (95.4-99.9)
HR HPV mRNA	57.1 (25.0-84.2)	93.4 (87.9-96.5)	30.8 (12.7-57.6)	97.7 (93.4-99.2)
Margin status	57.1 (25.0-84.2)	64.2 (55.8-71.8)	7.7 (3.0-18.2)	96.6 (90.6-98.9)
Cone containing CIN2+	71.4 (35.9-91.8)	22.8 (16.5-30.5)	4.5 (2.0-10.2)	93.9 (80.4-98.3)

Table IV B Accuracy parameters in prediction of treatment failure of low-grade disease or worse.

Test	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV
HR HPV DNA	53.3 (34.3-71.7)	84.4 (76.2-90.6)	48.5 (30.8-66.5)	86.8 (78.8-92.6)
Cytology at inclusion visit (ASC-US)[a]	73.3 (54.1-87.7)	99.1 (95.1-100)	95.6 (78.1-99.9)	93.2 (87.1-97.0)
HR HPV mRNA	23.3 (9.9-42.3)	94.6 (88.7-98)	53.9 (25.1-80.8)	82.2 (74.5-88.3)
Margin status	36.7 (19.9-56.1)	64 (54.3-72.9)	21.6 (11.3-35.3)	78.9 (69.0-86.8)

5.3 PAPER 3

5.3.1 HR-HPV DNA and APTIMA results at index cytology

Mean age of study participants was 32.0 years (range: 23-60 years (standard deviation (SD) 8.5 years)); half of the women were <30 years and no statistically significant difference in age was found between the ASC-US and LSIL groups (p=0.60). Mean age was 32.8 years (SD 9.0) and 31.9 years (SD 8.4) in the ASC-US and LSIL groups, respectively. In all, 190 (87.0%) women had index LSIL smears and 29 had ASC-US (13%).

All 219 women were HR-HPV DNA-positive. HPV16 was the most frequently detected HPV type in both the ASC-US (20.7%) and LSIL groups (31.1%). The second most common HPV type in the ASC-US group was HPV53 (17.2%), while HPV51 and HPV52 were most prevalent in the LSIL group (15.3%). HPV18 was found in 13.8% of the ASC-US group and 11.6% of the LSIL group. In the ASC-US group, 31.0% were HPV16/18-positive, compared with 41.1% in the LSIL group.

In all, 162 women (74.0%) were mRNA-positive on the APTIMA test, 17 (58.6%) from the ASC-US group and 145 (76.3%) from the LSIL group (Table 1). The majority of HPV16/18-positive women were APTIMA-positive, regardless of index cytology.

Fig 2. Study flow-chart

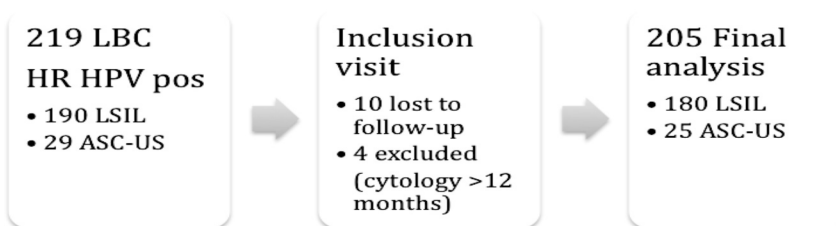


Table 1. Type-specific HPV DNA distribution in the ASC-US and LSIL groups by APTIMA status

	ASC-US group				LSIL group			
	APTIMA-		APTIMA+		APTIMA-		APTIMA+	
Type	**N**	**row %**	**N**	**row%**	**N**	**row %**	**N**	**row%**
16	2	33.3%	4	66.7%	16	22.5%	55	77.5%
18	0	0.0%	4	100.0%	6	25.0%	18	75.0%
31	1	25.0%	3	75.0%	2	7.7%	24	92.3%
33	2	50.0%	2	50.0%	3	25.0%	9	75.0%
35	0	-	0	-	4	23.5%	13	76.5%
39	1	50.0%	1	50.0%	4	16.7%	20	83.3%
45	0	0.0%	1	100.0%	5	20.0%	20	80.0%
51	3	75.0%	1	25.0%	4	12.5%	28	87.5%
52	1	50.0%	1	50.0%	7	20.0%	28	80.0%
56	0	0.0%	3	100.0%	1	4.3%	22	95.7%
58	2	50.0%	2	50.0%	2	15.4%	11	84.6%
59	0	0.0%	1	100.0%	2	10.0%	18	90.0%
66	1	33.3%	2	66.7%	0	0.0%	18	100.0%
68	0	0.0%	2	100.0%	3	37.5%	5	62.5%
26	0	-	0	-	1	50.0%	1	50.0%
53	1	20.0%	4	80.0%	5	25.0%	15	75.0%
73	1	50.0%	1	50.0%	6	27.3%	16	72.7%
82	0	0.0%	3	100.0%	0	-	0	-
16/18	2	22.2%	7	77.8%	22	23.7%	71	76.3%
Total	**12**	**41.4%**	**17**	**58.6%**	**45**	**20.6%**	**145**	**79.4%**

5.3.2 Cytological and histological results during follow-up

Altogether, 205 women came for follow-up visits within 12 months of index cytology and were included in the final analysis. Four women came for follow-up visits more than 12 months later and therefore their cytological test results could not be categorized as repeat cytology. Ten women were lost to follow-up for unknown reasons. The characteristics among those lost to follow-up did not substantially differ from those who were followed, although mean age was 30.3 years (slightly younger) (Fig 2).
Repeat cytology test results were available for 198 women. Seven women had only a biopsy test result available. If indicated, biopsies were taken by colposcopy.
Results for repeat cytology were as follows: normal cytology in 113 (57.0%) women, ASC-US in 16 (8.1%), LSIL in 52 (26.3%) and atypical squamous cells-cannot rule out high-grade lesions (ASC-H) in 17 (8.6%) women.
The histopathological results for the 209 women during follow-up were as follows: missing for 8 (3.8%) women, non-representative in 3 (1.4%), absence of CIN in 56 (26.8%), CIN1 in 69 (33.0%), CIN2 in 37 (17.7%) and CIN3+ in 36 (17.2%). Nine of 25 (36.0%) women in the ASC-US group and 64 of 180 (35.6%) women in the LSIL group developed CIN2+ during the 4 years of follow-up. Three of 25 (12%) among ASC-US were CIN3+ and 33/180 18%) among LSILs were CIN3+.

5.3.3 Accuracy of different triage options in the ASC-US group

APTIMA sensitivity to predict CIN2+ and CIN3+ was 77.8% (95% CI 40.0-90.0) and 100.0% (95% CI 40.0-100), respectively in the ASC-US group. Specificity to predict the absence of CIN2+ or CIN3+ was 50.0% (95% CI 30.0-70.0) and 45.5% (95% CI 30.0-60.0), respectively (Figure 1, Table 2).
Of the three triage approaches, APTIMA was the most sensitive test in the ASC-US group, but only reached statistical significance when using a repeat cytology cut-off of HSIL+ to predict CIN2+ (relative sensitivity 7.0, (95% CI 1.1-45.9)). APTIMA was significantly less specific than HPV16 DNA testing to predict CIN2+ and CIN3+ (relative specificity 0.6 (95% CI 0.3-0.9) and 0.6 (95% CI 0.3-0.9)), respectively, and significantly less specific than repeat cytology using a cut-off of LSIL+ and HSIL+ to predict CIN3+ (0.6 (95% CI 0.3-0.9) and 0.4 (95% CI 0.3-0.7)) (Table 3).
The positive predictive value (PPV) for all test options ranged from 29.4%-100.0% for CIN2+ and from 12.5%-100.0% for CIN3+. The PPV of APTIMA (46.7% (95% CI 24.8-69.9) for CIN2+ and 20.0% (95% CI 7.0-45.2) for CIN3+) was lowest among the three test methods, with the exception of repeat cytology when using a cut-off of ASC-US+ to predict CIN2+ (29.4%, 95% CI 13.3-53.1), and HPV 16/18 DNA testing to predict CIN3+ (12.5%, 95% CI 2.2-47.1), although the differences were not significant.
The relative PPV of APTIMA compared with repeat cytology using a cut-off of HSIL+ was 0.5 (95% CI 0.3-0.8) to predict CIN2+ and 0.2 (95% CI 0.1-0.6) to predict CIN3+ (Table 4), both of which were significant.
Risk of disease following a negative triage test (calculated as the complement of the negative predictive value (NPV): cNPV=1-NPV) ranged from 5.3%-11.8% for the outcome CIN3+, except for APTIMA, where no risk was detected. A negative APTIMA test resulted in a lower risk of disease compared with the other tests, but the difference was only significant when compared with repeat cytology using a cut-off of

LSIL+ and HSIL+ to predict CIN2+ (relative cNPV 0.6 (95% CI 0.4-0.9) and 0.6 (95% CI 0.4-0.9), for LSIL+ and HSIL+ respectively). (Table 4).

5.3.4 Accuracy of different triage options in the LSIL group

APTIMA sensitivity for predicting CIN2+ and CIN3+ in the LSIL group was 78.1% (95% CI 70.0-90.0) and 75.8% (95% CI 60.0-90.0) respectively. Specificity for predicting absence of CIN2+ and CIN3+ was 25.0% (95% CI 20.0-30.0) and 23.8% (95% CI 20.0-30.0), respectively (Table 2).
APTIMA was significantly more sensitive for predicting CIN2+ and CIN3+ compared with all other tests, except HPV16/18 DNA testing to predict CIN3+, where the difference between APTIMA and HPV16/18 DNA testing was not significant (Table 3). However, APTIMA was significantly less specific compared with all other tests.
PPVs ranged from 36.5%-76.9% for CIN2+ and from 18.2%-46.2% for CIN3+. The PPV of APTIMA was lowest among the three tests, but this was only significant when compared with repeat cytology using a cut-off of HSIL+, for which relative PPV was 0.5 (95% CI 0.3-0.7) for CIN2+ and 0.4 (95% CI 0.2-0.8) for CIN3+. (Table 4)
Risk of disease was still high when triage tests were negative (cNPV ranged from 28.0%-32.7% for CIN2+ and from 14.0%-18.6% for CIN3+). A negative APTIMA test did not result in or predict a decrease in risk of disease compared with other tests; risk of CIN3+ was significantly higher among women who were APTIMA-negative compared with women who were negative for HPV16 DNA, HPV16/18 DNA, or repeat cytology result using a cut-off of ASC-US+. (Table 4)
Most tests showed accuracy estimates that did not deviate strongly from the neutral diagonal line (LR+ and LR- near 1), indicating poor triage capacity (Figure 1).

Table 2. Overview of the sensitivity and specificity, PPV, NPV, the risk of disease[a] in case of a negative test (cNPV-1-NPV), DOR and LR

Triage group	Outcome	Test	Test cut-off	TP	FN	FP	TN	N	Sensitivity	Specificity	PPV	NPV	cNPV	DOR	LR+	LR-
ASCUS	CIN2+	APTIMA	+	7	2	8	8	25	77.8%	50.0%	46.7%	80.0%	20.0%	5.60	1.56	0.44
ASCUS	CIN2+	HPV16 DNA	+	3	6	2	14	25	33.3%	87.5%	60.0%	70.0%	30.0%	5.25	2.67	0.76
ASCUS	CIN2+	HPV16/18 DNA	+	4	5	4	12	25	44.4%	75.0%	50.0%	70.6%	29.4%	5.33	1.78	0.74
ASCUS	CIN2+	Repeat cytology	ASCUS+	5	4	12	4	25	55.6%	25.0%	29.4%	50.0%	50.0%	1.25	0.74	1.78
ASCUS	CIN2+	Repeat cytology	LSIL+	3	6	3	13	25	33.3%	81.3%	50.0%	68.4%	31.6%	4.33	1.78	0.82
ASCUS	CIN2+	Repeat cytology	HSIL+	1	8	0	16	25	11.1%	100.0%	100.0%	66.7%	33.3%	2.00	-	0.89
ASCUS	CIN3+	APTIMA	+	3	0	12	10	25	100.0%	45.5%	20.0%	100.0%	0.0%	2.50	1.83	0.00
ASCUS	CIN3+	HPV16 DNA	+	1	2	4	18	25	33.3%	81.8%	20.0%	90.0%	10.0%	3.00	1.83	0.81
ASCUS	CIN3+	HPV16/18 DNA	+	1	2	7	15	25	33.3%	68.2%	12.5%	88.2%	11.8%	1.67	1.05	0.98
ASCUS	CIN3+	Repeat cytology	ASCUS+	2	1	7	15	25	66.7%	68.2%	22.2%	93.8%	6.3%	3.75	2.10	0.49
ASCUS	CIN3+	Repeat cytology	LSIL+	2	1	4	18	25	66.7%	81.8%	33.3%	94.7%	5.3%	7.20	3.67	0.41
ASCUS	CIN3+	Repeat cytology	HSIL+	1	2	0	22	25	33.3%	100.0%	100.0%	91.7%	8.3%	11.00	-	0.67
LSIL	CIN2+	APTIMA	+	50	14	87	29	180	78.1%	25.0%	36.5%	67.4%	32.6%	14.36	1.04	0.88
LSIL	CIN2+	HPV16 DNA	+	24	40	32	84	180	37.5%	72.4%	42.9%	67.7%	32.3%	28.00	1.36	0.86
LSIL	CIN2+	HPV16/18 DNA	+	29	35	44	72	180	45.3%	62.1%	39.7%	67.3%	32.7%	26.43	1.19	0.88
LSIL	CIN2+	Repeat cytology	ASCUS+	34	30	39	77	180	53.1%	66.4%	46.6%	72.0%	28.0%	37.94	1.58	0.71
LSIL	CIN2+	Repeat cytology	LSIL+	27	37	33	83	180	42.2%	71.6%	45.0%	69.2%	30.8%	32.01	1.48	0.81
LSIL	CIN2+	Repeat cytology	HSIL+	10	54	3	113	180	15.6%	97.4%	76.9%	67.7%	32.3%	19.82	6.04	0.87
LSIL	CIN3+	APTIMA	+	25	8	112	35	180	75.8%	23.8%	18.2%	81.4%	18.6%	7.29	0.99	1.02
LSIL	CIN3+	HPV16 DNA	+	15	18	41	106	180	45.5%	72.1%	26.8%	85.5%	14.5%	26.95	1.63	0.76
LSIL	CIN3+	HPV16/18 DNA	+	18	15	55	92	180	54.5%	62.6%	24.7%	86.0%	14.0%	23.66	1.46	0.73
LSIL	CIN3+	Repeat cytology	ASCUS+	17	16	56	91	180	51.5%	61.9%	23.3%	85.0%	15.0%	21.49	1.35	0.78
LSIL	CIN3+	Repeat cytology	LSIL+	13	20	47	100	180	39.4%	68.0%	21.7%	83.3%	16.7%	19.40	1.23	0.89
LSIL	CIN3+	Repeat cytology	HSIL+	6	27	7	140	180	18.2%	95.2%	46.2%	83.8%	16.2%	24.71	3.82	0.86

PPV, positive predictive value, NPV: negative predictive value, DOR: diagnostic odds ratio, LR: likelihood ratio, TP: true positive, FN: false negative, FP: false positive, TN: true negative, N: number, ASCUS: atypical squamous cells of undetermined significance, CIN2+: cervical intraepithelial neoplasia grade 2 or worse, HPV: human papillomavirus, LSIL: low-grade squamous intraepithelial lesions, CIN3+: cervical intraepithelial neoplasia grade 3 or worse.
[a]The risks of disease cNPV=1-NPV

Table 3. Relative sensitivity and specificity of APTIMA compared to other tests to triage women with ASCUS or LSIL for the outcomes CIN2+ or CIN3+[a]

			Relative sensitivity			Relative specificity		
Triage group	**Outcome**	**Test**	**Estimate**	**lower CIB**	**upper CIB**	**Estimate**	**lower CIB**	**upper CIB**
ASCUS	CIN2+	HPV16 DNA	2.33	0.87	6.27	**0.57**	**0.34**	**0.96**
ASCUS	CIN2+	HPV6/18 DNA	1.75	0.78	3.93	0.67	0.38	1.17
ASCUS	CIN2+	Cyto at ASCUS+	1.40	0.71	2.77	2.00	0.75	5.33
ASCUS	CIN2+	Cyto at LSIL+	2.33	0.87	6.27	0.62	0.36	1.06
ASCUS	CIN2+	Cyto at HSIL+	**7.00**	**1.07**	**45.90**	0.50	0.31	0.82
ASCUS	CIN3+	HPV16 DNA	3.00	0.61	14.86	**0.56**	**0.34**	**0.91**
ASCUS	CIN3+	HPV16/18 DNA	3.00	0.61	14.86	0.67	0.39	1.14
ASCUS	CIN3+	Cyto at ASCUS+	1.50	0.67	3.34	0.67	0.39	1.14
ASCUS	CIN3+	Cyto at LSIL+	1.50	0.67	3.34	**0.56**	**0.34**	**0.91**
ASCUS	CIN3+	Cyto at HSIL+	3.00	0.61	14.86	**0.45**	**0.29**	**0.72**
LSIL	CIN2+	HPV16 DNA	**2.08**	**1.48**	**2.93**	**0.35**	**0.25**	**0.48**
LSIL	CIN2+	HPV16/18 DNA	**1.72**	**1.28**	**2.32**	**0.40**	**0.29**	**0.57**
LSIL	CIN2+	Cyto at ASCUS+	**1.47**	**1.13**	**1.92**	**0.38**	**0.27**	**0.53**
LSIL	CIN2+	Cyto at LSIL+	**1.85**	**1.35**	**2.54**	**0.35**	**0.25**	**0.49**
LSIL	CIN2+	Cyto at HSIL+	**5.00**	**2.79**	**8.97**	**0.26**	**0.19**	**0.35**
LSIL	CIN3+	HPV16 DNA	**1.67**	**1.09**	**2.54**	**0.33**	**0.24**	**0.45**
LSIL	CIN3+	HPV16/18 DNA	1.39	0.96	2.00	**0.38**	**0.28**	**0.52**
LSIL	CIN3+	Cyto at ASCUS+	**1.47**	**1.00**	**2.16**	**0.38**	**0.28**	**0.53**
LSIL	CIN3+	Cyto at LSIL+	**1.92**	**1.21**	**3.06**	**0.35**	**0.26**	**0.48**
LSIL	CIN3+	Cyto at HSIL+	**4.17**	**1.97**	**8.81**	**0.25**	**0.19**	**0.33**

HPV: human papillomavirus, ASCUS: typical squamous cells of undetermined significance, LSIL: low-grade squamous intraepithelial lesions, CIB: 95% confidence interval bound.

[a]Significant differences in bold.

Table 4. Relative risk of disease in case of a positive test (relative PPV) and in case of a negative test (relative cNPV) of APTIMA compared to other tests in the ASCUS and LSIL groups for the outcomes CIN2+ and CIN3+[a]

			Relative PPV			Relative cNPV		
Triage group	**Outcome**	**Test**	**Estimate**	**lower CIB**	**upper CIB**	**Estimate**	**lower CIB**	**upper CIB**
ASCUS	CIN2+	HPV16 DNA	0.78	0.32	1.91	0.67	0.44	1.02
ASCUS	CIN2+	HPV16/18 DNA	0.93	0.39	2.25	0.68	0.44	1.05
ASCUS	CIN2+	Cyto at ASCUS+	1.59	0.64	3.96	0.40	0.19	0.85
ASCUS	CIN2+	Cyto at LSIL+	0.93	0.36	2.45	**0.63**	**0.41**	**0.98**
ASCUS	CIN2+	Cyto at HSIL+	**0.47**	**0.27**	**0.80**	**0.60**	**0.39**	**0.91**
ASCUS	CIN3+	HPV16 DNA	1.00	0.13	7.57	0.00	.	.
ASCUS	CIN3+	HPV16/18 DNA	1.60	0.20	12.99	0.00	.	.
ASCUS	CIN3+	Cyto at ASCUS+	0.90	0.18	4.40	0.00	.	.
ASCUS	CIN3+	Cyto at LSIL+	0.60	0.13	2.74	0.00	.	.
ASCUS	CIN3+	Cyto at HSIL+	**0.20**	**0.07**	**0.55**	0.00	.	.
LSIL	CIN2+	HPV16 DNA	0.85	0.59	1.24	1.01	0.79	1.28
LSIL	CIN2+	HPV16/18 DNA	0.92	0.64	1.32	1.00	0.78	1.27
LSIL	CIN2+	Cyto at ASCUS+	0.78	0.56	1.09	1.16	0.91	1.47
LSIL	CIN2+	Cyto at LSIL+	0.81	0.57	1.16	1.06	0.83	1.34
LSIL	CIN2+	Cyto at HSIL+	**0.47**	**0.33**	**0.69**	1.01	0.80	1.27
LSIL	CIN3+	HPV16 DNA	0.68	0.39	1.19	**1.28**	**1.09**	**1.50**
LSIL	CIN3+	HPV16/18 DNA	0.74	0.43	1.26	**1.33**	**1.13**	**1.56**
LSIL	CIN3+	Cyto at ASCUS+	0.78	0.45	1.35	**1.24**	**1.06**	**1.47**
LSIL	CIN3+	Cyto at LSIL+	0.84	0.46	1.53	1.12	0.95	1.31
LSIL	CIN3+	Cyto at HSIL+	**0.40**	**0.20**	**0.79**	1.15	0.98	1.35

HPV: human papillomavirus, ASCUS: typical squamous cells of undertermined significance, LSIL: low-grade squamous intraepithelial lesions, CIB: 95% confidence interval bound.

[a]Significant differences in bold.

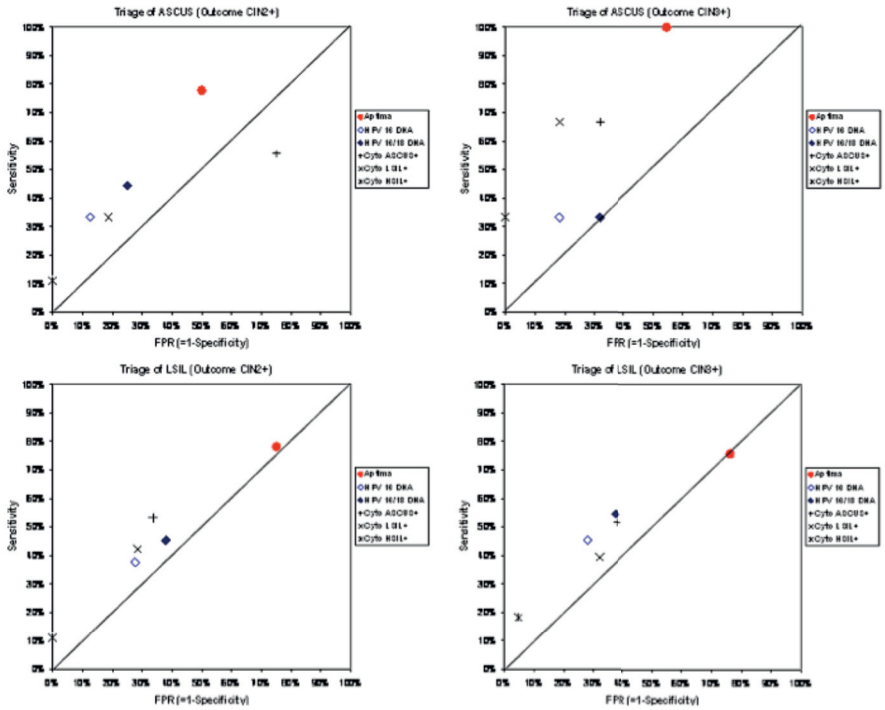


Figure 1. Sensitivity & FPR (False positive rate) of the different tests used to triage women with ASCUS (upper) or LSIL (lower) to detect CIN2+ (left) or CIN3+ (right). Red circle: APTIMA, rhombus without color HPV DNA 16, rhombus blue HPV16/18 DNA, cross: cytology AS-CUS as cut-off, diagonal cross: cytology with LSIL as cut-off, double diagonal cross: cytology with HSIL as cut-off. ASCUS-atypical squamous cells of undetermined significance, LSIL-low-grade squamous intraepithelial lesions, HSIL-high-grade squamous intraepithelial lesions CIN- cervical intraepithelial neoplasia HR-HPV: High-risk human papillomavirus

5.4 PAPER 4

5.4.1 Characteristics of the women in the study

39.5% of women with ASC-US were HR HPV-positive, as were 79.4% of women with LSIL. Overall, 214 (68.2%) women were positive for at least one of the 13 high-risk (HR) HPV types defined by IARC (104), but most infections were due to HPV type 16 and/or 18 (27.7%). 65 (20.7%) women were HPV16-positive and 26 (8.3%) HPV18-positive and the next most common HPV type in the group was HPV 51 (10.5%). Mean age of the study group was 34 years (median 32 years, range 23-60 years) and 184 (58.6%) women were older than 30 years. (Table 1)
Median follow-up time was 3.8 years (range 0.1-7.0 years).

Table 1. Distribution of background characteristics among 314 women with ASC-US/LSIL on index cytologies		
	Study population	Count of CIN2+
	n (%)	n
Age		
≤29	130 (41.4)	35
30-39	97 (30.9)	36
40-49	71 (22.6)	15
≥50	16 (5.1)	3
Baseline cytology result		
ASC-US	76 (24.2)	11
LSIL	238 (75.8)	78
HPV status		
HR HPV positive* (13 types)	214 (68.2)	85
HPV 16 and/or 18 positive	87 (27.7)	39
Other HR HPV positive**	127 (40.5)	46
HPV type-specific positivity		
HPV 16	65 (20.7)	32
HPV 18	26 (8.3)	11
HPV 31	31 (9.9)	18
HPV 33	15 (4.8)	10
HPV 35	13 (4.1)	4
HPV 39	22 (7.0)	9
HPV 45	21 (6.7)	8
HPV 51	33 (10.5)	8
HPV 52	31 (9.9)	7
HPV 56	28 (8.9)	8
HPV 58	17 (5.4)	7
HPV 59	20 (6.4)	7
HPV 68	9 (2.9)	2

*13 HR types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68
**Excluding types 16 and 18

5.4.2 Cumulative Incidence Proportion (CIP) of CIN2+

5.4.2.1 By age at baseline

A total of 89 (28.3%) women developed histologically confirmed CIN2+ during follow-up. The 7-year cumulative risk of developing CIN2+ was greatest among women aged 30-39 years at the start of follow-up (CIP 39.8% (95% CI: 30.3-51.0)) and among women younger than 29 at study start, the 7-year cumulative risk was 30.8% (95% CI: 22.6-21.0) (Figure 1). There were no significant difference between age categories (p=0.1499).

5.4.2.2 By index cytology

In the first two years of follow-up CIP increased quickly among women with a finding of ASC-US at baseline, then plateaued after two years. However, in the LSIL group, CIP increased steadily over the entire follow-up period. The 7-year CIPs of CIN2+ among women with baseline cytology of ASC-US or LSIL were 15.0% (95% CI: 8.6-25.5) and 37.3% (95% CI: 30.6-44.9), respectively. The difference was statistically significant (p=0.0049) (Fig. 2.).

5.4.2.3 By HR HPV status at baseline

HR HPV-positive women also had a higher cumulative risk of CIN2+ 7 years after the index test compared with HR HPV-negative women. CIP was 53.9% (95% CI: 40.6-68.3) for HPV16/18 and 38.5% (95% CI: 30.2-48.2) for other HR HPV types vs. 6.6% (95% CI: 2.3-18.1) for HR HPV negatives. The difference was statistically significant, p<0.0001. After 5 years of follow-up, the risk for CIN2+ continued to increase among

women who were HPV16/18 positive at baseline, while risk among women positive for other HR types did not increase after 5 years (Fig 3).

5.4.2.4 By index cytology and HR HPV status at baseline

Fig 4 shows CIP of CIN2+ by index cytology result stratified by HR HPV status. Overall, HR HPV-positive ASC-US/LSIL had a higher cumulative risk of CIN2+ compared with HR HPV-negative ASC-US/LSIL. CIP of CIN2+ for women with HR HPV-positive ASC-US was 35.2% (95% CI: 20.7-64.8) after 6.5 years of follow-up and the risk for women with HR HPV-positive LSIL was 45.8% (95% CI: 37.7-54.3) after 7 years of follow-up. CIP of CIN2+ increased rapidly among women positive for HR HPV at baseline and plateaued after 2 years among women with ASC-US, but continued to increase among women with LSIL. CIP of CIN2+ for HR HPV-negative women with ASC-US/LSIL remained similarly low during the first years, with only a few more cases in the LSIL group after 4.5 years of follow-up. The difference between ASC-US and LSIL when stratified by HPV status was statistically significant, $p<0.0001$. The 3-year risk of CIN2+ among HR HPV-negative ASC-US and LSIL was 2.0% (95% CI: 0.5-7.8). The 5-year risk among HR HPV-negative ASC-US remained 2.0%, but increased to 4.1% (95% CI: 1.2-12.9) for HR HPV-negative LSIL (Fig 4).

5.4.2.5 By index cytology and HR HPV16/18 genotype at baseline

Fig 5 shows CIP of CIN2+ among ASC-US and LSIL stratified by HPV 16/18, other HR HPV types and HPV-negativity. The highest cumulative risk of CIN2+ was observed among women positive for HPV16/18 (CIP for HPV16/18 positive ASC-US was 48.9% (95% CI: 23.8-51.1) at 5 years of follow-up; for HPV 16/18 LSIL the corresponding figure was 54.2% (95% CI: 40.2-45.8)) at 7-years of follow-up. CIP of CIN2+ for women with ASC-US who were positive for other HR HPV types was 27.6% (95% CI: 12.5-54.1) and for women with LSIL who were positive for other HR HPV types, 40.4% (95% CI: 31.1-51.1) at 6-years of follow-up. The risk for CIN2+ increased rapidly among women with HPV-positive ASC-US/LSIL during the first two years, but continued to increase throughout the entire follow-up period. However, risk among HPV16/18 positives was always greater than risk among women positive for other HR HPV types.

5.4.3 Risk associations with baseline characteristics

We evaluated age, cytological, and viral risk factors for developing CIN2+ in the study group. Unadjusted associations are shown in Table 2. Baseline risk factors significantly associated with developing CIN2+ included LSIL cytology (HR 2.4; 95% CI, 1.3-4.5) vs. ASC-US and HR HPV-positivity (HR 12.5; 95% CI, 4.6-34.1) vs. HR HPV-negativity. Testing positive for HPV genotype 16 and/or 18 (HR 2.4; 95% CI, 1.6-3.6), HPV16 alone (HR 2.6; 95% CI, 1.7-3.9) or one of the HPV types 31 (HR 3.0; 95% CI, 1.8-5.0) or 33 (HR 4.1; 95% CI, 2.1-7.9) was also significantly associated with developing CIN2+ compared with a negative test for that type.

In the multivariable model adjusted for age at baseline, index cytology and HPV status, the strength of association was greatest for HR HPV-positivity compared with negativity (Hazard Ratio HPV16/18 2.3 (95% CI; 1.5-3.5) $p<0.001$ (Table 3.)

Table 2. Unadjusted association between baseline characteristics and CIN2+		
Effect	HR (95% CI)	p-value
Age		
30-39 vs. ≤29	1.5 (0.9-2.4)	0.100
40-49 vs. ≤29	0.8 (0.4-1.5)	0.466
≥50 vs. ≤29	0.8 (0.3-2.7)	0.761
Baseline cytology		
LSIL vs. ASC-US	2.4 (1.3-4.5)	0.006
HPV status		
HR HPV positive* vs. HR HPV negative	12.5 (4.6-34.1)	<0.001
HPV 16 and/or HPV18 positive vs. HPV 16 and HPV 18 negative	2.4 (1.6-3.6)	<0.001
Other HR HPV positive** vs. Other HR HPV negative	1.7 (1.1-2.6)	0.010
HPV 16 positive vs. HPV 16 negative	2.6 (1.7-3.9)	<0.001
HPV 18 positive vs. HPV 18 negative	1.8 (1.0-3.4)	0.067
HPV 31 positive vs. HPV 31 negative	3.0 (1.8-5.0)	<0.001
HPV 33 positive vs. HPV 33 negative	4.1 (2.1-7.9)	<0.001
HPV 35 positive vs. HPV 35 negative	1.2 (0.4-3.3)	0.706
HPV 39 positive vs. HPV 39 negative	1.8 (0.9-3.5)	0.106
HPV 45 positive vs. HPV 45 negative	1.5 (0.7-3.0)	0.309
HPV 51 positive vs. HPV 51 negative	0.8 (0.4-1.7)	0.559
HPV 52 positive vs. HPV 52 negative	0.7 (0.3-1.5)	0.387
HPV 56 positive vs. HPV 56 negative	1.0 (0.5-2.1)	0.997
HPV 58 positive vs. HPV 58 negative	1.7 (0.8-3.8)	0.161
HPV 59 positive vs. HPV 59 negative	1.2 (0.5-2.5)	0.707
HPV 68 positive vs. HPV 68 negative	0.8 (0.2-3.1)	0.693

*13 HR types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68

**Excluding types 16 and 18

Table 3. Adjusted multivariable model - full study population		
Effect	HR (95% CI)	p-value
Age		
30-39 vs. ≤29	1.8 (1.1-2.8)	0.018
40-49 vs. ≤29	1.2 (0.6-2.2)	0.631
≥50 vs. ≤29	1.0 (1.5-3.5)	0.950
Baseline cytology		
LSIL vs. ASC-US	2.1 (1.1-4.0)	0.024
HPV status		
HPV 16 and/or HPV18 positive vs. HPV 16 and HPV 18 negative	2.3 (1.5-3.5)	<0.001

Figure 1. Cumulative Incidence Proportion of CIN2+ by Baseline Age

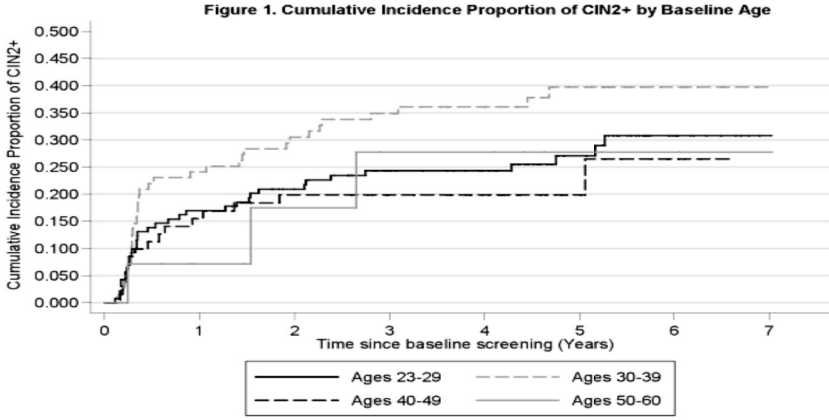


Figure 2. Cumulative Incidence Proportion of CIN2+ by Baseline Test Result

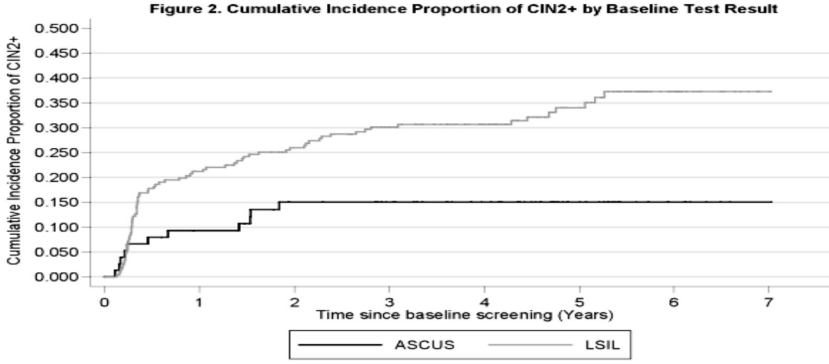


Figure 3. Cumulative Incidence Proportion of CIN2+ by Baseline HR HPV Status

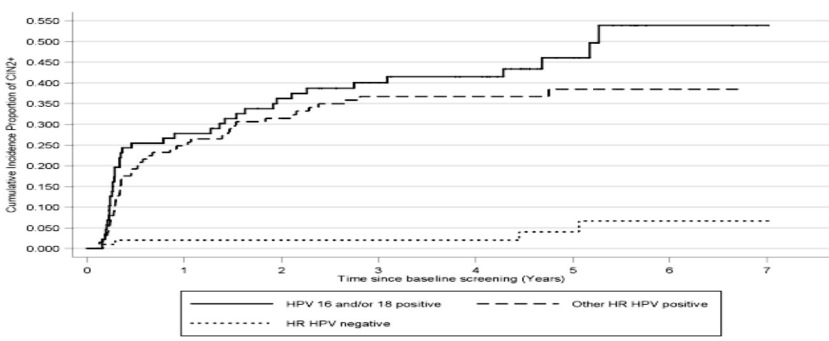


Figure 4. Cumulative Incidence Proportion of CIN2+ by Baseline Test Result & HR HPV Status

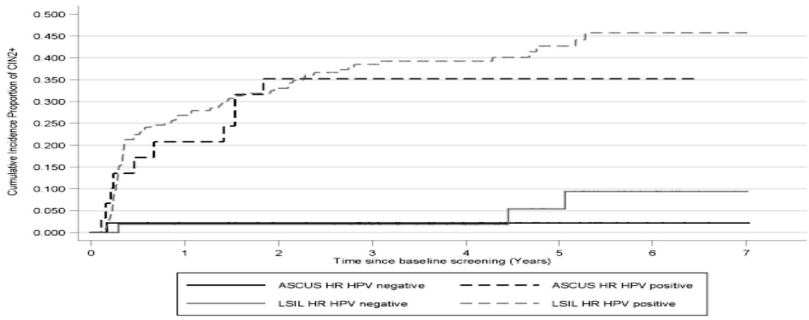
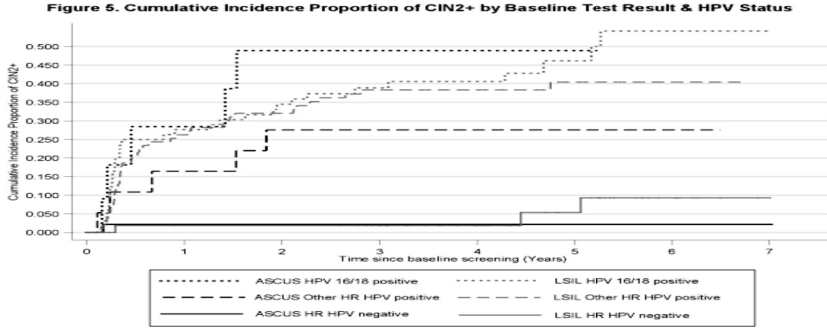


Figure 5. Cumulative Incidence Proportion of CIN2+ by Baseline Test Result & HPV Status



6 DISCUSSION

6.1 PAPER 1

6.1.1 Main findings and interpretations

The aim of this study was to compare the sensitivity of high-risk human papillomavirus (HR HPV) DNA testing for detection of high-grade cervical intraepithelial neoplasia (CIN) 2-3 with that of a second (repeat) Pap smear in women diagnosed with minor cytological abnormalities in their index Pap smear. The HPV DNA test was positive in 66% of the studied women and 43%(76) of these women had various degrees of CIN. The sensitivity to detect CIN2-3 was 82% (95% CI=67-91) for the HPV DNA test and 61% (95% CI=45-74) for the second Pap smear. The positive and negative predictive values of HPV DNA testing to detect CIN2-3 were 27% (95% CI=18-35) and 89% (95% CI=80-97), respectively. Our study demonstrates that HPV DNA testing with HC2 is a more sensitive method for detecting high-grade CIN lesions than Pap smear. Pap smear as the sole second screening test would have missed 39% (15/38) of the high-grade cervical lesions, providing false negative results. If an HPV test had been used instead, 18% (7/38) of these lesions would have been missed. If these two methods were combined, only 11% (4/38) of CIN2-3 cases would have been missed. The data also show that women < 30 years were more frequently HPV-positive (42%) without signs of CIN compared with older women (23%).
A follow-up repeat Pap smear was often obtained 4-6 months after the first smear that showed minor cytological abnormalities. However, management varies throughout Sweden and in Stockholm County all these women were referred for colposcopy and biopsy. Our results indicate that HR HPV DNA-testing with HC2 is a more sensitive method to select women who need referral for colposcopy and biopsy. For negative HPV DNA tests we proposed repeating the test in 1 year and if still negative, these women may return to population-based screening at 3-year intervals, thereby reducing psychological stress and healthcare costs.

6.1.2 Methodological considerations and validity

The study women were consecutively enrolled when diagnosed with minor cytological abnormalities (ASC-US or LSIL) detected through population-based screening. Swedish cytological diagnoses were converted into the Bethesda system. Sweden uses the old US cytological nomenclature by Koss, in which CIN1 is both a cytological as well as a histopathological diagnosis. The cytological term LSIL is a combination of LSIL and koilocytotic atypia. In the Koss system, a diagnosis of koilocytosis can be made in addition to any other diagnosis. Since only a small minority (0.85%) (18) of smears are reported as normal with koilocytosis as an ancillary diagnosis, the cytological diagnosis of CIN1 used in Sweden is essentially equivalent to the term LSIL (155).

There is a strong association between the LSIL diagnosis and HPV infection. In LSIL, 75-80% of lesions harbour oncogenic HPV types and another 10-15% are associated with nononcogenic HPV types. Only 5-10% are negative for HPV (151). In our study HC2 testing found that 93/125 (74%) cases were HPV-positive and 32/125 (26%) were HPV-negative LSIL. Zuna et al. question the existence of HPV-negative LSIL as a distinct biological entity and found no evidence to support its existence. Possible explanations for HPV-negative LSIL include a) infection with nononcogenic HPV types that are not targeted by HC2, b) cytological misinterpretation (false positive), c) false negative HPV tests and d) viral clearance between original finding and repeat cytology with enrolment for HPV testing. All women underwent colposcopy, which relies on subjective interpretation. Limiting factors in colposcopy include failure to identify an abnormality and failure to properly sample an identified abnormal area. Experienced gynaecologists performed all examinations, but studies have shown that even under enhanced imaging, an apparently normal cervix has been shown to harbour CIN2+ disease in 25% of women referred for colposcopy. This implies that colposcopy itself, rather than punch biopsy, is the limiting factor for detecting CIN2+ (195). Punched biopsies were carried out on all women regardless of presence of a visualised lesion and therefore verification bias could be limited/avoided. Studies have shown that increasing the number of biopsies and taking random biopsies from apparently normal cervical tissue increase detection rate of CIN3 (55, 57). A meta-analysis by Underwood et al. demonstrated that pooled sensitivity for a single punch biopsy is 90%; when one or more punch biopsies were performed sensitivity increased to 93%, and with multiple biopsies, sensitivity approaches 100%. The TOMBOLA trial identified a false-negative rate associated with punch biopsies, but concluded that it did not have an impact on clinical outcome because "missed cases would be picked up at the next round of screening" (196). Confirming the rate of true false negatives on colposcopy would require all women with negative punch biopsies to undergo loop electrosurgical excision procedures (LEEP), which is not feasible.

Other factors that influence the accuracy of colposcopy-directed biopsies are cytological findings before biopsy and screening history. All women in our study had a diagnosis of minor cytological abnormalities detected through the population-based screening programme, which essentially means there were no prior cytological abnormalities. Another confounding factor affecting accuracy is possible lesion regression during the waiting period for colposcopy.

Staff cytologists and pathologists, reflecting a real life setting, interpreted cytological samples and punch biopsies. No samples were reviewed, which is a practice that could alter the diagnosis, since histological interpretation of biopsy material is prone to error (49). On the other hand, reflecting routine clinical practice in the healthcare system could be a strength of the study since it does more closely duplicate the real life setting. We used histological findings from colposcopy as our reference standard, but did not accept "negative satisfactory colposcopy" as evidence for absence of high-grade CIN, as many studies do. When colposcopy was negative, a biopsy was taken at the 12 o'clock position. In many situations, however, an ethical and practical dilemma arises when performing a gold standard test that is invasive.

Presence of histologically confirmed high grade CIN was the disease endpoint. CIN3 is the most relevant clinical outcome since it is considered to be an obvious precursor to cervical cancer and is a more reproducible histological diagnosis than CIN2. CIN2+ is an intermediate condition containing over-called CIN1 and under-called CIN3.

We chose CIN2-3, which is the clinical cut-off for treatment of precancerous lesions and also generates a greater outcome in a small study population.
HC2 is an FDA-approved test and the system is calibrated using positive and negative control samples provided by the manufacturer. The test is known to cross-react with nononcogenic HPV types, which lowers specificity and results in higher rates of false positives. False-negative HPV test results may be due to errors during sample collection, technical problems with the assay process, or the presence of rare, low-risk HPV types that are not detected by the current test. Even highly sensitive tests like the HPV DNA test will not achieve perfect sensitivity. Another drawback of HC2 is that it has no internal cellular control to help adjudicate false negatives.
The external validity of our study, generalisability to other real-life settings, is good, since it utilises the real-life setting.
Disease prevalence is high and therefore the negative predictive value of the tests will not be affected.

6.1.3 Comparison with other studies

Our study preceded a randomised health services study of human papillomavirus-based management contra colposcopy of all low-grade cytological abnormalities in the Stockholm area in which they assessed safety and healthcare resource utilisation under a real-life healthcare policy using HPV DNA triage. This trial enrolled 3319 women who were diagnosed with ASC-US or CIN1 from 2003 to 2006. Clinics were randomised to either continue with prior policy (colposcopy of all women with ASCUS/CIN1) or to implement a policy using HPV triage and only perform colposcopy on HPV-positive women. The primary outcome measure of safety in a comparison between the two policies was detection rate of CIN2+. The secondary outcome measure was cost-effective use of healthcare resources. The proportion of histopathologically verified CIN2+ was similar for both approaches (22.5% for HPV triage and 20.3% for CIN2+ with colposcopy). In all, 64% of women with ASCUS and 77% of women with CIN1 were HPV-positive. HPV positivity was age-dependent: 81% of women below age 35 and 44% of women above age 45 tested HPV-positive. HPV triage was cost-effective only among women above age 35 (155); 32% fewer women were referred using the HPV triage policy (155).
The randomised triage trial ALTS (ASC-US LSIL Triage Trial) found that HPV triage of LSIL smears was not cost-effective since a majority of LSIL smears were HPV-positive. Several studies eventually formed the basis of new management guidelines recommending HPV testing to triage minor cytological abnormalities. In 2010, the Society of Obstetrics and Gynaecology Working Group for Cervical Cancer Prevention revised the Swedish guidelines and no longer recommend repeat cytology (39). The main reasons for the new recommendations were the high prevalence of CIN2+ in women with ASC-US and LSIL according to reports from both Sweden and abroad, along with the risk of decreased compliance with cytological surveillance over time (155, 197-199).
Our study is included in a recent Cochrane review of diagnostic test accuracy comparing HPV testing with repeat cytology for triage of minor cytological cervical lesions (135). This review corroborates conclusions from previous meta-analyses, which all indicated that HC2 triage of women with ASC-US predicts presence of underlying high-grade CIN with greater accuracy than repeat Pap smear (150, 154). In

triage of LSIL, however, the lower specificity of HC2 was confirmed, although a significant gain in sensitivity was demonstrated.
Recent European and American guidelines now recommend HPV triage of women with ASC-US, but not for management of women with LSIL. The recent Cochrane review demonstrated significantly higher sensitivity for HC2 to detect CIN2+ and CIN3+, which may justify recommending the use of HC2 when deciding whether to refer women for colposcopy. However, such recommendations should be based on local cost-effectiveness analyses, local prevalence of HPV in LSIL, performance of the HPV test used and patient compliance with follow-up recommendations. In addition, HC2 use should be restricted in situations where access to colposcopy referral is limited and/or expensive.

6.1.4 Implications for continued research and practice

We conclude that virological testing using HC2 is a more accurate method than repeat cytology to triage women with minor cytological abnormalities in our study population. We used conventional Pap smear, but if the collected cervical specimen is liquid-based (LBC), residual material from the same sample can be used for HPV testing, thereby avoiding additional medical visits. In the future newer assays developed for HPV self-sampling could be an option. Several studies have demonstrated a decline in HPV prevalence by age. In the health service study by Dillner et al. triage based on HPV testing was only cost-effective in women over age 35. Younger women with a high prevalence of HPV and profuse mild cellular abnormalities need a more specific marker that permits identification of those women at risk for high-grade CIN. Optimally, such a test would be as sensitive, but more specific, than HC2 and also more sensitive than repeat cytology. HPV testing may also be able to more effectively detect rare cytological abnormalities, such as atypical glandular cells or adenocarcinoma.

6.2 DISCUSSION PAPER 2

6.2.1 Main findings and interpretations

To our knowledge, the present study is the first to compare the HPV E6/E7 mRNA of 14 HR HPV types with HPV DNA testing for follow-up of women treated for CIN. This study identified high-grade residual/recurrent disease (CIN2+/HSIL+) in 7 (4.9%) women and low-grade disease (CIN1/LSIL) in 25 (17.5%) women during follow-up after treatment for CIN. These findings are consistent with residual/recurrent disease rates in other studies. A meta-analysis of 28 studies estimated treatment failure to range from 7.1% to 11.3% (134). Presence of HR HPV DNA predicted all (100%) cases of residual/recurrent high-grade disease with a specificity of 80.9%. These findings support previously known data and conclude that a positive HR HPV DNA test is a better predictor of treatment failure than cytology or positive resection margins (134, 154). Meanwhile, using the APTIMA HPV Assay to test for HR HPV E6/E7 mRNA showed low sensitivity for high-grade outcome, 57.1%, but high specificity, 93.4%. However, the Aptima missed three cases of high-grade disease and we concluded that this test was a poor predictor of treatment failure and was not useful for follow-up of women treated for CIN. Since women treated for CIN are at greater risk of developing SCC than the general population, careful surveillance is required. An ideal test in this

setting should have high sensitivity and high negative predictive value (NPV)(200, 201).

6.2.2 Methodological considerations and validity

Since the post-treatment population is at greater risk of disease, follow-up testing should have high sensitivity and high negative predictive value (NPV). Testing for HPV DNA fulfils these criteria, while significantly reducing follow-up visits and anxiety. However, the performance of most tests, such as the Aptima, has previously been evaluated in a different type of patient management setting, including colposcopy referral/triage populations in which HPV and disease prevalence are higher than in the post-treatment group. These factors could all have affected our results.

Our study confirms that high NPV (Aptima 97.7%) can be misleading since the HPV mRNA test missed 3 of 7 residual CIN2+ cases. The PPV and NPV of a diagnostic test depend on disease prevalence. In fact, regardless of diagnostic test, PPV falls with disease prevalence, while NPV rises. Simply put, low prevalence means that the person we are testing is unlikely to have the disease and therefore, based on this fact alone, a negative test is likely to be correct. In our study the prevalence of recurrent disease was only 7/143 (4.9%). The HPV mRNA test showed a high specificity and NPV, which could partly be explained by the fact that most of the study population had no residual CIN2+ according to our criteria. The sensitivity of cytology to detect residual disease post-treatment was 85.7%, but the mRNA test did not contribute significantly to improving detection of residual CIN2+. A larger study population with a higher rate of recurrence would lead to greater test accuracy.

The sensitivity of HPV mRNA (Aptima) for detection of high-grade disease was only 57.1%. This may be due to low viral load or low levels of E6/E7 mRNA transcripts in the sampled cells, even if the HPV type causing the residual lesion was included in the HR HPV mRNA test. Only two cases were positive for HPV types not included in the Aptima, HPV53 and HPV82, but the associated lesions were both low grade. HPV82 and HPV53 are categorised as group 2B, which despite limited epidemiological data has been proposed to designate types that are probably carcinogenic because of their close phylogenetic relationship with established carcinogenic types. The Aptima test was negative in three high-grade lesions (3/7, 43%). One of these lesions had a multiple infection with HPV16, 51 and 59, and the other two had single infections with HPV33 and HPV66, respectively. Lack of detection of mRNA may also be due to sampling technique, unevenly distributed oncogene expression in the lesions, or the fact that not all HPV-infected cases have transcriptionally active E6/E7 expression. Absent mRNA may also represent regressing dysplasia resulting from switched off E6/E7 (202), but since guidelines recommend treatment of all women over age 25 with a diagnosis of CIN2+, this potential regression cannot be investigated.

This study reflects a real-life setting, with clinical follow-up after surgical treatment for CIN. Pap smears were carried out during follow-up, colposcopy was performed and biopsies taken if an abnormality was diagnosed or suspected. If colposcopy or cytology were normal the women were considered disease-free. An optimal scenario would have been to biopsy (gold standard) every woman, but this is not ethically defensible and therefore verification bias may have affected our results.

Our outcome is based on a composite of both cytological and converted histological data for cases where only cytology was performed and where direct histological data

were absent. Therefore this study limits evaluation of the accuracy of cytological follow-up, because cytology was included in the definition of treatment outcome. Strength is that follow-up data was available from all women and that average follow-up time was 3.6 years.
The composition of our study population was heterogeneous since some women had been treated for more than 12 months before inclusion and others less than 12 months. Surgical treatment of CIN affects the clearance rate of HPV infection and is therefore a time-consuming process, which means our results may have been affected. How HPV mRNA is affected by conisation is unknown since to our knowledge this is the first study to investigate HPV mRNA after treatment. Aerssens (203) observed a pronounced decrease in presence of HPV immediately after women were treated with LEEP conisation or with cryotherapy. Six months, one year and two years after treatment the detection rates of HPV in the LEEP group were 20.3%, 15.3% and 8.4%, respectively. Kim et al. also concluded that HR HPV infection cleared gradually in most patients within 6 months of treatment and that a persistent HPV infection was a significant positive predictor of recurrence (204). A study protocol that determined exact intervals for clinical follow-up and testing would also have been useful when comparing results. Clinical performance on long-term follow-up may advantageously be judged by the cumulative incidence of CIN2+, since women may also undergo additional treatment such as repeat conisation during the follow-up period.
Specimen storage
False negative mRNA test results on the Aptima may possibly result from factors such as inappropriate storage conditions and excessive time since collection, which could lead to mRNA degradation and help explain the low sensitivity. mRNA is less stable than DNA. The liquid-based samples collected at the inclusion visit were stored in PreservCyt solution (ThinPrep) vials at room temperature and tested according to manufacturer's instructions. The Aptima HPV Assay is validated for specimens collected in ThinPrep Pap test vials containing PreserveCyt solution. Storage time ranged from a couple of months up to several years. Further, in our study, a positive test result was defined by an analyte signal-to-cut-off ratio (S/CO) of 1.00. According to current test interpretations an analyte S/CO>0.50 is considered positive. This difference may have resulted in lower sensitivity for high-grade disease in our study. However, two other studies, one from Clad et al. (205), the other from Waldstrom et al. (206), reported that the APTIMA is able to detect HPV high-risk mRNA with strong correlation to disease in retrospective LBC specimens stored at room temperature for up to three years.

6.2.3 Comparison with other studies

To our knowledge this study was the first to compare testing for HPV E6/E7 mRNA from 14 high-risk HPV types (APTIMA) with testing of high-risk HPV DNA for prediction of residual/recurrent disease in follow-up of women treated for CIN.
HPV DNA testing to predict cure or failure of treatment has been recommended in the Swedish national guidelines since 2010, but there is little data on the comparative performance of different HPV tests in a post-treatment setting. A Scottish study presented at the 2013 Eurogin meeting (European research organisation on genital infection and neoplasia) compared the performance of 5 HPV tests, including the Aptima HPV Assay, in a post-treatment setting. The mean follow-up period was 13.2

months. All tests were 100%-sensitive for detection of CIN3+ and specificity ranged from 75% to 84% according to assay. Detailed information about Aptima test performance is not available since this information came from a conference publication of abstracts. A Norwegian study by Tropé A et al. evaluated postconisation mRNA testing to predict residual high-grade disease. They demonstrated a sensitivity of 45.5% for the Pre-Tect HPV Proofer, which detects mRNA from 5 HR HPV types. They concluded that the Pre-Tect HPV Proofer did not appear to be suitable for short-term follow-up (207). In a recent summary of 15 studies, Arbyn et al. demonstrated the pooled sensitivity for HR HPV DNA testing (HC2 and PCR) to predict residual or recurrent CIN2+ after treatment to be 93% (95% CI: 85-97%), while sensitivity was 72% (95% CI: 66-78%) for cytology. The pooled specificities were 81% (95% CI: 74-86%) and 84% (95% CI: 74-86%) for HPV and cytology, respectively. HPV testing was significantly more sensitive (ratio 1.25) but not significantly less specific (ratio of 0.97) than cytology. These findings are consistent with our results (134). The Aptima test has not been evaluated in the post-treatment setting, but has demonstrated equal sensitivity but higher specificity compared with HPV DNA testing in other settings (triage and primary screening). In a study by Brismar et al. HPV testing predicted all recurrent disease while HPV genotyping increased the PPV with a lost in sensitivity (208). Söderlund-Strand, however demonstrated that only type-specific HPV persistence predicted recurrent or residual disease and that HPV genotyping was useful to improve that specificity post-treatment (161).

6.2.4 Implications for continued research and practice

In the future, one might envisage a follow-up strategy that includes both HPV DNA and cytology. Women who are HPV DNA-negative at 6 and 24 months after treatment could be safely referred back to the usual screening programme, while HPV DNA-positive women should be followed annually until negative.
Meta-analyses, or ideally large RCTs, are needed to compare HPV DNA testing with newer biomarkers such as HPV type-specific mRNA over an extended period of time to help establish an optimal follow-up strategy for women treated for CIN. Finding an indicator that predicts successful outcome and shortens the follow-up period would be especially helpful. One area of interest would be to determine the relevance of HPV positivity in the absence of cytological abnormality and how to manage these women who are referred back for colposcopy due to positive HPV status, but without signs of disease. It would also be of interest to evaluate a possible role for HPV self-testing after treatment and the efficacy of HPV vaccination to prevent recurrent disease.

6.3 DISCUSSION PAPER 3

6.3.1 Main findings and interpretations

Aptima was the most sensitive test to predict high-grade CIN among HPV-positive women with minor cytological abnormalities diagnosed with ASC-US on index cytology and among women with LSIL on index cytology, compared with the other tests. Aptima detected 100% of CIN3+ and 77.8% of CIN2+ in the ASC-US group and detected 76% of CIN3+ and 78% of CIN2+ in the LSIL group. Specificity to predict

absence of CIN2+ or CIN3+ was 50.0% and 45.5% respectively, in the ASC-US group and 25.0% and 23.8% respectively in the LSIL group. Specificity for CIN3+ is not clinically useful. CIN2 results would be considered to be false-positives, which is inappropriate since most clinical guidelines use CIN2 as the cut-off for treatment. Aptima was the least specific test in both groups with the one exception of repeat cytology, which achieved 25.0% specificity in the ASC-US group when ASC-US+ was used as a cut-off. Aptima specificity in the LSIL group is considered too low to be useful for triage since too many false positives would be generated.

The test accuracy of Aptima in the ASC-US group is consistent with findings from a meta-analysis of diagnostic accuracy for triage of women with ASC-US or LSIL, involving 8 studies where the pooled sensitivity and specificity of Aptima to detect CIN2+ was 95.7% (95% CI 91.5-97.2) and 56.4% (95% CI 44.7-67.5), respectively, and 96.2% and 54.9%, respectively, to detect CIN3+ (117). This meta-analysis showed greater specificity without loss in sensitivity for Aptima in triage of ASC-US and LSIL for detection of high-grade CIN compared with HC2, the most common HPV DNA test. Considering the widely accepted evidence for use of HC2 in triage of ASC-US and the similar sensitivity of Aptima, the higher specificity of Aptima would support the use of Aptima in triage of ASC-US cytology (without knowledge of HPV status). In our study, all women were HR HPV-positive and Aptima showed similar accuracy in the ASC-US group as in the meta-analysis; we therefore concluded that Aptima is suitable for triage in this group too. However, our ASC-US study group was small (n=25) and larger studies are needed to confirm these findings.

However, in triage of HPV-positive LSIL, our results show lower sensitivity (91.0% and 96.7%) and specificity (42.5% and 38.7%) for Aptima to detect CIN2+ and CIN3+ than the pooled results as reported in the aforementioned meta-analysis and therefore they cannot be used to efficiently triage women with HPV-positive LSIL.

The HPV16 DNA results from our study showed significantly higher specificity than Aptima to predict the absence of CIN2+, 87.5%, and CIN3+, 81.8%, in the ASC-US group, and 72.4% and 72.1%, respectively, in the LSIL group. No gain in specificity was observed with the addition of HPV18 DNA testing; on the contrary, specificity was less. HPV16 DNA testing identified women at substantial risk for cervical disease (PPV for CIN2+ 60.0% in the ASC-US group and 42.9% in the LSIL group), but sensitivity was low (33.3% and 37.5% for CIN2+ in ASC-US and LSIL, respectively), indicating a need for more aggressive follow-up of HPV16-positive women. Risk of disease among women positive for HPV16/18 was also substantial, albeit slightly lower (PPV for CIN2+ was 50% in ASC-US and 39.7% in LSILs). The >30% risk of disease (CIN2+) despite a negative HPV16 DNA or HPV16/18 DNA test indicates that these women should not return to the normal screening schedule.

Risk of significant disease among ASC-US cases despite a negative test (the complement of the negative predictive value, cNPV=1-NPV) was never low for any of the evaluated triage tests, with the one exception of Aptima in the triage of ASC-US for the outcome CIN3+ (zero risk). In LSIL triage, risk of disease remained high even when triage tests were negative (cNPV ranged from 28.0-32.7% for CIN2+ and from 14.0-18.6% for CIN3+), indicating that a negative triage test should not allow women to return to the normal screening schedule. In ASC-US triage, a negative Aptima test resulted in lower risk of disease compared with other tests (cNPV 20.0% for CIN2+); the difference was significant when compared with repeat cytology using LSIL+ or HSIL+ as a cut-off. In LSIL triage, a negative Aptima test did not decrease the risk of

disease. In fact, risk of CIN3+ was even significantly higher compared with a negative HPV16DNA, HPV16/18DNA or repeat cytology finding of ASC-US+.
Overall, in LSIL triage most tests showed accuracy estimates that did not deviate strongly from the neutral diagonal line (LR+ and LR- near 1), indicating poor triage for the LSIL group (Figure 1).

Repeat cytology

Considering ASC-US+ as the threshold for repeat cytology, sensitivity for CIN2+ was 55.6% and CIN3+ 66.7% among women with ASC-US on index cytology. The corresponding specificities were 25.0% and 68.2%. In the LSIL group the sensitivities for CIN2+ and CIN3+were 53.1% and 54.5%, and specificity for CIN2+ was 66.4%. Risk of CIN2+ was 28.0% even with negative repeat cytology. This study indicates that repeat cytology is inadequate for use in triage.
Our study population comprised HR-HPV-positive women, the majority of whom had a cytological diagnosis of LSIL (87.0%). 50% of the women were under the age of 30, which might have contributed to the observed low specificity since lesions in young women may be more prone to regression. A re-assessment of the data indicates that HPV66 is relatively common, though it is rarely found in cancers, which may decrease the specificity and PPV of assays in which this type is included (104). HPV66 is covered in the Aptima, which may also have contributed to low specificity. We found 5 cases of a single infection with HPV DNA type 66. The use of CIN2+ as an outcome has also come under discussion, since reproducibility of this diagnosis is considered to be poor (94).
The most prevalent HPV type in our study was HPV16, 20.7% in ASC-US and 31.1% in LSIL. The second most common type in the ASC-US group was HPV53 and in the LSIL group, HPV51 and HPV 52. Our finding that HPV16 was the most common type and HPV51 the second most common type in the LSIL group is consistent with HPV prevalence in another Swedish study by Söderlund-Strand (3).

6.3.2 Methodological considerations and validity

The HPV DNA test and the Aptima test were performed on cellular material stored in PreservCyt medium, validated for the aforementioned tests. Midwives collected the samples as part of the cervical cancer-screening programme (2005-2008). Regardless of HPV status, women with ASC-US/LSIL were referred for further work-up and colposcopy according to clinical guidelines and thus the clinicians who examined the women were blinded to the HPV and mRNA test results. Aptima testing was carried out in 2010 on residual material from LBC samples. Aptima detects mRNA, which is more fragile than DNA. Therefore test accuracy could be compromised by factors such as transport and storage of samples. Our specimens were stored for up to 5 years at room temperature in PreserveCyt medium before testing with the Aptima HPV Assay. According to instructions from the manufacturer, the Aptima test is validated for samples collected in PreservCyt medium, but recommended “storage at 2°C to 8°C, with no more than 30 days at temperatures up to 30°C. If longer storage is needed, the PreservCyt liquid Pap specimen or the PreservCyt liquid Pap specimen diluted into the Specimen Transfer Tube may be stored at -20°C or colder for up to 24 months.” We have previously reported on two other studies that demonstrated that the Aptima is retrospectively able to detect HPV high-risk mRNA in LBC specimens stored at room temperature for up to three years and retain strong correlation to disease (205, 206).

Our group has discussed the issue of RNA strand fragmentation; according to our laboratory expert, "if the mRNA strand is around 100 bp long, it is unrealistic to conclude that degradation might have caused false negative results." Aptima was FDA-approved in 2011, and has proven to be highly robust (114).
Ideally, to verify or rule out disease we would have obtained biopsies from all women regardless of colposcopy test results, but this was not feasible. Therefore a negative colposcopy was taken as absence of disease and when lesions were observed by colposcopy they were biopsied. This management introduces a type of verification bias known as the double gold standard bias, in which different gold standards are used for those with positive and negative test results, which increases both sensitivity and specificity. The best way to avoid such bias is to design a prospective study in which all patients receive definitive verification of disease status. The delay between sample collection (triage testing) and disease verification (clinical policy is 3 months) may also have influenced test accuracy since some lesions may have undergone interim regression. It would have been interesting to follow mRNA positive and negative lesions over time to see if negatives were more prone to regression, but this is not feasible due to current screening guidelines that use CIN2+ as a cut-off for treatment.
The ASC-US study population was small for drawing robust conclusions.
The strength of the present study lies in case verification and in the quality of follow-up data via medical records; in cases with insufficient information, data were supplemented with information from the Stockholm Oncology Centre. The long observation time (4 years) covers a full 3-year screening interval, allowing us to comment on the performance of the triage tests within the context of a programmatically relevant follow-up period.

6.3.3 Comparison with other studies

A triage test should be able to decide whether women need referral for colposcopy; in other words, able to differentiate a high-risk group for disease from a low-risk group. A test that maximizes sensitivity and specificity would allow effective triage.
One useful strategy may be to use the most sensitive test first (i.e. HPV DNA testing), followed by a more specific test (cytology or a new biomarker) afterwards to rule out false positives. In triage of LSIL cytology, more specific tests would be preferable, given the high prevalence of HPV. That might allow identification of the women at lowest risk and justify returning them to the regular screening program, but the problem so far has been low sensitivity and the risk of not detecting disease.
We studied a select group of women who were already at higher than average risk for precancer; the aim of a triage test in such a setting would be to identify the women at highest risk for immediate follow-up, since a negative test does not justify allowing these women to return to the regular screening programme.
Until now, most studies have focused on triage of ASC-US/LSIL cytology or HPV positivity. The Aptima HPV Assay is an attractive option due to its theoretical ability to detect oncogenic transforming infections of 14 HR HPV types, in contrast to the PreTect HPV-Proofer, which only detects HPV mRNA of 5 HR types (HPV16, 18, 31, 33 and 45). Aptima has been shown to be substantially more sensitive (ratio 1.91 (95% CI: 1.43-2.56)), but less specific (ratio 0.47 (95%CI: 0.34-0.63)) for predicting CIN2+, compared with the PreTect HPV-Proofer. Arbyn et al. proposed rules of thumb for test accuracy regarding the use of equivocal cytology for triage: a) Clinical sensitivity of at

least 90% for detection of CIN2+ with a relative sensitivity compared with HC2 not significantly lower than unity and a lower confidence interval (CI), but no lower than 0.90. b) Relative specificity not significantly below unity with a lower CI not below 0.95. Aptima has been shown to fulfil these criteria, but Arbyn writes "it should be recognised that the specificity for excluding high grade CIN2 (in the range 40-60% and consequently also the PPV (range 20-30% for CIN2+, range 10-15% for CIN3+)) is not optimal, allowing for the use of more specific markers, such as genotyping for HPV16 and 18 or RNA testing for 5 types. These markers allow identification of women at highest risk (PPV for CIN2+ >30%), warranting more intensive follow-up but women who test negative must not be referred back to routine screening, but require follow-up (117)." Arbyn et al. conducted the aforementioned meta-analysis of the diagnostic accuracy of Aptima vs. HC2 in women with either ASC-US or LSIL (HPV status was not known). Castel et al. also presented a risk management model for cervical cancer prevention according to which a triage test is considered feasible when the NPV is equal to or exceeds a predefined threshold of 98% (209).

Not many studies, to our knowledge, have evaluated triage of HPV-positive women with minor cytological abnormalities. Mesher et al. compared several HPV- (HC2, Abbot Real-time PCR, BD HPV test, Cobas 4800, PreTect HPV Proofer and Aptima) and p16INK4a-test strategies to triage women referred for low-grade cytological abnormalities in a fusion of the two Predictor studies. Five HPV tests were found to be highly sensitive and were able to reduce the number of referrals for colposcopy by 20%-30%. Aptima had sensitivity of 99% for detection of CIN3+, and a specificity of 34.7% for CIN2+. The sensitivities for detecting HPV16 ranged from only 66.0%-75.5% and the specificities from 81.3%-87.6%. Specificity could be improved by an additional 20%-30% if referrals were limited to women testing positive for HPV16 or p16INK4a. That strategy had little impact on sensitivity for CIN3+ but sensitivity for CIN2+ was slightly reduced. The authors concluded that short-term surveillance would be needed (210). A study by Rijkaart et al. compared 14 different triage strategies of HPV DNA-positive women for colposcopy referral rate, for detection of CIN3+. Triage with cytology, followed by repeat cytology at 12 months yielded a high negative predictive value (99.3% CI 98.1-99.8) and a markedly lower colposcopy referral rate of 33.4% (95%CI 30.2-36.7)(166). By comparison, triage with HPV DNA16/18 at baseline for detection of CIN3+ had a sensitivity of 65.4% and a specificity of 72.5%. Another study by the same author investigated whether HR HPV mRNA detection by Pretect HPV Proofer can be used as a reflex test to stratify HR HPV DNA-positive women with different cytological diagnoses for risk of CIN2+. The results showed that a positive mRNA test conferred an increased risk of CIN2+ in HR HPV DNA-positive women, especially among women with normal cytology (211). The mRNA test result did not influence risk of CIN2+ in cases of borderline or worse cytology. Rossi evaluated mRNA (Pretect HPV Proofer) as a prognostic biomarker for progression to CIN2+ following negative colposcopy or CIN1 histology among women referred for colposcopy because of minor cytological abnormalities, where the majority of women were HR HPV-positive. He found the absolute CIN2+ risk to be 18.4/1000 person/years in the mRNA-positive group and 3.6/1000 person/years in the mRNA-negative group. He concluded that mRNA is a good candidate for management of HR HPV DNA-positive women, especially to help reduce intensity of follow-up among women who test negative. However, only a small number of patients had the outcome CIN2+. A study by Benevolo et al. evaluated the diagnostic and prognostic

performance of the PreTect HPV Proofer among HR HPV-positive women with minor cytological abnormalities and found that mRNA positivity was significantly more associated with CIN2+ lesions than CIN2- lesions. Longitudinal specificity after 2 years of follow-up was high, 89%, but sensitivity low, 50%.
Methods with higher specificity and lower sensitivity, such as the PreTect HPV Proofer or genotyping for HPV16DNA or for HPV16/18DNA should perhaps be considered as a two-step management strategy, with referral for triage-positive women and repeat testing for triage-negative women. To date, the best evaluated triage strategy among HPV positives is repeat cytology. However, loss to follow-up may be a problem when implementing a screening strategy that involves a repeat test when reflex testing is not feasible. Rijkaart reported about a 40% loss to follow-up among HR HPV-positive women with normal cytology at baseline and other studies have reached similar conclusions. Therefore, adequate communication is important. In the POBASCAM trial (primary screening study for HPV) in the Netherlands, 77% of women followed up with repeat testing (167).

6.3.4 Implications for continued research and practice

At present we are experiencing an increasing trend of minor cytological abnormalities among younger women (135) who also have a high prevalence of HPV. In a Stockholm study, 64% of women with ASC-US and 77% of women with CIN1 were HPV-positive. HPV-positivity is age-dependent, with 81% prevalence among women below age 35 and 44% prevalence among women above age 45 (155). All young women (<30 years) referred for colposcopy because of minor cytological findings are HPV-positive. Colposcopy may lead to increased detection of lesions that normally would have regressed spontaneously. In addition, it leads to increased workload for gynaecological clinics, increased risk for overtreatment, perhaps with adverse obstetric outcomes, and increased anxiety among women.
We need to learn more so we can provide optimal clinical management of young women since their risk for cancer in general is extremely low. Studies of new biomarkers such as HPV16/18DNA, E6/E7mRNA, p16INK4a/Ki 67 or DNA methylation may contribute additional knowledge, but it is important to conduct more age-stratified analyses in the future. So far we lack data from long-term follow-up studies of HPV in primary screening for women under 30. Studies of women over age 30 have shown that HPV testing contributes to early detection of high-grade CIN without resulting in over-diagnosis.

6.4 DISCUSSION PAPER 4

6.4.1 Main findings and interpretations

HR HPV status had the significantly greatest impact on risk of developing high-grade cervical disease among women with minor cytological abnormalities.
We found that the long-term risk of developing CIN2+ following a negative HR-HPV DNA test was low during follow-up, regardless of ASC-US or LSIL diagnosis. For HR HPV-negative women with LSIL on index cytology, risk increased after 4.5 years of follow-up, suggesting that these women should be followed at intervals shorter than 4.5 years. The 3-year interval of the organised screening programme would seem

appropriate. The additional few cases identified among women with HPV-negative LSIL may reflect false negative cytology or newly acquired HPV infections during follow-up, but are less likely to be due to false negative HPV DNA tests.
Many studies have shown that the risk of developing pre-cancerous lesions and ICC is very low among women who are negative on both cytology and HR-HPV DNA testing (78, 174, 212). Therefore triage using HPV DNA testing for ASC-US cytology has been extensively evaluated and is now recommended to identify women at the highest risk of cervical disease who need referral for colposcopy and biopsy. A negative HPV DNA test is also reassuring and these women can wait at least one year for retesting.
Triage of LSIL was not previously recommended, since the most commonly used HPV DNA test for evaluation, Hybrid Capture 2 (HC2), was no more sensitive and substantially less specific than repeat cytology (154). However, a recent Cochrane review demonstrated that HC2 had significantly higher sensitivity, which could justify recommending HR-HPV DNA testing for triage of LSIL. Recommendations should be based on local cost-effectiveness analyses, local HPV prevalence and compliance with follow-up (134, 135). Swedish guidelines recommend HPV triage of all women with minor cytological abnormalities regardless of whether the diagnosis is ASC-US or LSIL, but recommend repeat cytology at one year among women who were HPV DNA-negative, just to be on the safe side (39, 155). Recent years have seen an increase in the diagnosis of minor cytological abnormalities, resulting in increased colposcopy referrals. Since referrals require significant time and resources from the healthcare system and can be stressful for women, this begs the question of whether HPV-negative women could return to the three-year screening interval. Our results demonstrate a consistently low risk for CIN2+ among HPV-negative women with minor cytological abnormalities during the first four years of follow-up and therefore (at least with the performance of the HPV test used in the present study) retesting after 1 year is not required.
We found that women diagnosed with HPV16/18 at baseline were at significantly higher risk of developing CIN2+ than HPV-negative women. Furthermore, our data imply that a positive HPV16/18 test at baseline is a better predictor than other HR-HPV types of who is at increased risk of developing high-grade lesions, even after 5 years. However, the risk of developing CIN2+ was substantial, even among women who were positive for HR-HPV types other than HPV16/18.
Risk stratification by genotyping in this group probably will not alter clinical management in any way because the risk of pre-cancer is so high that immediate colposcopy will be required. However, in populations with limited access to follow-up immediate treatment may be motivated. Identifying the presence of HPV16/18 may focus attention on this high-risk group, but may also cause considerable anxiety among these women. Commercially available FDA-approved HPV DNA tests such as the Cobas 4800 (Roche Molecular Systems, Pleasanton, CA, USA) can simultaneously detect HPV16 and HPV18 individually, as well as 12-pooled HR-HPV types (213). The rather high risk of developing CIN2+ among women with LSIL reflects the high prevalence of HR-HPV (79.4%) in this diagnostic category.

6.4.2 Methodological considerations and validity

A major strength of our study is the link to the National Quality Register for Cervical Cancer prevention (NKCx) from which we retrieved all follow-up data and to which all

cytological and histological results are reported. Correct links are ensured by using personal identification numbers for all women, which reduces loss to follow-up. Furthermore, our study reflects a real-life clinical setting in Sweden, as well as long-term follow-up in clinical practice. One study reported that most slides diagnosed as ASC-US in the US and UK is reported as normal in Sweden, which could affect comparisons of results between countries (26). Furthermore, we have a high prevalence of HPV among our ASC-US and LSIL cases, which could also affect comparability of cumulative risk estimates between countries. We have not adjusted for verification bias. Colposcopy biopsies were taken from visual lesions and women were considered disease-free if no lesions were seen. This could have affected our calculations, but it also allows our results to apply to other real-life settings. Some women might have been treated because of persistent low-grade disease, which may have censored outcome development. Because low and moderate CIN lesions often regress and the diagnosis is often poorly reproducible, ideally the outcome would have been CIN3+ or cancer (94), but that would have required a larger study population. However, in a clinical setting CIN2+ is of great interest since it represents the threshold for patient treatment (214).

6.4.3 Comparison to other studies

Several large randomised controlled studies (RCT) and longitudinal cohort studies have demonstrated that HPV negativity is protective against development of pre-cancer (78) (212, 215), though 100% assurance can never be guaranteed. Castle et al. reviewed case histories of 33 baseline HR-HPV negative CIN3 cases and found evidence that these cases were due to incident (new) cases (n=12), non HR-HPV (n=5), misclassified histology (n=8) and false negative HR-HPV (n=8) tests. They concluded that among women with cytological abnormalities, a few cases of cervical pre-cancer would be found that would test HR-HPV negative for one or more reasons (216). The low risk of HPV-negative ASC-US was confirmed in a large cohort study by Katki et al. that followed over one million women (217). They concluded that women with HPV-negative ASC-US were at similar 5-year risk for CIN2+/CIN3+ as women with a normal Pap smear and therefore could be managed similarly, namely with a 3-year retesting interval. In the same cohort, Katki et al. (218) also studied the risks of LSIL and found that HPV test results do modify risk, but only enough to consider repeat testing of HPV-negative LSIL at one year, rather than to refer for immediate colposcopy (219). Conclusions were based on a risk stratification concept introduced by Castle et al. that involves “equal management of equal risks” (209, 220), which states it is safe to return the patient to the regular screening programme if the 5-year risk of CIN3+ is <2%. If risk is 2%-10%, follow-up at one year is recommended, and if >10% referral for colposcopy is required. A study from Norway investigated a composite group of ASC-US and LSIL in delayed testing (6-12 months after screening detected ASC-US/LSIL cytology) in order to allow for viral clearance. They concluded that it is safe to return women with a negative HPV DNA test (Amplicore) to the regular 3-year screening schedule (221). The risk of CIN2+ was only 1.8% 3 years after a negative Amplicor HPV test. A recent study from the Norwegian Cervical Cancer Screening Programme (NCCSP) compared the short-time (6 months) and long-term (3 years) effectiveness of different HPV tests (Amplicore, HC2 and Pretect HPV Proofer) in the) and reported that the risk for CIN2+ among HPV-negative women with

persistent ASC-US/LSIL was over 2% and that returning these women to the normal screening programme was potentially unsafe. However, a normal repeat cytology (after 6 months) following an ASC-US/LSIL resulted in a low risk of severe abnormalities during the next screening round, justifying a return to the regular screening programme (222). Long-term follow-up of test accuracy after implementation of a new test in a prevention programme is of great importance. The Norwegian study illustrates the differences in the protective effect of different HPV tests, which underlines the importance balancing clinical sensitivity and specificity (clinical performance) to ensure safety for women participating in screening. It also highlights the importance of ongoing program evaluation when new screening routines are implemented. In a long-term follow-up of women with borderline and mild dyskaryosis (BMD), Kocken et al. reported that a negative HR-HPV test at baseline corresponded to a 5-year risk of CIN2+ of 9.9%, whereas a positive test increased the risk to 44.9%. They concluded that HR HPV-negative women may be referred for routine screening since their 5-year CIN3+ risk is negligible, which is also consistent with our results (223).
As in our study, several others have previously demonstrated high risks for pre-cancer among HPV-positive women and especially among those positive for HPV16 and/or 18 (77, 224). Kocken reported that women with borderline and mild dyskaryosis (BMD) who were positive for HPV16 were at higher risk for CIN3+ than women infected with other HR-HPV types. Their 5-year risk was 49.8%, vs. 29.8% among women infected with other HR-HPV types (223).

6.4.4 Implications for continued research and practice

In this fourth study we have taken advantage of the excellent Swedish register, NKCx and linked data from our own “biobank” to get 100% follow-up results. LBC is an excellent method, which allows sample material that is left over after cervical cytology slides preparation (with large amounts of cells with well-preserved DNA, RNA, proteins and cellular morphology) to be used for additional analyses. Systematised collection and storage of biological samples in biobanks, as well as linkage to nationwide registers such as the cancer registers or histopathology registers, provide invaluable resources for research in the future. Great effort should be dedicated to such initiatives. The Swedish Cervical Cytology Biobank (SCCB) is one such project that began a couple of years ago and will help ensure these endeavours for the future.
As discussed in other sections of this thesis, more knowledge of biological markers will hopefully help us to stratify risk in clinically challenging groups such as HPV-positive young women with minor cytological abnormalities, with CIN2+ lesions, or in discordant cases of high-grade dysplasia with normal or minor colposcopic findings.

7 CONCLUSIONS

7.1.1 Study I:

We found that testing for HR-HPV DNA with HC2 is a more sensitive method than repeat cytology with Pap smear to detect high-grade CIN. HR-HPV DNA testing for triage of women with minor cytological abnormalities has now become an established method. However, test specificity needs to be improved and will be a task for future research.

7.1.2 Study 2:

Our second study confirms the finding of previous studies that detection of HR-HPV DNA after treatment by conisation identified 100% of women with residual/recurrent high-grade disease. However, detection of HPV E6/E7 mRNA was a poor predictor of treatment failure. In this study we suggest that a negative HPV mRNA result cannot exclude the risk of malignant progression, and that HPV E6/E7 mRNA testing by APTIMA HPV assay is not useful to follow up women treated for CIN. More specific markers are needed to predict long-term results on follow-up after treatment.

7.1.3 Study 3

Our study was the first (to our knowledge) to investigate detection of HR-HPV E6/E7 mRNA by Aptima HPV Assay for triage of HR-HPV DNA-positive women with minor cytological abnormalities. Our results support Aptima triage of women with ASC-US cytology, but not with LSIL cytology. HR-HPV genotyping for HPV16 and HPV16/18, as well as repeat cytology were all more specific markers, but had low sensitivity and were associated with a higher than negligible risk for cervical disease, despite negative test results. Therefore these women could not return to the standard screening programme. Finding biological markers with the ability to distinguish between transient and persistent infection remains a challenge for future research.

7.1.4 Study 4

We found that HR-HPV DNA status had the greatest impact on development of high-grade cervical lesions among women with minor cytological abnormalities. The risk for cervical pre-cancer was low during the first 4.5 years of follow-up among HPV DNA-negative women, suggesting that these women could safely be returned to the standard screening programme instead of undergoing repeat testing after one year, as currently recommended. The highest risk of developing high-grade lesions was observed among HPV16/18-positive women, suggesting a need for a more aggressive follow-up.

8 FUTURE RESEARCH

Research on prevention of cervical cancer has progressed rapidly and extensively over the past 25 years. The understanding that HPV infection is the central and necessary cause of cervical cancer has inspired new approaches to disease prevention through improved screening methods and HPV vaccination. The first HPV vaccine was approved in 2006, and in 2008 Dr Harald zur Hausen received the Nobel Prize in Physiology or Medicine for his pioneering work concerning the role of HPV in the development of cervical cancer (225). Many areas in this field remain to be elucidated by future research, but I will concentrate here on a discussion of questions related to our studies. In current population-based cervical cancer screening programmes, the vast majority of abnormal findings are of minor grade. The risk of underlying high-grade cervical disease cannot be ignored, but the overwhelming majority have transient HPV infection. Clinical management of minor cytological abnormalities has recently changed course (HR-HPV DNA triage) in an effort to reduce the number of women requiring further examinations. However, our diagnostic tools are still too blunt at the price of heightened anxiety among affected women and a heavy burden on the healthcare system. Basic research on the natural history of HPV infection is important to elucidate the mechanisms involved in clearance or persistence of infection and how HPV evades the immune system. Specific biological markers are needed. One unanswered question is whether infections clear completely, or become latent, only to reappear later in life. Studies indicate that "clearance" simply reflects a dip in viral load below the threshold of detectability. HPV DNA testing is carried out on exfoliated cell smears, which sample the upper cellular layers of the ectocervix, and may therefore miss low viral load infections confined to the basal layers, making it difficult to verify whether infections have fully cleared or remained latent. Could reactivation occur later in life? If so, what are the triggers? Could this explain the second peak of HPV prevalence in the perimenopausal age? Or the higher risk of recurrence among women treated for CIN, which is higher still among women treated later in life, as a recent study has suggested? Perhaps we could also find an answer to the question about whether there might be an indication for vaccination to prevent recurrent disease/reinfection following conisation.

How do we improve the specificity of HPV DNA testing? Should thresholds for viral load in the tests be increased, or can new biomarkers help us? Again, the elucidation of these questions requires more knowledge of the biology and life cycle of HPV.

HPV typing is the most important predictor of the likelihood of lesion progression, especially in regard to HPV16, but even HPV18 makes a substantial contribution. Genotyping for HPV16/18 and perhaps for additional high-risk types improves the PPVs, but will it be safe and effective enough for screening or in triage, since risks associated with other HR-HPV types still remain?

Measurement of mRNA from the HPV E6 and E7 oncogenes provides high specificity for differentiating benign productive infections from those that have initiated neoplastic progression and those that are already cancerous. However, our studies, which reflect the real-life setting, were unable to confirm the usefulness of this approach. Although few studies have addressed the predictive value of mRNA testing following treatment for CIN, or among HR-HPV positive women with minor cytological abnormalities, the role of such mRNA testing is still worth elucidating through additional and larger

studies. However, it raises the question of whether measurement of HPV E6/E7 mRNA in clinical practice is sufficiently adequate to assess a transforming infection, or are other molecular events of greater importance? What triggers the irreversible step of HPV DNA integration? Is there an early indicator of this step? Are there other biomarkers that are more useful? Another interesting area of research that has received great attention in recent years involves epigenetic changes in HPV viral and host genes. The hypothesis is that methylation of genes affects expression through silencing and thereby the natural history of cervical neoplasia. This process may result from a host response to a foreign intracellular agent, or from a signal that indicates viral integration into the host genome. Our studies have confirmed the acceptance of histopathology as the gold standard, though even this method is prone to subjective interpretation. This field would also benefit from identifying more objective biological markers.

Last but not least, HPV positivity leads to patient anxiety and confusion related to guidelines, as does the shift from an oncologic to a sexually transmitted infection (STI)-directed approach. This shift will require educational programs for both healthcare providers and patients concerning HPV testing in the future (8, 226).

HPV transmission is increasingly common among young women worldwide and several markers such as TERC, specific proteins, and immunological markers may help us to develop more effective strategies for early detection and treatment of women with ICC. If we could identify women at risk of developing this cancer on the basis of a single marker, or a combination of different markers, such analyses would likely become a substitute for, or a complement to, cytological screening. Studies indicate that the first generation of HPV vaccines may prevent at least two-thirds of ICC. A combination of improved detection of precursor lesions by molecular markers in screening programs, and vaccination against HPV could make this the most preventable cancer on earth.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Incidensen av livmoderhalscancer har minskat med 60 % i Sverige sedan populationsbaserad vaginalcytologisk screening infördes men fortfarande drabbas ca 450, till övervägande del unga kvinnor, av denna cancertyp årligen.
Livmoderhalscancer orsakas av infektion med särskilda högrisk-typer av humant papillomvirus (HR HPV). Sådana infektioner är vanliga, särskilt bland unga kvinnor, men läker oftast ut spontant. Om infektionen blir kronisk kan den dock leda till cancerutveckling. De flesta cellförändringar övergår inte heller i cancer utan förblir stationära eller läker ut spontant. Målet med denna avhandling var att identifiera riskfaktorer för att kunna prognostisera en senare cancerutveckling hos kvinnor med lätta cellförändringar och hos kvinnor som genomgått behandling för cellförändringar. Genom utvärdering av nya känsliga diagnostiska metoder, ökad kunskap och ett förbättrat omhändertagandet av kvinnor med lätta cellförändringar skulle den gynekologiska hälsokontrollen kunna förbättras.
Den första studien publicerades redan 2005. Ett problem inom cellprovsbaserad screening är att merparten av alla avvikande prov består av lindriga cellförändringar (ASC-US -svårvärderad skivepitelatypi och LSIL -lätt dysplasi). Dessa förändringar är ospecifika. Det behövs bättre metoder för att identifiera riskpatienter, så att risken för överdiagnostik, överbehandling och onödigt psykiskt lidande kan minimeras. En möjlighet är att komplettera cellprovet med HPV-test, så kallat HPV-triage, för att kvinnor med ökad risk för framtida livmoderhalscancer skall kunna särskiljas från dem med reaktiva förändringar. HPV-test har en betydligt högre känslighet än cellprov att upptäcka behandlingskrävande cellförändringar (cervikal intraepitelial neoplasi grad 2 eller värre, CIN2+) och därmed även en god förmåga att förutse frånvaro av CIN2+. En nackdel är dess låga träffsäkerhet (specificitet), dvs. stor andel falskt positiva prover med avseende på CIN2+, som är mest uttalad bland unga kvinnor.
I den första studien vi har sammanställt resultat från 177 kvinnor med lindriga cellförändringar på livmodertappen. LSIL och ASC-US upptäcks hos mer än 5 % av kvinnor som kommer till gynekologisk cellprovtagning. Endast en liten del av dessa utvecklas till precancerösa och invasiva förändringar. Det har varit svårt att avgöra vilka lindriga cellatypier som behöver avlägsnas i profylaktiskt syfte. På de flesta håll i Sverige tog man enligt de rutiner som fanns ett nytt cellprov efter 3-4 månader om det första provet visat lindrig atypi. Logiskt sett borde kvinnor som är negativa för HPV inte behöva genomgå utredning och behandling. Med det arbetet jämförde vi HPV-testets och cellprovets känslighet (sensitivitet) vid uppföljningen av kvinnor med lindriga cellförändringar. Vi fann att HPV förekom i 66 % av dessa förändringar. Känsligheten för en upprepat cytologi prov var 61 % och för HPV-test -82 %. Resultatet av den studien och andra har visat att HPV testet är mycket bra på att sortera ut kvinnor med ökad risk för allvarliga cellförändringar bland kvinnor med lindriga cellförändringar och därför har riktlinjerna i Sverige sedan ett par år tillbaka ändrats. Numera kallas endast HPV positiva med lätta cellförändringar till utredning hos gynekolog medan HPV negativa inte utreds vidare. HPV negativa kvinnor kallas dock för säkerhets skull till ny cellprovs kontroll efter ett år. Detta har resulterat i vissa besparingar, både beträffande ekonomiska resurser och beträffande kvinnors psykiska hälsa.

Det är känt sedan länge att närvaro av HPV är en nödvändig, men inte tillräcklig faktor för utveckling av livmodershalscancer. Nu är det bevisat att en kronisk infektion med en HR-HPV typ är en betydande riskfaktor. De virala onkoproteinerna (cancerframkallande proteiner) E6 och E7 har störst betydelsen för omvandlingen av en normal cell till en cancercell och för upprätthållandet av cancerutvecklingen då de stör funktionen av cellens tumörsupressor proteiner (proteiner som normalt förhindrar att en cell omvandlas till en cancercell); pRb (retinoblastomprotein) och p53. Detta leder till en störd reglering av celldelningen och en minskad förmåga hos cellen att ”ta död på sig själv” vid allvarlig genetisk skada, vilket sammantaget kan leda till utvecklingen av cancer. Uttryck av de virala generna E6 och E7 har man kunnat mäta genom analys av så kallat E6/E7 mRNA (messenger ribonucleic acid) och det har visat sig användbart som markör för transformerande, cancerframkallande HPV infektioner. En ny metod för mätning av onkogen expression av E6/E7 mRNA från 14 HR-HPV typer (Aptima HPV Assay) har utvärderats i den andra studien. Förhoppningen var att testet med bibehållen känslighet och förbättrad träffsäkerhet (specificitet) skulle kunna identifiera tranformerande HPV infektioner för att bättre kunna hitta kvinnor med risk för utveckling av livmodershalscancer.
HPV E6/E7 mRNA (Aptima) testet utvärderades vid uppföljning av kvinnor efter behandling för att om möjligt bättre kunna förutsäga återfall i sjukdom. Alla kvinnor hade genomgått en så kallad koniseringsoperation, vilket innebär att en centimeter av livmodertappen tagits bort som behandling för allvarliga cellförändringar. När kvinnorna kom på sin första kontroll (143 st.) togs ett ”vanligt HPV test” (som mäter HPV DNA), ett HPV mRNA test (Aptima HPV Assay) och ett vanligt cellprov. Endast sju av 143 (5 %) kvinnor hade återfall sjukdom (CIN2+) under uppföljningstiden som i medeltal var 3,6 år. Det visade sig att HPV DNA testet var känsligast och upptäckte alla kvinnor med återfall i sjukdom, följt av cellprovet som upptäckte 86 %. Det minst känsliga testet var Aptima som missade tre kvinnor med behandlingskrävande cellförändringar (CIN2+). Vi konkluderade att Aptima inte lämpade sig som test för att förutsäga återfall efter behandling.
I det tredje arbetet utvärderades detektion av E6/E7 mRNA expression med Aptima HPV Assay hos kvinnor med lindriga cellförändringar. Eftersom metoden kan skilja mellan ofarliga HPV infektioner och farliga transformerande infektioner, skulle den kunna användas hos kvinnor under 30 år för att om möjligt reducera antalet gynekologiska besök med ca 70-80%. Särskilda HR HPV typer, som HPV16 och 18 har också visat sig speciellt betydelsefulla för utvecklingen av livmoderhalscancer och är därför intressanta. I studien testades 219 kvinnor med Aptima HPV Assay, HPV16, HPV16/18 och upprepat cellprov. Kvinnorna var alla HPV positiva och uppdelades i en ASC-US respektive LSIL grupp. 36 % av kvinnorna i varje grupp utvecklade behandlingskrävande cellförändringar under uppföljningstiden som var 4 år. Resultaten visade att Aptima var det känsligaste testet och upptäckte 78-100% av alla allvarliga cellförändringar (CIN2+) hos kvinnor med ASC-US men Aptima var inte lika känsligt hos kvinnor med LSIL (76-78%). Specificiteten var dock lägst och ett negativt Aptima test kunde heller inte i tillräcklig utsträckning utesluta frånvaron av CIN2+. Att testa för HPV16 och/eller HPV16/18 hade generellt låg känslighet men ett positivt test resultat innebar samtidigt en stor risk för CIN2+. Sammanfattningsvis visade studien att Aptima var tillräckligt bra för att rekommendera som triage-test av kvinnor i ASC-US gruppen, vilket också överensstämmer med resultat från en stor metaanalys från 2012(117). Inget av testerna var dock tillräckligt bra att rekommendera som triage-test

av LSIL då negativa test resultat inte kunde garantera att kvinnorna riskfritt kunde återgå till den normala screeningen.
I det fjärde arbetet studerade vi faktorer som har betydelse för utvecklingen av lätta cellförändringar till allvarliga (CIN2+) över tid. Vi följde 314 kvinnor i sju år med avseende på ålder, typ av lätt cellförändring (ASC-US/ LSIL), HR HPV status (positiv/negativ) och HPV typ (HPV16/18) vid studiens början. Information om kvinnorna utvecklade cellförändringar eller inte erhölls via det Nationella Kvalitetsregistret för Cervix Cancer Prevention (NKCx) vilket har 100 % täckning. Totalt utvecklade 28 % av kvinnorna CIN2+ under uppföljningstiden. Den största risken hade kvinnor som var HR HPV positiva vid studiens början och då särskilt om de var positiva för HPV16 och/eller HPV18. 54 % av HPV16/18 positiva utvecklade CIN2+ men hela 38 % av kvinnorna positiva för andra HR HPV typer utvecklade också CIN2+. HPV negativa hade den lägsta risken att utveckla allvarliga cellförändringar, särskilt under de första 4,5 åren. Endast ca 2 % av kvinnorna utvecklade CIN2+. Med detta som bakgrund föreslås att man överväger att avskaffa det ”kontroll test” som idag utförs efter ett år av HPV negativa och istället återför kvinnorna till det ordinarie screening programmet. Åldern hade ingen avgörande betydelse för utvecklingen till allvarlig cellförändring. Vårt övergripande syfte har varit att etablera klinisk användbara tidiga molekylära markörer för att förutsäga cancer progression hos kvinnor med lindriga cellförändringar vilket skulle kunna leda till en förbättring av den gynekologiska hälsokontrollen.
Sammanfattningsvis visar avhandlingen att HPV test kan användas för att identifiera kvinnor med störst risk för allvarliga cellförändringar och som därför bör utredas vidare av gynekolog. HPV mRNA test (Aptima HPV Assay) var i vår studie för okänsligt för att användas vid uppföljning efter behandling men fler och större studier bör utföras. Aptima visade sig däremot kunna användas för att sortera ut en högrisk grupp bland en subgrupp HR-HPV positiva kvinnor med lätta cellförändringar (ASC-US) men ett negativt testresultat kunde inte utesluta risken för sjukdom i framtiden. Infektion med högrisk HPV och då i synnerhet med HPV16/18 har störst betydelse för risken av utveckling från lätta cellförändringar till mer allvarliga. HPV negativa kvinnor har däremot så låg risk för CIN2+ att man kan överväga att avskaffa det nuvarande ”kontrolltest” som utförs efter ett år och istället återföra kvinnorna till den normala screeningen.

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