From the Department of Women's and Children's Health

Karolinska Institutet, Stockholm, Sweden

PREVENTION OF CERVICAL CANCER THROUGH USE OF LIQUID-BASED CYTOLOGY AND SUPPLEMENTARY HPV TESTING IN POPULATION-BASED SCREENING

Maria Fröberg

Karolinska Institutet

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To my family

For a chain to remain unbroken, each link must be strong.

ABSTRACT

The aim of this project is to evaluate an alternative screening strategy using liquid-based cytology (LBC), and supplementary detection of human papillomavirus (HPV) in cases of minor cytological abnormalities (atypical squamous cells of undetermined significance, ASCUS, and low-grade squamous intraepithelial lesion, LSIL).

Within population-based screening in Stockholm, Sweden, we alternated screening using LBC with supplementary HPV testing in ASCUS and LSIL (LBC+HPV testing, using Linear Array HPV genotyping Assay, Roche diagnostics) and conventional cytology (CC), from September 2005 to December 2006. LBC+ HPV testing (n=6075) and CC (n=4261) screening performance were compared. LBC+ HPV testing was evaluated over time (September 2005- December 2007). Diagnostic performance of HR-HPV detection as a triage test to identify high-grade precancerous lesions (CIN2+) in cases of ASCUS and LSIL was assessed, and age-specific HPV prevalence was studied. To assess the cost-effectiveness of HPV triage compared with immediate colposcopy or repeated cytology as a follow-up strategy for ASCUS and LSIL, an economic analysis was carried out from the perspective of the Swedish healthcare system, based on data from previous studies.

Comparing LBC+ HPV testing with CC, the adjusted OR for detecting CIN2+ and CIN3+ were 0.89 (95% CI: 0.64-1.25) and 1.02 (95% CI: 0.67-1.54) respectively. Detection of CIN2+ improved significantly over time for both methods. Positive predictive values were similar between methods for all endpoints. High-risk (HR-) HPV was found in 71% of LSIL and 49% of ASCUS cases (p=0.001) with similar prevalence between groups in women ≥30 years. HR-HPV prevalence was age-dependent in LSIL (p=0.01), with decreasing prevalence until age 50 years, followed by a slight increase. The negative predictive value of HR-HPV detection for histologically confirmed high-grade lesions was 100%. For women with ASCUS ≥30 years, HPV triage is the least costly alternative, whereas immediate colposcopy with biopsy provides the most effective option at a modest additional cost.

No obvious advantages of the LBC+ HPV testing strategy over CC were shown. However, detection of high-grade precancerous lesions improved significantly over time for both strategies. This finding suggests that introduction of LBC+ HPV testing may have lead to general improvement of expertise and increased vigilance in cytological interpretation. Observed changes underscore the importance of continuously monitoring rates of abnormal cytology to ensure balance and to guard against overdiagnosis or overconfidence in cytological evaluation. By using HPV reflex testing, additional extensive workup can safely be avoided in about 50% of all cases of ASCUS and LSIL among women ≥30 years. With highly sensitive HPV testing techniques at lower costs, HPV triage could become a cost-effective alternative. Cervical cancer screening will need to continuously adapt as HPV-vaccinated women reach screening ages and new potentially superior screening strategies are identified.

LIST OF PUBLICATIONS

I. MARIA FRÖBERG, Ingrid Norman, Bo Johansson, Anders Hjerpe and Sonia Andersson.
Liquid-based cytology with supplementary HPV testing versus conventional cytology in population-based screening to prevent cervical cancer.
To be submitted to British Journal of Cancer in April 2011.

II. MARIA FRÖBERG, Bo Johansson, Anders Hjerpe and Sonia Andersson.
Human papillomavirus "reflex" testing as a screening method in cases of minor cytological abnormalities.
British Journal of Cancer, 2008, 99, 563-568.

III. Sophia Brismar-Wendel, MARIA FRÖBERG, Anders Hjerpe, Bo Johansson and Sonia Andersson.
Age-specific prevalence of HPV genotypes in cervical cytology samples with equivocal or low-grade lesions.
British Journal of Cancer, 2009, 101, 511-517.

IV. Ellinor Östensson, MARIA FRÖBERG, Anders Hjerpe, Niklas Zethraeus and Sonia Andersson.
Economic analysis of human papillomavirus triage, repeat cytology, and immediate colposcopy in management of women with minor cytological abnormalities in Sweden.
Acta Obstetricia et Gynecologica Scandinavica, 2010, 89, 1316-1325.

CONTENTS

LIST OF ABBREVIATIONS

ICC	Invasive cervical carcinoma
SCC	Squamous cell carcinoma
ADC	Adenocarcinoma
TZ	Transformation zone
ASCUS	Atypical squamous cells of undetermined significance
AGUS/AGC	Atypical glandular cells of undetermined significance
ASC-H	Atypical squamous cells cannot rule out high-grade squamous intraepithelial lesion
AGC-H	Atypical glandular cells, favor neoplastic
HSIL	High-grade squamous intraepithelial lesion
AIS	Endocervical adenocarcinoma in situ
WNL	Within normal limits
CIN1	Cervical intraepithelial lesion grade 1 (low-grade dysplasia)
CIN2	Cervical intraepithelial lesion grade 2 (moderate dysplasia)
CIN3	Cervical intraepithelial lesion grade 3 (severe dysplasia, equivalent to carcinoma in situ)
CIN1+	Histologically confirmed CIN1 or a more advanced lesion
CIN2+	Histologically confirmed CIN2 or a more advanced lesion
CIN3+	Histologically confirmed CIN3 or a more advanced lesion
LBC	Liquid-based cytology
OR	Odds ratio
RR	Relative risk
CC	Conventional cytology
LBC+ HPV testing	Liquid-based cytology screening with supplementary HPV testing in cases of minor cytological abnormalities
PPV	Positive predictive value
NPV	Negative predictive value
CI	Confidence interval
HPV	Human papillomavirus
HR-HPV	High-risk (oncogenic) HPV
pHR-HPV	Probable high-risk HPV
LR-HPV	Low-risk HPV
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
STI	Sexually transmitted infection
RCT	Randomized controlled trial
CE	Cost-effectiveness
ICER	Incremental cost-effectiveness ratio
QALY	Quality-adjusted life years

1 POPULÄRVETENSKAPLIG SAMMANFATTNING

Livmoderhalscancer är globalt sett den tredje vanligaste cancerformen bland kvinnor. Varje år får mer än en halv miljon kvinnor diagnosen, och mer än en kvarts miljon kvinnor dör av sjukdomen. Symtom uppkommer vanligen sent i förloppet, då utsikterna för en effektiv behandling är dåliga. Cancerutvecklingen sker gradvis och förstadier till sjukdomen (cervikal intraepitelial neoplasi, CIN) finns vanligen i många år innan förändringen blir elakartad.

Välorganiserad screening med cellprov är ett effektivt sätt att förebygga utveckling av livmoderhalscancer, då avancerade CIN-förändringar, som kallas CIN2 och CIN3, kan upptäckas och behandlas. Med konventionell metodologi (CC=conventional cytology) tas cellprovet genom att celler som skrapats från livmodertappen stryks ut på glas, färgas och undersöks i mikroskop. Det finns två huvudsakliga former av cervixcancer: skivepitelcancer och körtelcellscancer. Insjuknandet i skivepitelcancer har minskat dramatiskt i Sverige sedan allmän screening med cellprov infördes. Screeningprogrammet tycks dock ha nått sin begränsning då ingen tydlig trendminskning av livmoderhalscancer setts de senaste 10 åren.

Infektion med särskilda högrisk-typer av humant papillomvirus (HR-HPV) är nödvändig för utveckling av livmoderhalscancer. Sådana infektioner är vanliga, särskilt bland unga kvinnor, och läker vanligen ut spontant. Om infektionen blir kronisk kan den dock leda till cancerutveckling.

Ett problem inom cellprovsbaserad screening är att merparten av alla avvikande prov består av lindriga cellförändringar. Lindriga cellförändringar kan indelas i två diagnosgrupper: ASCUS (atypiska skivepitelceller av oklar signifikans) och LSIL (lindrig intraepitielial skivepitelsförändring). Dessa förändringar är ospecifika, och CIN2 eller mer avancerade förändringar (CIN2+) finns bara i en minoritet av dessa fall. Det behövs bättre metoder för att identifiera de kvinnor som löper ökad risk för framtida livmoderhalscancer, så att överdiagnostik, överbehandling och onödigt psykiskt lidande kan minimeras. En möjlighet är att komplettera cellprovet med HPV-test, så att kvinnor med ökad risk för framtida livmoderhalscancer kan särskiljas från dem med reaktiva förändringar, så kallad HPV-triage. HPV-test har en betydligt högre känslighet än cellprov för att upptäcka CIN2+, och därmed en god förmåga att förutse frånvaro av CIN2+. Det begränsas dock av sin dåliga förmåga att förutse sjukdom, framför allt bland unga personer där självläkande HPV-infektioner är vanliga.

Ett enstaka cellprov innebär en begränsad möjlighet för att upptäcka CIN2+. Screeningens skyddseffekt är därför beroende av relativt tätt upprepade screeningprov. Cellprov är särskilt begränsat då det gäller att upptäcka förstadier till körtelscellscancer. Detta bidrar till att livmoderhalscancer ibland utvecklas även hos kvinnor som tidigare deltagit i screening och haft normala cellprov. En alternativ metod med vätskebaserat cellprov (liquid-based cytology=LBC) har utvecklats för att förbättra cellprovets kvalitet och underlätta bedömningen. Provtagningstekniken är densamma som vid CC, med skillnaden att cellerna slammas upp i fixerande vätska, och appliceringen på objektglas sker på cytologlaboratoriet. En fördel med LBC är att man enkelt kan göra kompletterande analyser, t.ex. HPV-test, utan att kvinnan behöver kallas för ett nytt prov, till skillnad från CC. Vissa studier visar att LBC har en bättre känslighet för att upptäcka förstadier till livmoderhalscancer.

Målet med det här projektet var att uvärdera en alternativ screeningstrategi med vätskebaserat cellprov och kompletterande HPV-test vid lindriga cellförändringar inom det svenska screeningprogrammet.

Inom screeningprogrammet i Stockholm alternerades LBC med kompletterande HPV-test vid lätta cellförändringar (LBC+HPV-test) och CC från september 2005-december 2006. Screening med LBC+ HPV-test och CC jämfördes avseende förmåga att upptäcka CIN, för olika grad av avvikelse indelat i grupperna CIN1+, CIN2+ och CIN3+. Metodernas positivt prediktiva värde, d.v.s. deras förmåga att förutse en onormal förändring, jämfördes. Screening med LBC+HPV-test utvärderades också över tid (september 2005-december 2007). HPV-testets diagnostiska förmåga att påvisa CIN2+ undersöktes. HPV-förekomsten i olika åldersgrupper vid ASCUS och LSIL studerades. För att undersöka kostnadseffektiviteten för HPV-triage vid uppföljning av ASCUS och LSIL gjordes en ekonomisk analys baserad på tidigare studier inom området.

Med LBC+HPV-test ökade andelen upptäckta CIN2+ med 45 % (från 1,1 till 1,6 %) under första året, och förmågan att förutse CIN2+ ökade markant. Andelen upptäckta förstadier till cancer ökade betydligt över tid, för båda metoderna. Skillnaden mellan metoderna var inte betydande (justerad oddskvot för CIN2+ 0,89 med 95 % konfidensintervall 0,64- 1,25; justerad oddskvot för CIN3+ 1,02 med 95 % konfidensintervall 0,67- 1,54). De positivt prediktiva värdena för CIN var likvärdiga för metoderna. Högrisk-HPV fanns i 71 % av alla LSIL- och i 49 % av alla ASCUS-fall. Bland kvinnor över 30 år var skillnaden i högrisk-HPV-förekomst inte betydande. Ingen högrisk-HPV-negativ kvinna hade CIN2+ inom 2 år från cellprovet. Alltså var HPV-testets förmåga att förutse frånvaro av CIN2+ var utomordentligt god. För kvinnor över 30 år vars screeningprov visat ASCUS, var HPV-triage det billigaste uppföljningsalternativet, medan omedelbar undersökning av livmodertappen i mikroskop med riktade vävnadsprov var en mer effektiv metod för att detektera CIN2+, till en marginellt högre kostnad.

Sammanfattningsvis påvisades inga uppenbara fördelar med att använda LBC+ HPV-test i screening jämfört med CC. Över tid fanns dock fanns en markant ökning av andelen upptäckta förstadier till livmoderhalscancer, för båda metoderna. Det är möjligt att införandet av den nya tekniken med LBC+ HPV-test ledde till en generell kompetenshöjning och en ökad vaksamhet vid bedömningen av cellprov. Med LBC är det nämligen lättare att se även subtila förändringar, som kan vara svåra att upptäcka med CC. De förändringar som observerades under studieperioden understryker vikten av att övervaka den totala andelen avvikande cellprovsfynd; detta för att balansera överdiagnostik mot övertro på den egna förmågan att visuellt kunna särskilja reaktiva förändringar från möjliga förstadier till cancer. Genom att använda kompletterande HPV-test kan ytterligare omfattande utredningar undvikas för ca 50 % av alla kvinnor vars screeningprov visat ASCUS och för dem över 30 år med LSIL. Om HPV-test med god diagnostisk förmåga används och kostnaderna för HPV-test reduceras kan kompletterande HPV-test bli en kostnadseffektiv strategi vid uppföljning av lindriga cellförändringar. Screeningprogrammet måste kontinuerligt anpassas allteftersom HPV-vaccinerade kvinnor når åldrar som omfattas av screening och nya lovande screeningmetoder blir tillgängliga.

2 BACKGROUND

2.1 The uterine cervix

The uterine cervix is the lower third of the uterus and is composed of dense fibromuscular tissue, is about 3 cm long and has a diameter of about 2.5 cm. The outer part of the cervix (ectocervix) protrudes into the vagina, whereas the upper two-thirds (endocervix) of the cervix lies above the vagina (Figure 2.1).

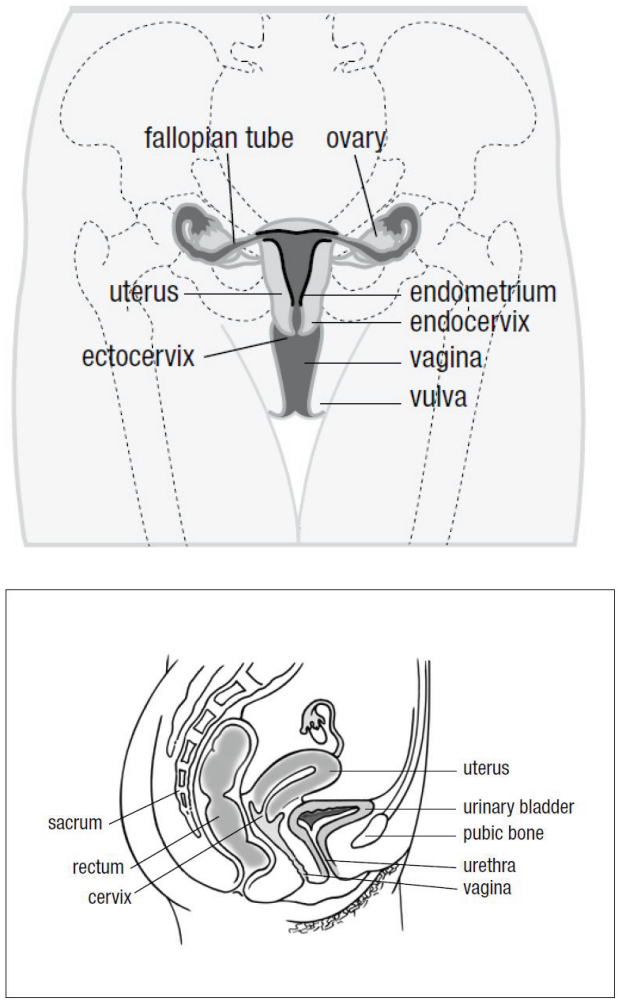


Figure 2.1. Front and side-view of female internal organs. From Comprehensive Cervical Cancer Control: a guide to essential practice. Integrating Health Care for Sexual and Reproductive Health and Chronic Diseases, World Health Organization, Figure 2.2, page 29, (2006). http://screening.iarc.fr/planningguides.php (latest accessed on 16th of March 2011). Reproduced with kind permission from the World Health Organization.

The cervical canal runs through the cervix. The endocervical canal is lined by thin glandular epithelium which meets the stratified squamous epithelium of the ectocervical surface. The opening of the canal into the uterus is called the internal os, and the opening towards the vagina the external os. The transition between the monolayer of glandular epithelium (also called columnar) and the stratified squamous epithelium creates the original squamocolumnar junction (SCJ) (Figure 2.2). Its location in relation to the external os depends on the age and hormonal status of the woman; in young women it can be visualized at the ectocervical surface, whereas in older women it is often located within the endocervical canal.

When the thin glandular epithelium is exposed to the acidic environment of the vagina, the columnar epithelium is gradually replaced by metaplastic squamous epithelium (which derive from glandular reserve cells) before it meets the true stratified squamous epithelium and gives rise to the new SCJ. The transformation zone (TZ) is the area between the original and the new SCJ. The TZ is a crucial area in the pathogenesis of cervical cancer (2006 ; Richart, 1969; Sellors J.W., 2003).

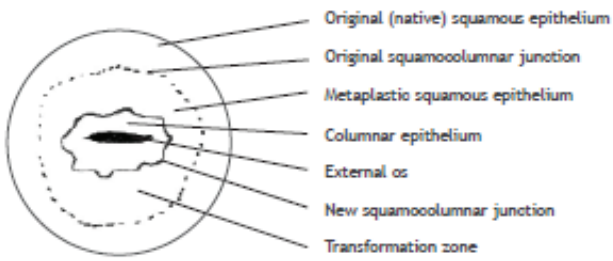


Figure 2.2. A schematic diagram of the transformation zone. From Sellors J.W. and Sankaranarayanan R. Colposcopy and Treatment of Cervical Intraepithelial Neoplasia. A Beginner's manual. Lyon, France, IARCPress, 2003. http://screening.iarc.fr/doc/Colposcopymanual.pdf (latest accessed on 16th of March 2011). Reproduced with kind permission from the International Agency for Research on Cancer, WHO.

2.2 Cervical cancer

2.2.1 Epidemiology

Cervical cancer is a preventable disease. In spite of this fact it is one of the leading cancers among women worldwide. In 2008 it was the third most common cancer in women after breast and colorectal cancer, with an estimated 529 000 new cases and 275 000 deaths from cervical cancer, of which 88% were in developing countries. In some regions it is the number one cause of cancer-related deaths (Ferlay *et al*). Like other cancers which are mainly caused by infectious agents, such as liver cancer and stomach cancer, the disease is markedly overrepresented in less developed countries (Jemal *et al*). More than 85% of the global burden of cervical cancer occurs in developing countries. The primary high-risk areas are Eastern and Western Africa with cumulative incidence risk (0-74 years) of 3.8%, Southern Africa (2.9%), South Central Asia (2.6%), Middle Africa and South America (2.5%).

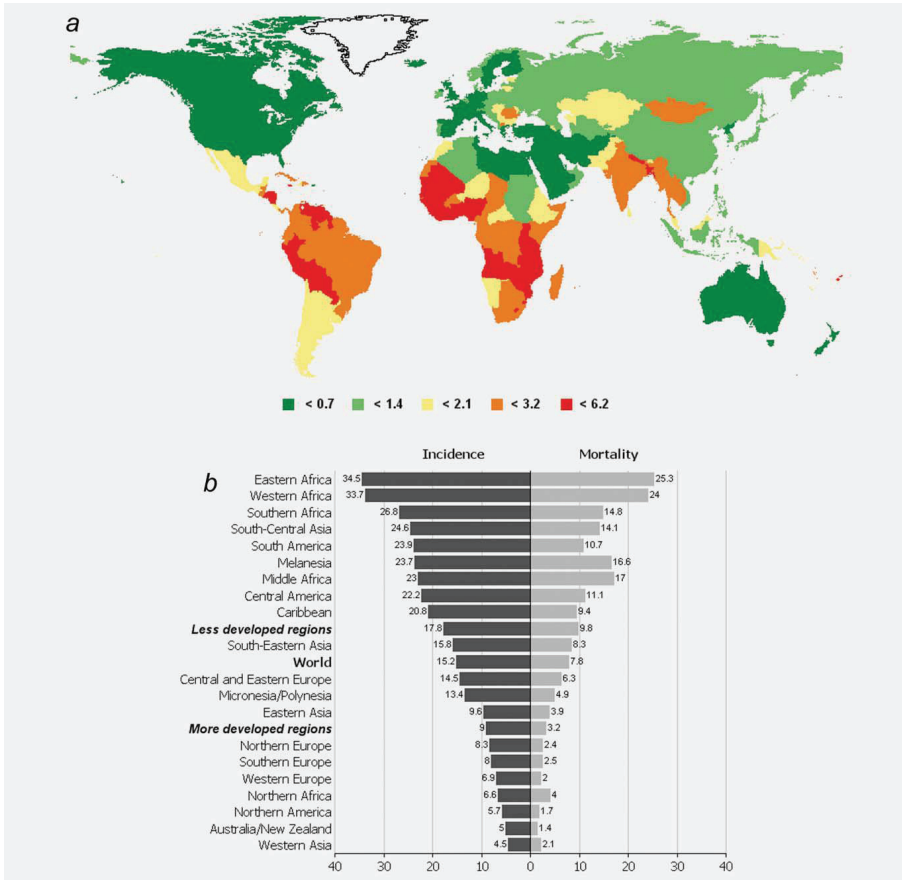


Figure 2.3. (a) Estimated cumulative incidence risk (0-74 years): cervix uteri cancer. (b) Estimated age-standardized incidence and mortality rates for cervix uteri cancer. From "Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008", by Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM, Int J Cancer, 127, 2008 (Figure 12 a and b, page 2913), 2010 UICC,. This material is reproduced with kind permission of John Wiley & Sons, Inc.

Areas where the cumulative incidence risk of cervical cancer is very low (0.5%) include Western Asia, Northern America and Australia (Figure 2.3) (Ferlay *et al*).
The huge variation in incidence between regions reflects differences in availability of preventive programs against cervical cancer, lifestyle and the position of women in society, whereas the variation of the mortality: incidence ratio (also called fatality) reflects the inequalities in accessibility of well-functioning health services delivery to assure detection and appropriate treatment of cervical precancer and cancer (Jemal *et al*; Sankaranarayanan & Ferlay, 2006). Before onset of screening in developed countries, incidence of cervical cancer was similar to that of developing countries (Gustafsson *et al*, 1997).

In Sweden, population-based cervical cytology screening has been offered since the late 1960's, the major reason that cervical cancer is a rare disease today (Andrae *et al*, 2008; Bergstrom *et al*, 1999; Gunnell *et al*, 2007). Cancers of the breast, lung, colorectum, uterine body, and ovaries are all more common among Swedish women. In 2009, 437

new cases were diagnosed, representing 1.7% of all cancers among women. Of these women, 44% were 20-49 years old (Socialstyrelsen, 2010). Cervical cancer is indeed a disease of young women. In more developed countries the age standardized incidence rates (ASR) generally begin to increase earlier (probably due to earlier detection) and increase until 40 years of age after which a plateau is reached, whereas in less developed regions ASR continues rising to a much higher incidence level until an age of about 60 years (Kamangar *et al*, 2006).

2.2.2 Clinical manifestations

Development of cervical cancer is a multistep process, which is almost always induced by a persistent oncogenic human papillomavirus infection (high-risk (HR) HPV) that disrupts control over cell growth and which, over years or even decades, leads to asymptomatic precancerous lesions that can progress into invasive cancer (zur Hausen, 2009). Pathophysiology will be described in greater detail in Chapter 2.3. Small invasive cancers that are not clinically detectable, also called microinvasive cancers, are also usually asymptomatic. These cancers can only be detected through a screening test for abnormality. However, most cancers are not discovered until they become symptomatic, which often occurs at a relatively advanced stage, especially if the woman is not sexually active. For early detection of cervical cancer, women who present with one or more of the following complaints should be evaluated for possible cervical cancer: irregular bleeding, postcoital bleeding and persistent vaginal discharge (especially if persistent after treatment for bacterial sexually transmitted infection) (2006). Early and late symptoms and signs of cervical cancer are listed in Table 2.1.

Early	• Vaginal discharge, sometimes foul-smelling • Irregular bleeding (of any pattern) in women of reproductive age • Postcoital spotting or bleeding in women of any age, even young women • Postmenopausal spotting or bleeding • In the case of abnormal perimenopausal bleeding, cervical cancer should always be considered, particularly if the bleeding fails to respond to appropriate treatment
Late	• Urinary frequency and urgency • Backache • Lower abdominal pain
Very late	• Severe back pain • Weight loss • Decreased urine output (from obstruction of the ureters, or renal failure) • Leakage of urine or faeces through the vagina (due to fistulae) • Swelling of the lower limbs • Breathlessness (due to anaemia or, rarely, lung metastases or effusion)

Table 2.1. Symptoms of invasive cervical cancer. From Comprehensive Cervical Cancer Control: a guide to essential practice. Integrating Health Care for Sexual and Reproductive Health and Chronic Diseases, World Health Organization, Table 6.1, page 169, 2006. http://screening.iarc.fr/planningguides.php (latest accessed on 16 of March 2011). Reproduced with kind permission from the World Health Organization.

2.2.3 Prognosis and treatment

The clinical suspicion of cervical cancer requires examination including a biopsy or diagnostic conization for a definitive histological diagnosis. The main forms of cervical cancer are squamous cell carcinoma (SCC), adenocarcinoma (ADC) and a mixed form called adenosquamous carcinoma. Cancers of non-epithelial origin also occasionally occur in the uterine cervix. Once the diagnosis is confirmed, the stage of the disease needs to be determined to formulate a suitable treatment (2006).

A widely used and recommended system has been elaborated by the International Federation of Gynecology and Obstetrics (FIGO), and is described in Table 2.2 (2006 ; Pecorelli *et al*, 2009; Quinn *et al*, 2006). It is based on the spread of the disease in the pelvis and to distant organs. The extent of growth of the cancer is assessed clinically including a speculum examination of the vagina and a rectal examination, often made under general anesthesia. Microinvasive carcinomas are an exception since these cases are not clinically detectable and instead staged according to pathological criteria of the depth and width of the invasive lesion. Intravenous pyelogram or an abdominal ultrasound can be performed to detect occlusion of the ureters. Additional examinations can be helpful for staging, such as cystoscopy, proctoscopy, an endocervical curettage or smear, a chest X-ray or a skeletal X-ray or bone scan. Magnetic resonance imaging or a computerized tomography scan of the pelvis and abdomen is encouraged for more objective determination of tumor size in (Pecorelli *et al*, 2009). One should also consider associated diseases, such as anemia, HIV/AIDS and syphilis.

Tumor stage is the most reliable predictor of clinical outcome for patients with invasive cervical cancer. In developing countries only 5% of cervical cancers are found at an early stage (including FIGO stages IA1-IIA) (2006). The results of a nationwide audit of the Swedish screening program (Andrae *et al*, 2008), including all cases of invasive cervical cancers reported to the high-quality Swedish Cancer Registry from 1999 until the end of 2001 found that among these women (n=1230), 305 (25%) cases were assumed to be screen-detected whereas 925 (75%) cases were assumed to be symptomatic. Among screen-detected cancers, only 9% were advanced-stage cancers (FIGO stage II or higher), whereas among symptomatic women 48% were advanced cancers.

Lymphovascular space invasion, tumor spread to regional lymph nodes, and large tumor size worsen the prognosis across all FIGO stages, especially in earlier stages (Pecorelli *et al*, 2009; Quinn *et al*, 2006). Non-SCC generally have worse prognosis than ADC across all FIGO stages. Age ≥50 years is a predictor of poor prognosis only in women with FIGO stage I cancer (Quinn *et al*, 2006).

FIGO stage	Extension of tumor	5-year survival
Stage I	Carcinoma confined to the cervix.	
IA	Microinvasive carcinoma, not clinically visible. Can only be diagnosed by microscopy.	
IA1	Stromal invasion <3mm in depth and <7 mm horizontal spread	~98%
IA2	Stromal invasion ≥3≤5 mm in depth and < 7 mm horizontal spread	~95%
IB	Carcinoma clinically visible; or a microscopic lesion greater than IA2	
IB1	≤4 cm in greatest dimension	~85%
IB2	≥4 cm	~75%
Stage II	Carcinoma spread beyond the cervix, but not as far as the lower third of the vagina or the pelvic wall	
IIA1▪	Tumor size <4 cm with involvement of less than the upper two-thirds of the vagina, but not to tissues surrounding the uterus (parametria).	~75%
IIA2▪	Tumor size ≥4 cm with involvement of less than the upper two-thirds of the vagina and without invasion of parametria.	
IIB	Parametrial invasion, but not as far as the pelvic wall or the lower third of the vagina.	~65%
Stage III	Tumor extends to pelvic wall or involves the lower third of the vagina, or causes hydronephrosis or non-functioning kidney	
IIIA	Extension to the lower third of the vagina, with no extension to the pelvic wall and no hydronephrosis or non-functioning kidney	~30%
IIIB	Extension to the pelvic wall, hydronephrosis or non-functioning kidney.	~30%
Stage III	Tumor has spread.	
IVA	Spread to involve the mucosa of the bladder or rectum.	~10%
IVB	Spread to distant organs, such as extrapelvic lymph nodes, kidneys, skeleton, lungs, liver and brain.	<5%

Table 2.2. Summary of the FIGO stages with prognosis figures for cases managed with optimal treatment (adapted from Comprehensive Cervical Cancer Control: a guide to essential practice. Integrating Health Care for Sexual and Reproductive Health and Chronic Diseases, World Health Organization (2006), page 172-176. http://screening.iarc.fr/planningguides.php (latest accessed on 16 of March 2011) ▪ Subdivision of Stage IIA according to Pecorelli et al, 2009.

In cases of tumors >2 cm, protocols with neoadjuvant chemotherapy to shrink tumors before fertility-sparing surgery, have been used. For tumors >2cm, fertility-sparing surgery is still considered an experimental procedure and needs further evaluation (Rob *et al*). If preserved fertility is not a high priority, simple hysterectomy (surgical removal of the uterus including the cervix) with or without lymphadenectomy is indicated for

FIGO stage 1A1 and sometimes IA2 cancers. For cancers of FIGO stage up to IIA1, standard treatment includes vaginal or abdominal radical hysterectomy (surgical removal of the uterus, parametria, and upper 2 cm of the vagina) and lymphadenectomy (2006 ; Herzog, 2003). For more advanced cancers, treatment including radical radiotherapy or a combination of chemotherapy and radiotherapy is preferred. Radiotherapy and chemotherapy can also be used to supplement surgery (2006).

2.3 Pathogenesis

2.3.1 The causative role of human papillomavirus infection

The Italian surgeon and researcher Rigoni-Stern, born in 1810 in Verona, Italy, systematically recorded data on cancer incidence and mortality. He found that uterine cancer (without distinction between the uterine cervix and body) was common among married women, widows and prostitutes but uncommon among nuns and virgins, and suggested that the disease was related to sexual activity (Rigoni-Stern, 1842).

More than 100 years later, an association between human papillomavirus (HPV) infection and cervical cancer was first suspected, from anecdotal reports of conversion of condyloma accuminata into SCC (zur Hausen, 1977). The hypothesis was strengthened by the failure to detect the long suspected etiologic agent herpes simplex virus, in cervical cancer biopsies (zur Hausen *et al*, 1974b). Pioneering experimental work was made by Harald Zur Hausen and co-workers in the 1970's and onwards. In 2008 he was awarded the Nobel Prize in Physiology or Medicine for his work.

An early experiment aimed to examine the relation of human wart virus with a number of benign and malignant human tumors (zur Hausen *et al*, 1974a). Viral DNA from plantar warts was isolated. With the aid of Escherichia coli RNA polymerase, radioactive complementary RNA (cRNA) was produced and used as a probe for detection of viral DNA. High viral DNA concentrations were found in plantar warts, and found to a lower extent in verrucae vulgares, whereas laryngeal papillomas, condyloma accuminata and cervical cancer, with the exception of one case with cRNA binding slightly above background values, were negative. The hypothesis of the possible existence of different types and subtypes of HPV was presented, and additional studies were launched to identify and characterize novel HPV types in human tumors.

Presence of HPV DNA in cervical cancer biopsies was first demonstrated in 1982 (Green *et al*, 1982). The year after, HPV 11 DNA was found in two cases of cervical carcinoma *in situ* and in one case of invasive cervical cancer out of totally 24 biopsies from *in situ* or invasive genital carcinomas (Gissmann *et al*, 1983). By using HPV 11 as a probe, a novel HPV type could be isolated from a cervical cancer biopsy (Durst *et al*, 1983). This HPV type was designated as HPV 16, and was found in 61% (11/18) of all cervical cancer biopsies from German patients and 35% (8/23) of such samples from Kenya and Brazil. HPV 16 was frequently detected in cervical intraepithelial neoplasia (CIN) , but was uncommon in cases of common genital warts (Crum *et al*, 1984). This breakthrough was followed by the isolation of the second main oncogenic HPV type,

HPV 18, which was detected in cervical cancer biopsies and several cervical cancer derived cell lines, but not in benign tumors (reviewed in (zur Hausen, 2009)).

In the 1980s and 1990s central carcinogenic properties of specific HPV types were uncovered, and will be presented in greater detail in paragraph 2.3.5.

Global epidemiological studies identified HPV 16, 18, and a few other HPV types as key etiologic factors in cervical cancer (Munoz et el 1992, Bosch et al. 1992 (Bosch *et al*, 1995). With newer sophisticated techniques for HPV detection, i.e. polymerase chain reaction (PCR) and histopathological re-evaluation of both HPV-positive samples and the small portion of initially HPV-negative samples, HPV could eventually be identified in virtually all (99.7%) adequate cervical cancer samples (Walboomers *et al*, 1999).

2.3.2 Classification of papillomaviruses

In 2010, 189 different papillomavirus (PV) types had been identified (Bernard *et al*). This figure has rapidly increased since 2004, when the number was 118 (de Villiers *et al*, 2004). PVs are small (~8kb), non-enveloped DNA viruses that infect epithelium of vertebrates. The PV types known today have been isolated from humans (120 types), non-human mammals, birds, and reptiles.

Classification of the different PVs in the *Papillomaviridae* family is based on nucleotide sequence similarities with certain medical and biological properties (de Villiers *et al*, 2004). The L1 gene encodes for the major viral capsid protein L1, which together with the L2 protein, comprises the complete capsid. The viral structure is described in greater detail in section 3.3.4. L1 is overall the most conserved open reading frame (ORF) of the PV genome. Comparisons of whole genome sequences lead to a similar distribution of inter-related HPV types, as found when comparing L1 ORFs (de Villiers *et al*, 2004). Classification of PVs follows the guidelines below:

- *Genera*: higher-order clusters of PV types, e.g. genital HPVs. Different genera have less than 60% nucleotide sequence homology in the L1 ORF. Within a genus, full-length sequences of whole genomes have more than 23%, but less than 43% nucleotide homology.

- *Species:* lower order clusters of PV. Species within a genus share 60-70% nucleotide identity, and have identical or similar biological properties.

- *Type*: a novel isolated PV type is recognized as such if the L1 ORF nucleotide sequence differs by more than 10% compared with the most similar previously known PV type. The traditional PV types within a species share 71-89% nucleotide identity within the complete L1 ORF.

- *Subtype*: If the difference in L1ORF nucleotide sequence is 2-10% compared with the most similar previously known PV type, it is considered a new subtype, and a *variant* if the difference is <2%.

The main PVs infecting the anogenital tract of humans belong to the Alpha genus, which includes ~70 different HPV types (Bernard *et al*).

A recent large meta-analysis including 30 848 cases showed that the most frequently occurring HPV types in invasive cervical cancers are HPV 16, 18, 58, 33, 45, 31, 52, 35, 59, 39, 51, and 56, in decreasing order of frequency. HPV 16 and/or 18 were associated with 73% of all cervical cancer cases (Li *et al*). This is in accordance with the HPV types defined as carcinogenic by the IARC(Bouvard *et al*, 2009). Of these, the eight HPV types that are most frequent in cervical cancer accounted for 91% of 8977 HPV-positive cervical cancers of epithelial origin in a worldwide retrospective cross-sectional study on HPV genotype distribution (de Sanjose *et al*). Four carcinogenic HPV types are less frequently found in cervical cancer (HPV 39, 51, 56, and 59), but evidence is considered sufficient to define them as carcinogenic (Bouvard *et al*, 2009). The carcinogenic HPV types belong to species 5 (HPV 51), 6 (HPV 56), 7 (HPV 18, 39, 45, and 59), and 9 (HPV 16, 31, 33, 35, 52, and 58).

HPV 68 was classified as "probably carcinogenic to humans." Other HPV types were classified as "possibly carcinogenic" due to limited evidence of carcinogenicity or their close phylogenetic relationship with carcinogenic types (Bouvard *et al*, 2009). Low-risk risk HPVs cause benign and low-grade mucosal lesions (de Villiers *et al*, 2004).

2.3.3 Genital HPV infection prevalence

Infection with HPV is the most common sexually transmitted infection. About 50-80% of sexually active men and women are estimated to become infected with low- or high-risk HPV sometime in their lives (reviewed in (Stanley)). From a 2007 meta-analysis based on reports of HPV prevalence from 1995 to 2005 (de Sanjose *et al*, 2007), an estimate was made of the worldwide age-specific HPV infection spectrum among women with normal cytology. The overall adjusted prevalence was 10.4%, with a large variation between geographic areas; the lowest observed prevalence was in Southeastern Asia (6.2%) and the highest in Eastern Africa (31.6%). Adjusted prevalence in Northern Europe was 7.9% including studies from Sweden, Denmark, and the UK, a figure close to the 6.8% (n=6123) HR-HPV-positive women aged 32-38 years in a population-based screening in Sweden (Forslund *et al*, 2002) and to a similar cohort, where 7.1% to 11.0% were HR-HPV positive (n=12527), depending on the HPV detection assay used (Wahlstrom *et al*, 2007).

The overall genital HPV prevalence in men is lower than in women, suggesting that penile tissues may be more resistant to HPV infections (Partridge & Koutsky, 2006).

HPV 16 was the most prevalent HPV type in all regions except Eastern Africa, Japan, and Taiwan, where it was the second most prevalent type after HPV 52 (de Sanjose *et al*, 2007). Prevalence was high among young women (>20%) irrespective of the level of development of each country, except in Asia where figures were lower. After the age of 25 years, HPV prevalence was higher in less developed countries than in more developed countries (Figure 2.4). The most significant reduction of HPV prevalence

was seen after age 35 years, using broad age groups as shown in Figure 2.4. After age 45 years, HPV frequency increased except in Asia, where the decline continued with increasing age.

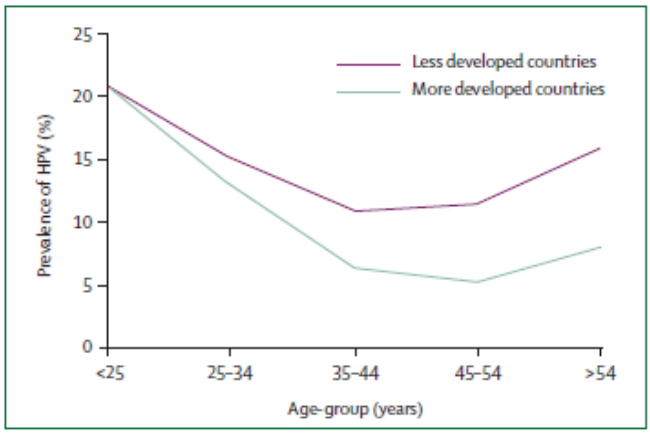


Figure 2.4. Worldwide estimates of age-specific HPV prevalence by country-specific development status in women with normal cytology. Reprinted from, The Lancet Infectious Diseases, 7, de Sanjose S, Diaz M, Castellsague X, Clifford G, Bruni L, Munoz N, Bosch FX, Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis, 453-9, 2007, with permission from Elsevier.

2.3.4 HPV transmission and viral life cycle

Sexual intercourse is the main route of transmission of genital HPV infection, although skin-skin and skin-mucosa transmission also occur (Burchell *et al*, 2006; Winer *et al*). Most HPV infections are cleared by the immune system without causing any symptoms. However, a small fraction of all these infections persist, and may over time cause genetic damage that leads to loss of control of the cell cycle and accumulation of harmful mutations, which eventually lead to formation of precancerous lesions and progression to invasive cancer (zur Hausen, 2002).

The TZ (Figure 2.2) is, as mentioned, an important site for HPV infection, and cervical cancers are considered to arise mainly in this region. The site is a fragile spot where the virus can penetrate the epithelial lining through microscopic wounds to reach the basal layer cells (2006 ; Horvath *et al*; Richart & Barron, 1969; Sellors J.W., 2003). The virion probably enters the basal epithelial cell by primary binding of the major viral capsid protein L1 to the basement membrane, followed by infectious transfer and binding to heparan sulphate proteoglycans present on the cell surface of keratinocytes (and many other cell types). This binding causes conformational change of the viral capsid that enables cleavage of the minor capsid protein L2 and viral binding to a more specific co-receptor, mediating internalization of the virus (reviewed by (Horvath *et al*)). A more detailed description of viral genetics is presented in paragraph 2.3.5.

In the basal layer of the epithelium, viral gene expression is low, with only modest expression of genes that promote proliferation and lateral expansion of the cells (Figure 2.5). The virus uses the differentiation program of the epithelium for its replication, and in early infection, whole infectious viral particles are shed with the desquamating superficial epithelial cells (reviewed in (zur Hausen, 1996; zur Hausen, 2002).

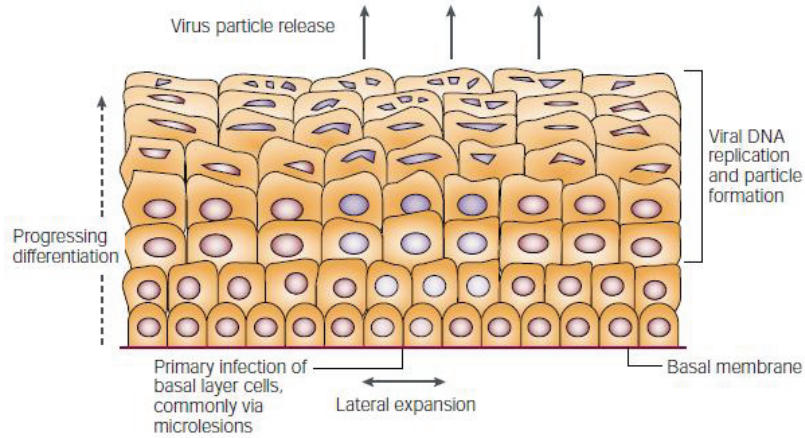


Figure 2.5. The human papillomavirus life cycle. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Zur Hausen H, Papillomaviruses and cancer: from basic studies to clinical application, pages 342-50), 2002.

Incubation time (infection without viral replication or carcinogenic effects) varies from 3-4 weeks to years of latency. The virus evades detection and elimination through several mechanisms. Important factors include absence of viremia, low levels of viral protein expression, and absence of cytolysis. The virus replicates and assembles in cells that are already destined to die and desquamate, consequently producing no "danger signal" to the immune system. Moreover, the interferon response, which is key to antiviral protection, is suppressed by the E6 and E7 proteins of HR-HPV (see below). The E7 protein downregulates the Toll-like receptor 9, contributing to evasion of innate immunity, which delays activation of adaptive immunity. However, eventually most HPV infections are cleared within 18 months. Only about 10-20% of infections persist and may lead to development of cervical precancerous lesions that can progress to invasive cancer (reviewed in (Stanley)).

2.3.5 Mechanisms of HPV-induced carcinogenesis

The ~8kb genome is an episome, i.e. a circular double-stranded DNA molecule (Figure 2.6). It carries an upstream regulatory region (URR) and eight ORFs of six early genes (E1, E2, E4, E5, E6, and E7) and two late genes (L1 and L2), named according to "early" or "late" expression in the viral life cycle. Although LR- and HR-HPV types have a similar genetic structure, their biological functions are different. The mechanisms listed below are described for HR-HPV, and are often absent or weak for LR-HPV types (Pim & Banks). The oncogenic potential also varies between individual HPV types, as reflected by the different extent to which they induce cancer.

In early productive infection and CIN1, only weak transcription of viral early genes is seen in the epithelial basal layer (Figure 2.5), and expression increases in the more differentiated cells of the superficial layers. Onset of expression of late genes takes place in the differentiated cells of the most superficial layers. In high-grade CIN and invasive cervical carcinoma (ICC), stronger expression of viral early genes is seen in the basal layers, and distribution is more eventhroughout the epithelium, whereas the expression of late genes is low or absent and viral replication is low (Durst *et al*, 1992).

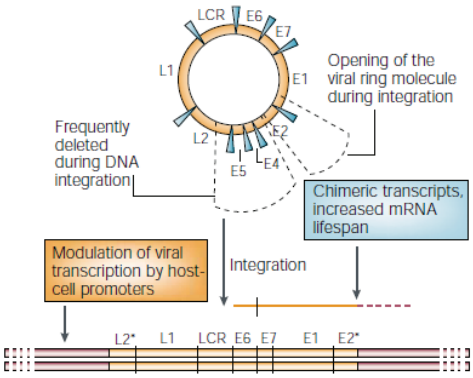


Figure 2.6. The organization of episomal (circular) HPV DNA and its integration into host DNA. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Zur Hausen H, Papillomaviruses and cancer: from basic studies to clinical application, pages 342-50), 2002.

The E1, E2, and E4 proteins are often referred to as regulatory proteins. E1 is a helicase and is recruited to the viral origin of replication in the URR by E2 where it forms DNA replication machinery together with cellular proteins (Yugawa & Kiyono, 2009). During S-phase in the basal epithelial cells, the episomal DNA replicates together with cellular DNA, and the viral episomes become equally distributed in daughter cells after cell division. E2 seems to have a crucial role for correct segregation, anchoring the viral episomes to the mitotic chromosomes (Johansson *et al*, 2009). E2 also regulates early viral gene expression, and is important for genome amplification. The increased levels of E2 needed for viral replication may eventually lead to the repressive downregulation of E6/E7, resulting in loss of the environment needed for viral DNA synthesis. This has been interpreted as a timing mechanism for viral replication. The assembly of viral particles in the superficial epithelial layers is thought to require E2 in addition to L1 and L2. The structural proteins L1 and L2 comprise the viral capsid, and are usually not expressed in advanced precancerous lesions and cancer.

Less is known about the E4 protein. It accumulates in the cell at the time of viral genome amplification. Loss of E4 has been observed to disrupt late events in viral replication. It has also been observed that E4 can relocate cyclin/cyclin dependent kinase complexes from the nucleus to the cytoplasm, thereby preventing progression through mitosis (reviewed in (Doorbar, 2006)). It has also been suggested that E4

influences E2 function, varying the amount of E2 available in the cell (Davy *et al*, 2009).

The genes E5, E6, and E7 possess proliferation-stimulating properties. The E5 protein is most important in early stages of infection. It is a transmembrane protein present mainly in the endoplasmic reticulum. It is thought to increase recycling of growth factor receptors on the cell surface, thereby increasing epidermal growth factor-mediated signaling, leading to maintenance of a replication-competent environment in the upper epithelial layer (Doorbar, 2006). In addition to epidermal growth factor receptor, E5 interacts with the platelet-derived growth factor-beta and the colony-stimulating factor 1 receptor (Hwang *et al*, 1995). Moreover, E5 seems to be an important factor in immune evasion: it reduces HLA class-I molecules present on the cell surface, thereby leading to reduced recognition of infected cells by cytotoxic T-lymphocytes (Campo *et al*).

HR-HPV E6 and E7 are considered the main viral oncogenes, with the tumor suppressor proteins p53 and pRb as their main targets (Figure 6). The functions of E6 and E7 differ between HPV types. Carcinogenicity in humans has only been proved for the twelve HPV types designated as HR-HPV (Bouvard *et al*, 2009).

The transcription of E6 and E7 in cervical cancer was first described in 1985 (Schwarz *et al*, 1985). They are consistently expressed in cervical cancer, and inhibiting their expression blocks the malignant phenotype of the cells (Munger *et al*, 1989). The binding capability of the E7 protein to tumor suppressor protein pRb (Dyson *et al*, 1989) and that of the E6 protein to tumor suppressor protein p53 (Werness *et al*, 1990) were discovered only a few years later. Acting one by one, E6 and E7 are able to induce keratinocyte immortalization and transformation, but acting together they potentiate the transforming effect (Munger *et al*, 1989). Both E6 and E7 contribute to evasion of immune response (McLaughlin-Drubin & Munger, 2009).

As a regulator of the cell cycle checkpoints G1/S and G2/M, p53 is a key player in tumor suppression, also called “the guardian of the genome.” DNA damage leads to activation of p53 that induces high expression of p21, resulting in cell cycle arrest and apoptosis. E6 counteracts these functions of p53 through complex formation with p53 with subsequent ubiquitin-mediated degradation. Lack of p53 function leads to accumulation of mutations and aneuploidy, leading to cell transformation (Longworth & Laimins, 2004). It has also been suggested that loss of p53 leads to inhibition of differentiation through reduced activation of Notch-1, a determinant of keratinocyte differentiation (reviewed in (Yugawa & Kiyono, 2009)). E6 can also indirectly downregulate p53 activity, through association with its co-activator p300/CBP. Moreover E6 blocks apoptosis through p53-independent pathways, e.g. by blocking the mitochondrial proapoptotic Bak, which results in apoptosis resistance and an increase in chromosomal instability (zur Hausen, 2002). E6 probably also interacts with the PDZ protein family, involved in maintenance of cell polarity, formation of cell-cell adherence junctions, and cellular signaling. The binding of E6 with such proteins lead to degradation of the PDZ protein, which is suggested to contribute to transformation and immortalization. Moreover the E6 protein has the ability to activate telomerase, an enzyme that prolongs the telomeres at chromosome ends. Telomeres normally shorten

with each cell division, eventually leading to senescence. Telomerase activity is normally absent in somatic cells. E6 also activates telomerase indirectly by acting on other factors, as through ubiquitin-mediated degradation of NFX1-91, a telomerase repressor, following which telomerase can be activated through complex formation involving E6 and c-myc and other factors ((Longworth & Laimins, 2004; Yugawa & Kiyono, 2009).

The ability of the E7 protein to associate with the pRb protein family is central to its immortalizing and transforming capability. pRb proteins are negative regulators of cell cycle progression from G1 to S-phase. The unphosphorylated form of pRb represses initiation of S-phase through complex formation with the E2F/DP1 transcription factors which stimulate expression of gene products involved in transition into S-phase. Normally, pRb is phosphorylated by cyclin-kinase complexes during progression from G1 to S-phase, leading to release of E2F, which stimulates transcription of genes involved in DNA synthesis. Binding of E7 to pRb leads to release of the same transcription factors and constitutive activation of the same genes. E7 can also cause degradation of pRb through ubiquitin-mediated degradation and might enhance phosphorylation of pRb through interaction with cyclins and cyclin-dependent kinases (CDKs). This effect can be enhanced by blocking the activities of the CDK inhibitors p21 and p 27, leading to an uncontrolled cell cycle (Longworth & Laimins, 2004; Yugawa & Kiyono, 2009). Moreover, E7 induces abnormal centrosome numbers, leading to disorganized segregation of chromosomes during cell division, contributing to genomic instability (McLaughlin-Drubin & Munger, 2009).

In the course of carcinogenesis, viral DNA becomes integrated into the host cell genome, a process that disrupts the E2 gene (Schwarz *et al*, 1985). Due to their location, the E4, E5, and part of L2 are sometimes partially deleted after integration. Emergence of cells with high-level expression of E6 and E7 through integration of viral DNA and loss of E2 is considered to be a rate-limiting step for cervical carcinogenesis (Yugawa & Kiyono, 2009). In the carcinogenic process, integration occurs randomly over the whole genome with predilection for genomically fragile sites, and may cause deregulation of cellular genes (Wentzensen *et al*, 2004). Deregulated E6 and E7 expression can lead to increasing genomic instability with increased risk of accumulating genetic and epigenetic changes. As a result of activation of oncogenes and/or inhibition of tumor suppressor genes, cellular clones with a growth advantage may arise and undergo malignant progression (Yugawa & Kiyono, 2009).

2.3.6 Precancerous lesions of the cervix

Cervical carcinogenesis is a stepwise process, and 15-30 years are usually required for the increasingly abnormal (dysplastic) cervical epithelial lesion to progress into invasive cancer. The cervical intraepithelial neoplasia (CIN) terminology is based on the histological appearance and biological behavior of cervical lesions (Figure 2.7), in which CIN1 denotes mild dysplasia in which abnormal cells are seen in up to one third of the epithelium, CIN2 moderate dysplasia with abnormal cells in up to two thirds of the epithelium, and CIN3 corresponding to carcinoma in situ with abnormal cells in the full thickness of the epithelium (Richart, 1969). When cells of the lesion break through

the epithelial basement membrane it is by definition invasive. Since the glandular epithelium of the cervix is a monolayer, there is no such stepwise division of potentially precancerous lesions; precancerous lesions that arise from these cells are called adenocarcinoma in situ (AIS) or glandular intraepithelial neoplasia (GIN).

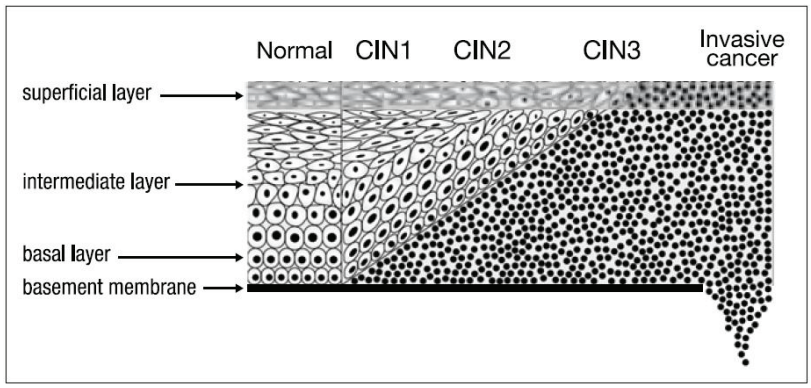


Figure 2.7. Progress from normal epithelium to invasive cancer. From Comprehensive Cervical Cancer Control: a guide to essential practice. Integrating Health Care for Sexual and Reproductive Health and Chronic Diseases, World Health Organization, Figure 2.8, page 38 (2006), http://screening.iarc.fr/planningguides.php (latest accessed on 16 of March 2011). Reproduced with kind permission from World Health Organization.

The risk of progression to cervical cancer increases with the grade of dysplasia. Several attempts have been made to clarify the prognosis related to the different grades of CIN, with attendant difficulties presenting reliable data since management of such lesions usually involves at least taking a biopsy, thereby interfering with the natural course of the disease. Moreover, it is ethically unacceptable to passively await possible occurrence of invasive cancer in cases where CIN is suspected.

One review was based on studies including prospective follow-up of women abnormal cytology, with results based on cytology, histology, and/or colposcopy (Ostor, 1993). Follow-up time was highly variable between studies, and most studies ended with detection of CIN3. For CIN1, the rate of regression was 60%, persistence 32%, progression to CIN3 was 10%, and progression to invasion 1%. For CIN2, the corresponding figures were 43%, 35%, 22%, and 5%, and for CIN3 rates were 33% regression, 56% persistence, and >12% progression to invasion. The latter figure is probably an underestimate due to design of the included studies, and because detection of CIN3 calls for action.

A large cohort study was undertaken of Canadian women screened 1962-1980, during which time management was usually conservative if cervical cytology showed mild and moderate dysplasia. Results showed a high rate (88%) of regression to normal in cases of mild dysplasia, and a 10% risk of progression to severe dysplasia or worse, within a 10 year follow-up period (Holowaty *et al*, 1999). For screening cytology showing moderate dysplasia, regression to normal occurred in 54% of cases and progression to CIN3 or worse in 32%, within the same follow-up period.

Lessons were learned from an unethical study conducted in New Zealand 1965-1974 (McCredie *et al*, 2008), aimed at proving the theory of CIN3 as a precursor to cervical cancer to be wrong. Treatment with curative intent was withheld from women with histologically confirmed CIN3. Medical records were reviewed in 2001-2002 for the 1229 women included in the study following the judicial inquiry. In 143 women managed with punch or wedge biopsy alone, the cumulative incidence of invasive cancer of the cervix or the vaginal vault was 31% (95% CI: 23-42%), and 50% (95% CI: 37-65%) in the subset of 92 women who had persistent CIN3 for 24 months.

A diagnosis of CIN2 or more advanced lesions is often referred to as a "high-grade cervical lesion" or CIN2+. Grouping these diagnoses together may be questionable. A Costa Rican population-based study of CIN and HPV infection including pathologic review showed that CIN3 was confirmed in 81% of patients compared with 61% for CIN2. CIN3 was associated with HR-HPV positivity in 93% of cases, compared with 72% for a CIN2 diagnosis (Carreon *et al*, 2007).

2.3.7 Risk factors

Only a small fraction of all HR-HPV infections will lead to development of cancer, implying that additional risk factors contribute to carcinogenesis. Risk factors are often related to HPV infection, e.g. through increased exposure to the infection, biological factors facilitating transmission, persistent HPV infection, cell immortalization, transformation, tumor progression, or host susceptibility to the carcinogenic effects of HPV.

Lack of availability of effective cervical screening and treatment for precancerous lesions is a crucial risk factor for developing cervical cancer and dying from the disease due to late diagnosis, as is underscored in paragraph 2.2.1 by the extreme variations in ICC incidence and mortality. In countries where well-organized population-based cervical screening programs exist, unscreened or underscreened women are a high-risk group and will be discussed in paragraph 2.4.3.

Immunosuppression due to HIV infection or immunosuppressive treatment after organ transplantation increases the risk of malignancies, especially for cancers with a known infectious etiology, including all HPV-related cancers: cervical, vulvovaginal, penile, anal, and oropharyngeal cancer (Grulich *et al*, 2007). HIV-positive women exhibit higher rates of HPV infection with multiple oncogenic types, higher prevalence of CIN, and are at increased risk of cervical cancer. They also present 10-15 years earlier with invasive disease compared with HIV-negative women. The possible benefit of HAART (highly effective antiretroviral therapy) is still unclear (reviewed in (Pantanowitz & Michelow)).

Sexual and reproductive factors are closely related to HPV infection and risk of cervical cancer. Young age at first full-term pregnancy and number of full-term pregnancies (FTP) have been found to be strongly associated with the risk of ICC with RR=1.77 (95% CI: 1.42-2.23) for first FTP at age <17 years vs. ≥25 years, and

RR=1.76 (95% CI: 1.53-2.02) for ≥7 vs. 1-2 FTPs. Results were similar when restricted to HPV positive and negative cases and controls (2006).

Lifetime number of sexual partners and age at first intercourse as risk factors for CIN3 and invasive cervical cancer have also been studied in a collaborative re-analysis of epidemiological studies (2009). All analyses were conditioned on age and study (and study center for multicenter studies). RRs for ICC increased with increasing numbers of lifetime sexual partners; the RR was 2.78 (95% CI: 2.29-4.52) for ≥6 versus 1 partners, after additional conditioning on age at first intercourse, number of full-term pregnancies, and age at first full-term pregnancy. RRs for ICC increased with decreasing age of first intercourse, showing an RR=2.05 (95% CI: 1.54-2.73) for age ≤14 years vs. ≥25 years, conditioned on number of lifetime sexual partners and reproductive factors as above. Interestingly, conditioning on reproductive factors strongly attenuated the RR for age at first intercourse. This latter factor was closely linked to age at first full-term pregnancy in many studies. However, an association between age at first intercourse and ICC was also seen for nulliparous women. The increased risk of ICC conferred by numerous sexual lifetime partners and low age at first sexual intercourse is most likely related to the risk of acquiring HPV infection and potential duration of that infection, since many young women acquire HPV infection soon after sexual debut (Winer *et al*; Winer *et al*, 2005).

Factors related to sexual partners of women also play an important role for the risk of CIN and ICC. A Spanish study including husbands of women involved in two case-control studies of CIN showed that the risk of ICC was strongly related to the husband's number of lifetime sexual partners, but especially to the number of sexual partners during marriage (adjusted OR 11.0 (95% CI: 3.0-40.0) for >21 vs. 1 woman) and to the number of prostitutes as extramarital sex partners (adjusted OR 8.0 (95% CI:2.9-22.2) for >10 women vs. none). Young age at first intercourse also increased the risk of ICC. A strong association was also seen for penile HR-HPV infection in the husband, especially for type 16, and ICC (Bosch *et al*, 1996). The finding in this study of similar figures when restricting the analyses to husbands whose wives were monogamous underscores the role of men as vectors of HPV infection. A pooled analysis of the IARC HPV Prevalence studies also found an increased prevalence of HPV infections related to the husband's extramarital affairs after correcting for lifetime number of sexual partners of the woman and her age (OR 1.45: CI 95%, 1.24-1.70) (Vaccarella *et al*, 2006). The importance of male sexual behavior for HPV infection is also underscored by a study from Pakistan, showing stronger association with the husband's extramarital affairs and with the age difference between husband and wife than with number of lifetime sexual partners of the woman (Raza *et al*).

Male circumcision has been proven to reduce prevalence and incidence and to increase clearance rates of male genital HPV infection (Auvert *et al*, 2009; Gray *et al*; Tobian *et al*, 2009). It also reduces the prevalence and incidence of HPV infections in their female partners (Wawer *et al*). Among men with multiple partners it decreases the risk of ICC in their partner (Castellsague *et al*, 2002). However, the protective effect is only partial.

Condom use is protective against HPV transmission, though not completely (briefly discussed further in 2.4.1.). Non-use/ low use of condoms is often taken into account as a potential confounding risk factor when studying other factors.

Smoking has been defined as a carcinogen in cervical cancer by the IARC (2004). The International Collaboration of Epidemiological Studies on Cervical Cancer combined data from 23 epidemiological studies on the relation between smoking and development of ICC. Current smokers had an increased risk of SCC compared with never smokers (RR: 1.60 CI: 1.48-1.73). This relation was stronger with lower age for smoking onset and dose. The increased risk was still present, but weaker, for former smokers. Considering only HR-HPV positive women, the risk of SCC was still significantly increased for current smokers. No relation between smoking and ADC could be demonstrated (Appleby *et al*, 2006). The role of smoking as an independent risk factor for SCC was confirmed by a Nordic epidemiologic study (Kapeu *et al*, 2009). Moreover, a prospective study of young women, controlling for cervical HPV status (Birmingham, UK), showed that smoking increases the risk of developing CIN2 or 3 after becoming sexually active (Collins *et al*). Smoking has been reported to impair antibody response to HPV infection (Simen-Kapeu *et al*, 2008).

A collaborative re-analysis of 24 studies dealing with oral contraceptives as a risk factor for cervical cancer showed an increased risk for cervical cancer, with a relative risk of 1.90 (95% CI: 1.69-2.13) for use >5 years versus 'never use' (Appleby *et al*, 2007). This effect was independent of HR-HPV status. However, risk decreases with time from last use to a level comparable with that of 'never users' after 10 years. Estimation of long-term risks of oral contraceptives (10 years' use) shows only small increases in cumulative incidence, at least if use is limited to younger age groups. The mechanism by which oral contraceptive use is linked to cervical cancer is not fully understood.

Increased risk of cervical cancer has been observed for women with low socioeconomic status. Studies indicate that the relation between low socioeconomic status and CIN3/ICC is due to confounders, such as HPV infection, age, study center, sexual behavior, and husband's sexual behavior (including sexual contact with prostitutes), history of cervical screening, and smoking (de Sanjose *et al*, 1997; Khan *et al*, 2005). One study hypothesized that the sexual behavior of men is a major contributor to increased risk of ICC for these women, based on the observed high rate of risky sexual behavior among men in this group (de Sanjose *et al*, 1997).

Sexually transmitted infections other than HPV have been suggested as risk factors of ICC. In pooled analyses of Herpes simples virus-2 (HSV-2) , HSV-2 seropositivity was associated with an increased risk of ICC among HPV-positive women after adjustment for potential confounders, suggesting that HSV-2 may act in conjunction with HPV to modestly increase the risk of ICC (Castellsague *et al*, 2006; Smith *et al*, 2002). However, a Nordic longitudinal nested case-control study used 1974-1993 data and compared those results with results from a meta-analysis of studies. Smoking and HPV-adjusted analyses showed no association of HSV-2 with ICC (Lehtinen *et al*, 2002). In a study conducted in Jamaica, analyses adjusted for potential confounders including

HPV status showed no association of either HSV-2 or Chlamydia Trachomatis (CT) with grade of cervical lesion (CIN3+ vs. CIN1) (Castle *et al*, 2003).

More consistent positive correlations with cervical cancer have been seen for CT. One population-based case-control study found that the relative risk of ICC associated with past CT infection was 17.1 (95% CI: 2.6 to infinity), after adjustment for HPV-infection (Wallin *et al*, 2002). A pooled analysis of the IARC multicentric case-control study on CT and ICC found an increased - yet much more modest - risk of CT for SCC (OR 1.8: 95% CI, 1.2-2.7) but not for adeno- or adenosquamous carcinoma (Smith *et al*, 2004). More recent results have lead to similar conclusions (Madeleine *et al*, 2007). A study carried out in Taiwan, with prospective follow-up of women for 9 years, found no statistically significant overall risk of ICC, but a significant association with incident ICC (OR 2.9, 95% CI, 1.2-7.4) (Naucler *et al*, 2007a). The overall general impression from studies is that a modest effect of CT on ICC is plausible. However, a recent study showed no association between CT and CIN2+, arguing that previous results may have been related to an increased susceptibility to HPV infection caused by CT (Safaeian *et al*).

HSV-2 and Chlamydia Trachomatis may act as cofactors in development of ICC. This concept is more questionable for HSV-2 than for CT. The failure to detect them in tumors or prediagnostic cervical smears suggests that the effect comes early in carcinogenesis (Naucler *et al*, 2007a; Tran-Thanh *et al*, 2003). CT has been shown to increase expression of the proliferation marker Ki-67 antigen in cervical epithelium, and interfere with growth factor signaling (Fischer, 2002). There are also findings to support that CT possesses effective mechanisms to evade adaptive immunity.

Chromosomal aberrations are increasingly common with progressive disease and have been found in 19% of CIN1and 90% of CIN3 lesions (Wentzensen & von Knebel Doeberitz, 2007). Observed chromosome losses include 2q,3p, 4p, 4q, 6q, 11q, and 18q, while those gained include 1q, 3q, 5p, and 8q (Duensing & Münger, 2004). The most consistently noted aberration is gain of chromosome 3q and along with it, genomic amplification of the RNA component of the human telomerase gene (hTERC*)*, which resides on cytoband 3q26 (Heselmeyer *et al*, 1997; Heselmeyer *et al*, 1996). Genomic amplification of this gene is therefore likely to play an important role in progression from low-grade dysplasia to high-grade CIN and invasive cancer. In a previous study, progression was never observed in the absence of genomic amplification, and, inversely, extra copies of this gene were not present in lesions that spontaneously regressed (Heselmeyer-Haddad *et al*, 2005). This marker has shown promise to be highly sensitive and specific for identification of CIN3 and ICC (Andersson *et al*, 2009) and evaluation in large-scale screening is planned.

A genetic link to cervical cancer was first demonstrated in a Swedish epidemiological study published in 1999, showing a familial clustering of this disease specific to biologically related individuals. For example, there was an increased risk of ICC for biological mothers of cases compared with controls, whereas there was no difference in risk for adoptive mothers (Magnusson *et al*, 1999). One suggested genetic factor is a polymorphism at codon 72 of the p53 gene, resulting in either proline or arginine. Some researchers have seen increased risk of HPV–induced carcinogenesis in homozygous individuals, but study results have been somewhat conflicting (Andersson *et al*, 2001;

Bertorelle *et al*, 1999; Oliveira *et al*, 2008). Genetic variations related to adaptive immunity factors such as major histocompatibility complex class polymorphisms and cytokine signaling have also been suggested as contributive risk factors in HPV-associated carcinogenesis (Ghaderi *et al*, 2000) .

Nutritional factors have also been suggested to play a role in acquisition of HPV infection and carcinogenesis. A prospective study (24 months) of women at risk of HPV infection investigated the possible protective effects of folate (folic acid) for HPV infection, while controlling for vitamins B12, A, E, and C and total carotene, as well as taking into account known risk factors. Higher folate status was inversely related to the risk of acquiring HPV infection and individuals with higher folate status were more likely to be associated with becoming HPV–test negative during the follow-up time. Protective effects against CIN have also been observed for folate, as well as for riboflavin, thiamine, and vitamin B12 (Hernandez *et al*, 2003). High intake of fruits and vegetables, as well as specific micronutrients such as B vitamins including folate, appear to be associated with protective effects against persistent HPV infection and cervical carcinogenesis, though this field needs further study to establish robust evidence (Garcia-Closas *et al*, 2005).

A case-control study conducted in Honduras among women of low socioeconomic status, and taking into account HPV infection and additional risk factors for cervical cancer, showed an independent increase in risk from chronic (>35 years) exposure to wood smoke from wood fires in the kitchen (OR 9.42 (95% CI, 1.16-7.74) (Velema *et al*, 2002). The relationship was dose-dependent. Since wood smoke exposure is significant for many women in developing countries, this association merits further investigation.

2.4 A preventable disease

The well-known infectious etiology and associated risk factors, the availability of prophylactic HPV vaccines, and the usual 10-20 years required for detectable and easily treatable precancerous lesions to progress into invasive cancer, all offer excellent prospects for prevention of cervical cancer.

2.4.1 Behavioral prevention strategies

Primary prevention of cervical cancer means prevention of HPV infection and cofactors known to increase the risk of cervical cancer. Education is needed to improve knowledge about the disease, in order to reduce risky sexual behavior. Strategies to achieve less risk-taking behavior need to be adapted to local context. Moreover, cancer control programs should include efforts to reduce smoking, a known risk factor for cervical cancer and other cancers (2006). Early smoking initiation has also been associated with more lifetime sexual partners, earlier sexual debut, younger age at first pregnancy, and less condom use compared with never smokers, suggesting that this

group represents a target for health information (Hansen *et al*). Primary prevention through HPV vaccination is discussed in the following section.

Risky sexual behavior with a high number of sexual partners or concurrent sexual relationships is not necessary for HPV transmission. HPV infection is the most common STI and is prevalent even among individuals with few sexual partners. However, the risk of becoming infected depends on various factors. In countries where early marriage is customary, girls/young women commonly have their sexual debut at an earlier age than boys/men. Men spend more of their sexually active lives unmarried, and premarital sexual relations are more common among men than women. Hence, women may be at risk of HPV exposure even if they only have sex with their husband. A global trend is rising age at first marriage, and more widespread premarital sexual relationships among both men and women. Overall, single men report more numerous sexual relationships than do single women – however it is unclear whether bias in reporting contributes to these differences. Generally, among all sexually active men and women, men tend to report multiple sexual partners more frequently than women. In summary, these patterns suggest that men are a source of STIs, even within marriage. It is not only individual behavior that affects risk of HPV infection and other STIs, but the position of the individual in a sexual network (reviewed in (Bosch *et al*, 2008).

Consistent male condom use has proven to partially protect the female partner from HPV infection. Protective effects against CIN have also been observed (Veldhuijzen *et al*; Winer *et al*, 2006). Condom use is an attractive option to prevent HPV transmission, since it also protects against other STIs, including HIV, which is a risk factor for early, aggressive cervical cancer (2006).

In men, most HPV infections seem to clear within one year (reviewed in (Burchell *et al*, 2006)), with a mean duration of infection of about 7.5 months and 12.1 months for HPV 16 (Giuliano *et al*). Education to achieve increased awareness about the male role in HPV transmission, as well as recommendations for preventive measures, may also be one way to involve men in protecting women from future cervical cancer.

2.4.2 HPV vaccination

Technical advances in the 1990s enabled successful production of the major viral capsid protein L1 in eukaryotic expression systems. The ability of the L1 protein to self-assemble into highly immunogenic virus-like particles (VLPs) was also demonstrated (Kirnbauer *et al*, 1992; Zhou *et al*, 1991). Natural HPV infection does not elicit strong immune responses, but about 50-70% of women with incident HPV infection seroconvert within 18 months. Type-specific variation showed lower seroconversion levels for HPV 16 and 18 than for HPV 6 (Carter *et al*, 2000). In many women, low levels of anti-L1 antibodies are present several years after infection (af Geijersstam *et al*, 1998).

Today, one quadrivalent vaccine against HPV 16, 18, 6, and 11 (Gardasil, by Merck and Co, Inc) and one bivalent vaccine against HPV 16 and 18 (Cervarix, by GlaxoSmithKline Biologicals) are available on the market. Incorporation of HPV

vaccination of young girls 10- 12 years into the vaccination program, and catch-up vaccination of girls aged 13-17 years began in 2010. In Sweden, a central decision was made to use Cervarix, though this question is still under debate.

Both vaccines are given by intramuscular injection, with three doses given within 6 months. Delivered via the intramuscular route, both vaccines elicit a strong immune response compared with natural infection, and result in nearly 100% protection against incident HPV infection with the HPV types found in the vaccine (reviewed in (Stanley)).

For Gardasil, effective protection against CIN caused by vaccine HPV types has been documented. Such protective effects have also been shown among women with seropositivity for vaccine HPV types and simultaneous HPV DNA negativity, indicating protection against disease caused by re-infection or re-activation of latent infection (Olsson *et al*, 2009). Effective protection is also seen against vulvar and vaginal intraepithelial neoplasia (VaIN/VIN) and external condylomas caused by any of the vaccine HPV types. An RCT on the effects of Gardasil on HPV-associated genital disease in women aged 16-26 years at enrollment, with an average follow-up time of 3.6 years, was published in 2010. In women negative for 14 HPV types (12 HR-HPV types and LR-HPV 6 and 11) who had received at least one vaccine dose, the reduction of HPV 16/18/6 or 11-related disease was 98% for CIN1, 100% for CIN2, CIN3, and AIS, 96% for genital warts, and 95% for VaIN/VIN1 and VaIn/VIN1-3. In the intention-to-treat-population (HPV-exposed and non-exposed), the reduction of HPV 16/18/6 or 11-related disease was 69% for CIN1, 55% for CIN2, 45% for CIN3, 60% for AIS, 80% for genital warts, and 76-79% for VaIn/VIN. Considering genital lesions caused by any HPV type, reduction of incident genital disease rates among the population negative for 14 HPV types were: CIN 1+ 29%, CIN2+ 43%, genital warts 83%, and VaIN/VIN2-3 77%. In the intention-to treat-population, the reduction of incident genital disease rates caused by any HPV type were as follows: CIN1+ 19%, CIN2+ 19%, genital warts 62%, and VaIN/VIN2-3: 50% (Munoz *et al*).

For Cervarix, an RCT enrolling 15-25 year old girls/women, with average follow-up of almost three years, also showed a high degree of efficacy against CIN2+ related to HPV 16 and 18 and to nonvaccine HPV types (Paavonen *et al*, 2009). In a cohort of women (irrespective of HPV status) who had received at least one vaccine dose, HPV 16 or 18-related CIN2+ was reduced by 53% and CIN3+ by 34%. For CIN2+ related to any HPV type, the reduction was 30%, and for CIN3+ 25%. In a cohort of women naive for 14 HPV types who had received at least one vaccine dose, HPV 16 or 18-related CIN2+ was reduced by 98% and CIN3+ by 100%. For CIN2+ related to any HPV type, the reduction was 87%. Cross-protection has also been seen against CIN2+ caused by HPV 31, 33, and 45. Significant cross-protection against persistent infection with non-vaccine HPV types has been seen for both vaccines (Lu *et al*).

In summary, the two prophylactic HPV vaccines are safe, well-tolerated (Lu *et al*; Wacholder *et al*), and effective in preventing HPV infections and lesions associated with vaccine HPV types in young women (Lu *et al*; Munoz *et al*; Paavonen *et al*, 2009). In a perfect world, the current HPV vaccines may prevent up to 70-76% of all ICC (de Sanjose *et al*; Li *et al*); about 66-70% of SCC, and about 78-82% of ADC (de

Sanjose *et al*). According to estimates of figures for high-grade precancerous lesions (CIN2-3/HSIL), HPV 16 and/or 18 account for about 47-67% (Clifford *et al*, 2006; Smith *et al*, 2007). The potential preventive effect may be even higher, depending on the degree of cross-protection induced against disease caused by close relatives of HPV 16 and 18. To achieve optimal preventive effect, the vaccine should be administered before sexual debut, but preventive effects have also been seen against re-infection and re-activation of HPV infection and associated diseases.

Once the vaccine program is fully implemented, efficacy and safety will need to be followed prospectively. Given the incomplete protection against future cervical cancer and uncertainty regarding duration of protective effects, screening will need to continue in some form even in vaccinated cohorts. A major challenge will be to inform vaccinated individuals about the continued importance of screening (Sundstrom *et al*).

2.4.3 Screening – a success story

Secondary prevention of cervical cancer involves organized screening programs to target the appropriate age group, as well as effective links between all levels of care, including appropriate follow-up for diagnosis and treatment of women with positive screening results (2006).

2.4.3.1 History of cervical screening

Since the middle of the last century, cervicovaginal cytology screening with Papanicolau (Pap) smears has been used in many regions worldwide, saving lives of millions of women. The method is named after its innovator, Dr. Papanicolau, born in Greece in 1883. He was a physician with a many talents. The idea of taking vaginal smears emanated from his research that involved collecting oocytes from guinea pigs. In order to evaluate timing of ovulation, Dr. Papanicolau started analyzing cells collected with vaginal smears. In the 1920s he started analyzing vaginal smears from humans. When he first observed malignant cells in a vaginal smear he realized that he had made an important discovery. However, the scientific community showed little interest in the method (reviewed in (Michalas, 2000)). The breakthrough did not come until 1941, after many years of observation and robust correlation with cervical cancer (Papanicolau GN, 1941). In this paper, Dr. Papanicolau emphasized the need for a simple inexpensive method that could be applied to large numbers of women for early diagnosis of cancer.

2.4.3.2 Cytological evaluation and classification

The rationale for cytological screening to prevent cervical cancer is as simple as it is ingenious. By evaluating cells collected from the cervix, precancerous lesions in asymptomatic women can be identified and treated, thereby interrupting the possible progressive evolution of the lesion toward malignancy. In addition the procedure may identify women who have already developed invasive cancer at an early stage, which improves prospects of successful treatment.

Most abnormalities detected in cytological screening are subtle and non-specific, with a low positive predictive value for detection of CIN2+ and CIN3+. There is also a high degree of interobserver variability in cytological assessment, resulting in highly variable test accuracy (Fahey *et al*, 1995; Scott *et al*, 2002). Pronounced cytological abnormalities are highly specific, with high positive predictive value for such lesions. However, sensitivity of cytology screening to detect precancerous lesions is limited, with a high degree of variation between screening clinics, cytolopathologists and cytotechnologists (Cuzick *et al*, 2008).

Different cytological classification systems exist. The Swedish classification system can easily be translated into the Bethesda classification system (Solomon *et al*, 2002), the most commonly used system in the scientific community today (Table 2.3). About 600 000-700 000 cytology smears are sampled every year, of which 57-70% are carried out under the population-based screening program. About 80% of all abnormal smears are non-specific low-grade abnormalities (ASCUS, AGUS/AGC or LSIL), and only 20% are diagnosed as the high-grade abnormalities (ASC-H, ACG-H or HSIL) that usually account for histologically verifiable high-grade lesions (Sparén, 2008).

Summary of the Besthesda 2001 system	
Specimen adequacy:	
Satisfactory for evaluation (including statement on presence/absence of endocervical cells)	
Unsatisfactory for evaluation	
Interpretation:	
Negative for intraepithelial lesion or malignancy	
Epithelial cell abnormality:	
Squamous cell	Glandular cell
ASC-US (atypical squamous cells of undetermined significance	AGC (specify endocervical, endometrial or not otherwise specified)
ASC-H (atypical squamous cells cannot exclude HSIL)	AGC-H (atypical glandular cells, favor neoplastic)
HSIL (high-grade squamous intraepithelial neoplasia)	AIS (endocervical adenocarcinoma in situ)
Squamous cell carcinoma	Adenocarcinoma

Table 2.3. Cytological classification according to a summarizd version of the Bethesda 2001 terminology (Solomon et al, 2002).

2.4.3.3 Follow-up and treatment

Following an abnormal screening result, the gold standard investigation to diagnose CIN includes a pelvic examination by a specially trained clinician (colposcopist), colposcopic assessment of the cervix and vaginal fornices, biopsy of suspected lesions, and endocervical brush sample for endocervical sampling, alternatively endocervical curettage if the patient is under anaesthesia. Colposcopy involves microscopic examination of the cervix, before and after application of acetic acid and iodine (2006 ; Andrae B, 2010). The colposcopist assesses for the presence of acetowhite areas including punctuation and mosaic appearance, presence and characteristics of epithelial capillaries, size of visible lesions, and iodine uptake (Walker *et al*, 2003). The examination should determine the location of the transformation zone and identify suspected precancerous and cancerous lesions. In relevant cases, a decision should be taken about the most suitable treatment depending on patient characteristics such as age, fertility status, desire for future pregnancy, and risk profile (2006 ; Andrae B, 2010; Wright *et al*, 2007b).

Colposcopy complemented with biopsy and histological assessment is a gold standard examination with certain disadvantages. Performance of colposcopy is highly variable between examiners. In one study involving seven colposcopists, sensitivity for detection of histologically verified CIN3+ varied from 29 to 93% (p=<0.001).Sensitivity increased with the number of biopsy samples, up to 4 "random" biopsies, and with endocervical curettage in 6/7 colposcopists (Pretorius *et al*), thus underlining the risk of sampling error. There is strong scientific support for random biopsies and endocervical sampling in absence of visually obvious pathology (reviewed in (Chase *et al*, 2009)). Both over- and underdiagnosis in histological evaluation may occur and are most pronounced with less advanced lesions (Carreon *et al*, 2007).

If a diagnosis of CIN2 or CIN3 is made by histological assessment, surgical removal of the lesion is always indicated in cases of CIN3, while there are exceptions in CIN2 (see below). In cases of histological findings of CIN1, treatment is indicated in HR-HPV positive women who are not likely to plan future pregnancies. A relative indication for treatment of CIN1 is persistent CIN1 for >2 years and a direct indication in women >40 years (Andrae B, 2010).

Side-effects are associated with treatment of CIN. A Cochrane review published in 2010 assessed CIN treatment options including laser conization, large loop excision of the transformation zone (LLETZ), knife conization, cryotherapy, and laser ablation in regard to residual disease detected on follow-up examination, severe perioperative pain, severe primary and secondary bleeding, cervical stenosis at follow-up, vaginal discharge, incomplete colposcopy at follow-up, extent of thermal artifact histological specimen, and duration of treatment. The authors concluded that no technique is obviously superior to other in terms of treatment failure or operative morbidity, but LLETZ appeared to provide the most reliable histological specimen with the least morbidity (Martin-Hirsch *et al*). A major drawback of ablative methods is that no tissue specimen is available for histological analysis.

Moreover, prior treatment of CIN requires long-term follow-up due to the increased risk of future CIN3 and cervical cancer (Melnikow *et al*, 2009; Strander *et al*, 2007b). The risk is particularly high in women treated with cryotherapy (Melnikow *et al*, 2009; Strander *et al*, 2007b). Treatment of CIN is also associated with increased risk of perinatal morbidity and mortality. A meta-analysis studied the effect of different excisional and ablative CIN treatment techniques on perinatal mortality, preterm delivery, and low birth weight. The highest risks are seen for cold knife conization. Destruction using laser ablation and cryotherapy is associated with fewer perinatal side-effects than excisional techniques (Arbyn *et al*, 2008b). Ablative methods are second line therapy according to Swedish guidelines (Andrae B, 2010). They are considered suitable only if the histological result from a colposcopically directed biopsy confirms suspicion of CIN2, and invasive disease can be excluded beyond all reasonable doubt, and in women with an overt or assumed desire to become pregnant in the future. In young women with CIN2, a wait and see approach is an alternative due to the high rate of spontaneous regression in this group. Women younger than 40 years diagnosed with persistent CIN1 should not be treated, but followed-up (2006 ; Andrae B, 2010; Wright *et al*, 2007a).

Management guidelines for CIN treatment are based on a relatively crude estimate of future cancer risk; overtreatment is significant since many of the lesions that are treated would have regressed spontaneously. Improved knowledge of prediction of progression and invasion is needed to minimize side-effects of screening, follow-up, and treatment of potentially precancerous lesions. Therapeutic vaccination (e.g. against viral oncoproteins) represents an attractive option. Experiments in animals and humans have generally shown a high tolerability for these vaccines, a few of which have also induced significant immune responses. Proof of clinical efficacy in premalignant and malignant disease in humans is still limited (Decrausaz *et al*; Trimble & Frazer, 2009), but some vaccines have induced regression of CIN in a large percent of patients (van der Burg *et al*).

2.4.3.4 Preventive effects of conventional cytology screening in Sweden

Previous studies have shown that, although insufficient participation in screening and poor management of abnormal screening findings are major contributors to cervical cancer in Sweden, some cancer cases develop in spite of previous normal screening results (Andersson-Ellstrom *et al*, 2000; Stenkvist & Soderstrom, 1996). Reliability of cytological screening therefore depends on repeated sampling.

In Sweden, a population-based screening program was progressively established in the 1960s and 1970s. Today, women are invited to participate in screening from the year they turn 23 years, then every three years until the age of 50, after which screening is scheduled every five years until age 60. Screening has been successful, reducing incidence of SCC by about two thirds and overall cervical cancer-induced mortality even more (Bergstrom *et al*, 1999; Gunnell *et al*, 2007). The incidence of ADC, however, has increased since initiation of the screening program despite an increase in the number of cases of adenocarcinoma in situ (AIS) found by screening (Gunnell *et al*,

2007). The protective effect of cervical cytology screening against ADC has been called into question (Dahlstrom *et al*).

A nationwide audit of the Swedish screening program was recently undertaken, including all adequately reported cervical cancer cases diagnosed 1999-2001 (n=1230) and five age-matched controls per subject (Andrae *et al*, 2008). Results showed that non-participation was associated with an increased risk of developing non-squamous cervical carcinoma (mainly ADC) with OR 1.59 (95% CI: 1.30-2.11), whereas the corresponding figure for SCC was 2.97 (95% CI: 2.51-3.50). These results argue for a weaker but nevertheless protective effect of screening even for ADC. The increased risk of non-participation in screening was seen in all age groups (21-29, 30-65, and >66 years). Non-participation was also associated with a higher risk of presenting with an advanced cancer stage (FIGO IA: OR 1.70, 95% CI, 1.28-2.26; FIGO IB: OR 2.10 95% CI, 1.71-2.59; FIGO ≥II 4.84, 95% CI, 3.61-6.44).

These results are supported by an analysis comparing the characteristics of a prescreening and screening cohort of women treated for cervical cancer at Radiumhemmet Oncology Clinic in Stockholm, Sweden, during the periods 1944-1957 and 1990-2004 respectively. In the screening cohort, women were older (mean age 55 vs. 49 years) with a larger portion of women older than 69 years (27% vs. 5%) compared with the prescreening group. In the prescreening cohort, only a minority of patients presented with early stage (FIGO stage I) cancer (17%) whereas in the screening cohort 55% of cases were early cancers. Adenocarcinoma comprises a proportionally larger group in the screening cohort compared with the prescreening cohort (26% vs.5%) (Accepted in International Journal of Gynecological Oncology (Pettersson Folke, 2011)).

Inadequate management of abnormal smears is relatively common in the history of women diagnosed with cervical cancer in spite of screening participation. This is particularly common for low-grade cytological abnormalities, among which a significant number of all high-grade precancerous lesions are detected (Kinney *et al*, 1998) . Repeated cytology has been proven to be an inadequate strategy for follow-up of low-grade abnormalities. Women with low-grade abnormalities are at increased risk of cervical cancer. Histological assessment leads to a reduced risk compared with cases managed with repeat cytology alone (OR: 0.46, 95% CI, 0.24-0.89) (Silfverdal *et al*; Stenkvist & Soderstrom, 1996).

In conclusion, non-participation in cervical screening and inadequate follow-up and treatment of abnormal screening findings are major reasons why women develop cervical cancer and advanced disease with ensuing high mortality. Screening coverage in Sweden is 70-80%, with significant variation among different regions (Sparén, 2008). The aim set by the European Union is to reach at least 85% screening coverage. However, no EU country has achieved this goal through organized screening; coverage varies from 10-79% (Anttila *et al*, 2009). Improving screening coverage and clinical management of women with abnormal screening results can further reduce cervical cancer incidence and mortality even in countries with established organized population-based screening programs.

Today, a variety of screening methods and strategies exist, many of which are related to detecting the presence of HR-HPV infection (see 2.5). During 2010, prophylactic HPV vaccination was incorporated into the Swedish vaccination program for girls aged 10-12 years along with catch-up vaccination for girls aged 13-17 years. Up to this point in time, vaccination of girls up to 17 years was subsidized, but not free of charge. Within six years, cohorts of vaccinated young women will reach screening age and strategies will have to be adapted to fit this new situation.

2.5 Alternative screening options

2.5.1 Liquid-based cytology

Liquid-based cytology was developed as an alternative cytology method and several different brands are commercially available. The method is described in section 4.2.2. In some parts of the world, e.g. the US and the UK, LBC is the predominant cytology method in use.

One advantage of LBC is that after cytology slide preparation, supplementary analysis can be carried out, such as reflex HPV testing for high-risk human papillomavirus (HR-HPV) DNA. Detection of HR-HPV can distinguish between nonspecific minor cytological abnormalities and abnormalities that may be associated with a high risk of current or future CIN2+ (Arbyn *et al*, 2006; Froberg *et al*, 2008). The number of abnormal cytology findings can thereby be reduced, allowing healthcare resources to be allocated to women who may better benefit from follow-up and treatment.

Moreover, several studies have shown that LBC reduces the rate of unsatisfactory screening samples (Davey *et al*, 2007; Kirschner *et al*, 2006; Ronco *et al*, 2007a; Strander *et al*, 2007a). In settings with high rates of unsatisfactory smears, this advantage is important, since unsatisfactory smears entail re-sampling. In Sweden, however, rates of unsatisfactory smears are low (1%) (Sparén, 2008) and therefore this advantage alone would not motivate a change to LBC in population-based screening. The explanation can likely be found in the fact that highly skilled midwives sample virtually all screening smears in Sweden, whereas in other settings screening is performed by more diverse categories of medical staff with less focused training and less experience in sampling technique.

Previous studies have noted an increased rate of abnormal cytology findings using LBC compared with conventional cytology (Davey *et al*, 2007; Kirschner *et al*, 2006; Ronco *et al*, 2007a; Strander *et al*, 2007a). Other studies have interpreted such findings as an indication of increased sensitivity for detection of CIN, without gold standard examination with colposcopy and biopsy. The question of whether LBC increases sensitivity for detecting precancerous lesions, including glandular lesions, is controversial. Previous studies of liquid-based cytology (LBC) screening performance have yielded contradictory conclusions. A systematic review published in 2006 (Davey *et al*, 2006) investigated the relative performance of these methods, and how

performance was influenced by study design and quality, including factors such as validity, type of reference standard used, allocation of screening test, and blinded evaluation of reference standard. The authors concluded that study design of previous studies was generally poor, and only a few studies used adequate methods to compare tests (Bossuyt *et al*, 2004). Existing data did not support superior sensitivity for CIN2+ detection, and authors called for large-scale RCTs with adequate reference standards and methodology.

A meta-analysis with rather strict but well-motivated inclusion criteria (Arbyn *et al*, 2008a) concluded that LBC was not superior to CC in either sensitivity or specificity for detection of CIN2+. Pooled relative sensitivity, when screening tests show ASCUS or more advanced abnormalities, was 1.03 (95% CI, 0.97-1.09) and relative specificity 0.91 (95% CI, 0.84-0.98). Some studies with histologically confirmed study endpoints have shown improved detection of high-grade lesions (CIN2+) compared with CC (Davey *et al*, 2007; Halford *et al*; Hussein *et al*, 2005; Hutchinson *et al*, 1999; Strander *et al*, 2007a). Others found no difference or even somewhat lower positive absolute or relative sensitivity for LBC (Coste *et al*, 2003; Hutchinson *et al*, 1999; Mattosinho de Castro Ferraz Mda *et al*, 2004; Ronco *et al*, 2007a; Siebers *et al*, 2009; Taylor *et al*, 2006).

A previous Swedish RCT (not included in the meta-analysis by Arbyn *et al*) comparing performance of LBC with CC in a population-based screening setting concluded that the detection rate of histologically verified CIN2+ increased markedly, by about 40%, with LBC (Strander *et al*, 2007a). Such findings led to increased use of the newer cytology method in the Swedish screening program. Since the publication by Strander *et al*, large-scale RCTs in population-based screening settings have been carried out in the Netherlands (Siebers *et al*, 2009) and Italy (Ronco *et al*, 2007a). Neither of these studies has shown significantly improved sensitivity for CIN2+ or CIN3+. However, an interesting finding was that the study by Siebers *et al* showed a proportionally large but statistically insignificant increased detection rate of carcinoma (adjusted detection rate ratio: 1.69: 95%, 0.96-2.99). One large-scale split-sample study in Australia (Davey *et al*, 2007) concluded that LBC was somewhat more sensitive for CIN2+, with 1.29 more cases found per 1000 women screened. This figure is based on histological follow-up diagnosis of discordant screening samples and the overall detection rates of CIN2+ were not addressed.

In summary, LBC facilitates supplementary analysis (e.g. HPV reflex testing), reduces rates of unsatisfactory screening results, and increases the rate of abnormal cytology findings, but the question of whether it improves diagnostic accuracy of cytology screening remains unclear.

2.5.2 Supplementary HPV testing

2.5.2.1 Clinical relevance and diagnostic performance

In Sweden, about 80% of all abnormal screening results are low-grade abnormalities (Sparén, 2008). Although the positive predictive value of low-grade cytology findings for detection of high-grade cervical lesions is low, a significant portion of all high-grade cervical intraepithelial neoplasia (CIN2+) detected through screening can be found in this group (Kinney *et al*, 1998). Colposcopy with directed biopsy is the gold standard method for detection of CIN, and was previously (since 2001) used for follow-up of all abnormal cervical screening results in Stockholm County Council. Disadvantages of this strategy for minor cytological abnormalities include unnecessary psychological stress, risk of overdiagnosis and overtreatment with subsequent adverse events, and elevated healthcare costs for the rather large number of women without clinically significant cervical lesions (Arbyn *et al*, 2008b; Chase *et al*, 2009; Freeman-Wang *et al*, 2001).

The causal relationship between HR-HPV infection and cervical cancer, along with highly sensitive methods for HR-HPV detection, have made detection of viral DNA an attractive approach to distinguish clinically irrelevant minor cytological abnormalities from those which may be associated with an increased risk of developing cervical cancer (2000; 2003a; 2003b; Andersson *et al*, 2005a; Arbyn *et al*, 2006; Dillner *et al*). The idea of this strategy is to achieve more targeted management by referring only those women who may actually benefit from more extensive work-up and treatment. This strategy is called HPV triage when used as a supplement to CC and entails a second patient visit for HPV sampling and testing. If LBC is used in screening, supplementary HPV testing can be carried out directly from residual material following cytology slide preparation, so-called “reflex” testing. This strategy does not necessitate patient recall, and reduces sampling error.

Cytology-based triage of ASCUS and LSIL (one or more repeated cytology smears and re-referral to screening if cytology samples are negative, otherwise work-up including colspocopy) has been a widely used follow-up strategy in Sweden and in other countries. The observed increased risk of cervical cancer among women with minor cytological abnormalities in their screening history, who had cytological but not histological follow-up, emphasizes the limitations of this follow-up strategy (Silfverdal *et al*, 2009). The low sensitivity of repeat cytology for detection of precancerous lesions as a follow-up method for minor cytological abnormalities is well-documented (Arbyn *et al*, 2006). The role of repeat cytology for follow-up of minor cytological abnormalities is now insignificant or substantially reduced in current guidelines (Andrae B, 2010; Arbyn *et al*; Wright *et al*, 2007a).

Because of the high sensitivity and negative predictive value of HR-HPV detection for high-grade precancerous lesions, HPV triage has now become an uncontroversial approach for management of ASCUS (2003b; Arbyn *et al*, 2006; Cuzick *et al*, 2008). HPV triage/reflex HPV testing has not been generally recommended in cases of

LSIL, due to the high prevalence of HR-HPV, which leads to poor specificity for HPV triage in this group. Results from a meta-analysis show that overall HPV test positivity varies substantially among studies, but is overall significantly lower in ASCUS (43%: 95% CI, 40-46%, range 23-74%) than in LSIL (76%: 95% CI, 71-81%, range 55-89%). The highest rate of HPV-positivity seen in women with ASCUS aged <30 but > 20 years was 72% (Arbyn *et al*, 2009a).

There is increasing support for HPV triage in older women with LSIL since HPV test sensitivity is usually high even in this group, and important reductions of the HR-HPV test positivity rate after age 30 improved test specificity (Arbyn *et al*, 2009b; Brismar-Wendel *et al*, 2009; Dillner *et al*; Evans *et al*, 2006; Froberg *et al*, 2008; Ronco *et al*, 2007b). A large-scale Italian trial also concluded that HPV triage using Hybrid Capture 2 performed better in 35-60 year old women than in 24-34 year old women, both for ASCUS and LSIL (Ronco *et al*, 2007b). An age limit of 40 years has also been discussed (Cotton *et al*).

2.5.2.2 Cost-effectiveness

The exclusion of HR-HPV-negative women from intensive follow-up could result in lower costs for the Swedish screening program. Colposcopy is resource-intensive, and several studies have shown that it results in high costs and is associated with risk of adverse effects, which indicates a need for a more efficient and less aggressive alternative (Cantor *et al*, 1998; Johnson *et al*, 1993; Kim *et al*, 2002; Myers *et al*, 2000; Smith, 1999)

A recent trial conducted in Stockholm, Sweden, concluded that HPV triage to follow up ASCUS and LSIL was equally effective as immediate colposcopy for detection of CIN2+. The authors observed equally high (about 80% or more), and even somewhat higher HPV test positivity in ASCUS compared with LSIL, in women up to 31 years of age. An associated cost-effectiveness analysis showed that the least costly strategy for follow-up of ASCUS and LSIL was immediate colposcopy in women < 35 years and HPV triage in women ≥35 years (Dillner *et al*). A study performed in the US showed that reflex HPV testing in ASCUS (regardless of the woman's age) was superior to both immediate colposcopy and repeat cytology (Kim *et al*, 2002).A German study concluded that HPV triage with "two follow-up visits" (as a supplement to CC), was cost-effective compared with repeated cytology and immediate treatment in women with Pap III and Pap IIId (roughly corresponding to ASCUS and LSIL/CIN1 to HSIL/CIN2 respectively) (Sheriff *et al*, 2007). One cost-effectiveness study showed that HPV "reflex" testing is more effective and less costly than HPV triage with "two follow-up visits", repeat cytology and immediate colposcopy in women with ASCUS (Kim *et al*, 2002). Similar results were found in other cost-effectiveness analyses of HPV "reflex" testing in cases of ASCUS, which

concluded that this strategy was cost-effective in women of all ages (Kim *et al*, 2005; Kulasingam *et al*, 2006).

Since 2010, Swedish guidelines recommend immediate colposcopy with biopsy for management of ASCUS and LSIL for women younger than 35 years and HPV triage for women ≥35 years (Andrae B, 2010). However, repeat cytology was still used in 2010 as a follow-up method in some parts of Sweden.

2.5.3 Primary HPV testing

A single HPV test has higher sensitivity (~96%) for detection of high-grade CIN than a single cervical cytology sample (50-75%), although it is somewhat less specific (Cuzick *et al*, 2006). Considering this fact, it seems logical to use the more sensitive test for primary screening and the less sensitive but more specific test to triage for further measures.

Primary HPV testing (alone, in combination with concomitant cytology, with cytology triage, or repeated HPV testing to target persistent HPV infection) has gained strong support as a highly effective screening strategy for prevention of cervical cancer. Accumulating evidence from a number of large-scale RCTs shows that HPV DNA detection as a primary test in population-based screening, increases detection rates of CIN2+ and CIN3+ compared with cytology-based screening (Bulkmans *et al*, 2007; Cuzick *et al*, 2003; Leinonen *et al*, 2009; Naucler *et al*, 2007b; Ronco *et al*). According to a study investigating possible screening strategies using data from the Swedish trial by Naucler *et al* (including women aged 32-38 years), the gain in sensitivity from using a combination of primary HPV testing and cytology was limited. The authors found that the optimal trade-off between sensitivity and a high PPV for detection of CIN3+ was achieved by complementing HPV testing with cytology triage and repeated HPV testing in women who are HPV positive but cytology negative, at baseline (Naucler *et al*, 2009).

One British study comparing combined HPV test and cytology with cytology screening alone, found equal detection rates of precancerous lesions at baseline. In the second screening round, however, the combined resulted in a 50% lower detection rate of CIN3+ compared cytology alone (Kitchener *et al*, 2009). Additional studies have also found similar reductions in detection rates of CIN3+ in subsequent screening rounds (Bulkmans *et al*, 2007; Naucler *et al*, 2009; Ronco *et al*). The Italian trial by Ronco *et al* also demonstrated a significant reduction in invasive cancer incidence following primary HPV screening compared with conventional cytology. Similar findings were obtained in a study conducted in rural India in a setting that lacked organized cervical screening. In that study, screening with a single HPV test in women aged 30-59 years resulted in significant reductions in incidence of advanced cervical cancer (FIGO stage II or more advanced stages) and cervical cancer-related mortality compared with routine practice, which included information on cervical cancer and how to seek screening within the existing healthcare system. Screening with a single cytology

sample or VIA (visual inspection of the cervix with acetic acid) did not result in any significant benefits compared with routine practice (Sankaranarayanan *et al*, 2009).

Given the high HPV prevalence among young women and the high rate of regressive cervical lesions, primary HPV testing in this group leads to overdiagnosis and overtreatment (Leinonen *et al*, 2009; Ronco *et al*, 2007b). An age limit of about 30-35 years is probably suitable, but this may need to be reconsidered when cohorts of HPV-vaccinated women reach screening age. HPV genotyping allows identification of type-specific persistent infection, and may represent an attractive approach to target women at highest risk of high-grade CIN and future ICC.

Prolongation of screening intervals could be considered if HPV testing is used in primary screening, since a negative HPV test (alone or in combination with cytology) is a more reliable predictor of long-term absence of high-grade CIN than cytology alone (Cuzick *et al*, 2008; Mesher *et al*). Cost-effectiveness studies indicate that HPV testing in primary screening is an attractive option in relation to today's screening policies (Bistoletti *et al*, 2008; Goldie *et al*, 2004; Kim *et al*, 2005).

3 AIMS

3.1 General aim

The objective of this study was to evaluate liquid-based cytology (LBC) and supplementary HPV testing in Sweden's current cervical cancer screening program. An ideal screening strategy should be:

- Highly sensitive (with low risk of false negative results) to identify true precancerous lesions.
- Highly specific (with low risk of false positive results) to avoid unnecessary examinations and worry for women as well as unnecessary waste of resources.
- Cost-effective, i.e. costs should be reasonable in relation to health-related outcome.

3.2 Specific aims

3.2.1 Paper I

The aim of this study was to compare the combination of LBC plus HPV reflex testing in cases of minor cytological abnormalities (LBC+ HPV testing) with conventional cytology (CC) in regard to their ability to identify precancerous lesions in a population-based screening setting.

3.2.2 Paper II

The aim of this study was to determine the value of HPV genotyping as a reflex test in cases of minor cytological abnormalities.

3.2.3 Paper III

Since prevalence of HPV infection is age-dependent, with high prevalence and low discriminative value among younger women, we searched for a suitable age limit for HPV reflex testing. The purpose was also to define the age-specific HPV infection spectrum before implementing prophylactic HPV vaccine in the vaccination program.

3.2.4 Paper IV

The purpose of this study was to assess the cost-effectiveness of HPV triage as an alternative follow-up strategy to immediate colposcopy with directed biopsyor repeat cytology alone, for management of minor cytological abnormalities.

4 MATERIAL AND METHODS

4.1 Study subjects and study design

4.1.1 Paper I

4.1.1.1 Enrollment and management of different cohorts

This prospective clinical trial invited women who were included in the population-based cervical screening program at six sampling centers in suburban Stockholm to participate. Over the course of one year (September 2005-September 2006) women were assigned to screening either with conventional cytology (CC, n=4261) or with liquid-based cytology (ThinPrep®, Hologic, Marlborough, MA, USA) and supplementary HPV testing in cases where minor cytological abnormalities (ASCUS/LSIL) were found (LBC+ HPV testing, LBC1, n=4059). Screening methods were alternated every other week. To gain more experience using the new method, LBC+ HPV testing exclusively was performed between September and December 2006 (LBC2, n=2016). In all, 6075 women were assigned to LBC+ HPV testing and 4216 women to screening with CC from September 2005 to December 2006. The main analyses in this study were based on the results from screening these women. To maintain proficiency in the use of LBC, LBC+HPV testing continued at two of the six sampling centers (A and B), while awaiting a definitive administrative decision regarding choice of screening method. For the purpose of evaluating the LBC+ HPV testing screening strategy over time, women screened in 2007 were included (LBC3, n=3383) along with the 2653 women screened at these centers from 2005 to 2006.

4.1.1.2 Follow-up of abnormal screening results

Women with abnormal screening results, using ASCUS as a cut-off, were routinely referred to a gynecology clinic for work-up including a pelvic examination, colposcopy with directed biopsies of suspicious areas, and repeat Pap smear including an endocervical brush sample. In clinical practice blind biopsies are encouraged if there is any doubt regarding absence of pathology, but management may vary. Follow-up cytological and histological samples were assessed within the regular healthcare system. Clinicians had no information on which cytology method was used.

All women with abnormal screening results were followed up for two years from the time of the original screening occasion. Results were retrieved via laboratory and medical records, supplemented with data from the Stockholm Oncology Center (Onkologiskt Centrum Stockholm). Within the follow-up period most women will have had at least one colposcopy with subsequent biopsies and treatment with conization if indicated. Most treated women will also have had a post-treatment follow-up visit. Moreover, the follow-up time falls well within one screening cycle.

4.1.1.3 Outcomes

The primary endpoint of this study was detection rate of histologically confirmed CIN2+ (CIN grade 2 or more advanced lesions) yielded by LBC+HPV testing versus CC. Detection rates of CIN using CIN1+ and CIN3+ as cut-offs (CIN1 or more advanced lesions and CIN3 or more advanced lesions) were also compared for the sake of thoroughness. We compared PPV of the two different screening strategies to detect CIN1+, CIN2+, and CIN3+. Secondary endpoints were the rates of abnormal cytological findings including both high-grade cytology and low-grade cytology.

4.1.2 Paper II

This prospective trial evaluates HPV reflex genotyping of LBC samples from population-based screening showing minor cytological abnormalities. Histological follow-up results obtained within one year from screening were used as the reference standard, with CIN2+ as the primary endpoint.

Six screening units (one of which comprised two nearby clinics) in the southern suburbs of Stockholm participated in the study. From September 2005-September 2006, about 4000 consecutive liquid-based cytology samples were obtained from routine screening. There were 149 cases of minor cytological abnormalities (ASCUS/AGUS n=53 and LSIL n=93). Mean age was 33 years, median age 30 years, and age range 22-59 years. Women with ASCUS as a screening result were older than women whose screening sample showed LSIL; mean ages were 35 and 31 years, respectively. Age ranges were 22-58 years and 22-59 years respectively.

All women with minor cytological abnormalities were referred for gynecological investigation within the regular healthcare system. Follow-up guidelines recommended colposcopy, directed biopsies, and a repeat Pap smear including endocervical sampling. If no colposcopically visible lesion was seen, blind biopsies were encouraged, but management varied. Follow-up data were traced through medical and laboratory records and supplemented with data from the Stockholm Oncology Centre. All calculations were based on the 112 women for whom histological follow-up results were registered within the predefined follow-up period.

4.1.3 Paper III

This cross-sectional study on the age-specific HPV infection pattern in women with screening results showing minor cytological abnormalities included 343 cases from population-based screening, with 120 cases of ASCUS (including 3 cases of AGUS) and 223 cases of LSIL. Age data were not accessible for one ASCUS and one LSIL case, and therefore age was recorded for only 341 of 343 women. Mean age was 33.6 years (median 32, range 22-60 years). Mean age of women with screening results showing ASCUS was 35.7 years and for women with LSIL, 32.4 years. Women with ASCUS were older than women with LSIL; 38% versus 21% were older than 40 years. Women were grouped according to age at 5-year intervals from age 20-49 years; the oldest women, aged 50-60 years, were placed into a single group of comparable size.

4.1.4 Paper IV

4.1.4.1 Study population

This cost-effectiveness analysis is mainly based on data from a Swedish clinical trial (Andersson *et al*, 2005a) comparing HPV triage with repeat cytology for management of ASCUS and LSIL, using colposcopy with directed biopsies as the gold standard. Primary screening was performed using conventional cytology. The study group consisted of a total of 117 women referred for gynecological investigation because of screening results showing ASCUS (n=52) or LSIL (n=125). These women underwent repeat conventional cytology sampling, HPV testing (Hybrid Capture 2®, Digene Corporation), and colposcopy with at least one biopsy. The most severe lesion found on biopsy or in subsequent cervical cone material determined the histologic diagnosis with which HPV test and Pap smear results were compared. Mean age of patients was 34 years (median: 31 years; range 23-60 years).

4.1.4.2 Description of follow-up strategies for ASCUS and LSIL

1. Repeat cytology (Cytology) means referral for gynecological consultation and repeat conventional cytology smear only. In cases of cytological abnormality, with ASCUS as the cut-off, women are referred for a second follow-up visit to a gynecologist for colposcopy with biopsy. If the follow-up Pap smear is within normal limits, the woman is referred back to the primary screening program for a new Pap smear three or five years later, depending on patient age.

2. HPV-based triage (HPV triage) means referral for a follow-up visit to a midwife for HPV testing. HR-HPV-positive women are referred for a second follow-up visit to a gynecologist for colposcopy with biopsy. HR-HPV-negative women are referred back to the primary screening program.

3) Immediate colposcopy (Colposcopy with biopsy) means immediate referral for gynecological examination including colposcopy and directed biopsies. If no colposcopically visible lesion is seen, a biopsy is taken close to the squamocolumnar junction at 12 o'clock. Sensitivity and specificity of this strategy are considered to be 100%, since by definition this approach constitutes the reference examination for diagnosing CIN.

The primary endpoints of these strategies are sensitivity and specificity to detect histologically confirmed CIN2+ lesions.

4.2 Cytology

Cytological diagnoses were defined using a modified Bethesda nomenclature(Solomon *et al*, 2002, as shown in Table 2.3(Solomon *et al*, 2002). The Bethesda classification was modified according to Swedish recommendations that define samples with

koilocytosis without cellular atypia to be within normal limits (WNL). Therefore, LSIL only includes cases of mild dysplasia.

In all studies including LBC (Paper, I, II and III) all cytological screening samples were prepared and assessed at the Department of Clinical Pathology and Cytology, Karolinska University Hospital Huddinge. Cytological diagnoses were determined at joint sign out sessions and the final classifications were made by cytopathologists trained in LBC interpretation (three-day course, as for cytotechnologists) and with previous participation in at least ten LBC sign out sessions under the supervision of a more experienced colleague.

4.2.1 Conventional cytology (Paper I, IV)

Cells were collected from the ectocervix using a wooden Ayre spatula and from the endocervix using a soft brush. In the conventional method, cells are smeared on a glass slide that is placed in an alcohol bath for cell fixation. The cells are then stained according to a specific protocol--the Pap method--in order to study the various components of the epithelium (Papanicolau GN, 1941).

4.2.2 Liquid-based cytology (Paper I, II, III)

Liquid-based cytology has been developed as a refinement of conventional cytology. Sampling is similar to that of conventional cytology, except for use of a plastic spatula. All midwives involved in the study received training in LBC sampling. Cells were directly stirred into a fixative medium in a small plastic container and cytology slides were prepared using the ThinPrep® 2000 processor (Hologic). The processor senses the quantity of cells, applies them to the slide in a thin layer with a minimal amount of overlap, and filters away mucus, blood, and inflammatory cells that may obscure the cells of interest. Pap staining was carried out as for CC, with the sole exception that the ammonium bath concentration was five times greater to optimize color intensity, which was weaker for LBC than for CC with the original staining protocol.

Staff had limited experience of LBC screening at the beginning of this study, but did have extensive experience with conventional cytology. A split-sample study involving 137 patients had previously been conducted to compare LBC with CC (Zhu *et al*, 2007). All screening samples (LBC and CC) were assessed by the same four cytotechnologists who had completed a three-day course in LBC interpretation held by the manufacturer. Before independent interpretation of LBC, all cytotechnologists needed to interpret at least 200 LBC samples from routine cases under the supervision of a more experienced colleague.

From September 2005 to September 2006, HPV test results did not affect cytological evaluation. Women with any cytological abnormality were referred for gynecological follow-up, regardless of HPV test results. This workup allowed evaluation of HR-HPV

detection using LA as a reflex test in cases of minor cytological abnormalities (Froberg *et al*, 2008). Beginning in September 2006, HPV test results were taken into account when making the final cytological diagnosis in cases of preliminary diagnosis of ASCUS or LSIL. The final diagnosis was considered to be LSIL in HR-HPV-positive samples, and considered to be WNL in HR-HPV-negative samples, unless special reasons were found to consider the sample to be abnormal despite HR-HPV-negativity. In the present paper, cases of ASCUS and LSIL identified by LBC were assessed as LSIL/low-grade cytology only if HR-HPV-positive, and assessed to be WNL in HR-HPV-negative cases of minor cytological abnormalities.

To simplify presentation of results, abnormal cytological diagnoses were sub-divided into low-grade cytology (ASCUS, AGC/AGUS, and LSIL) and high-grade cytology (ASC-H, AGC-H, HSIL and AIS).

4.3 HPV testing

4.3.1 HPV detection and genotyping (Paper I, II, III)

Of the cell suspension remaining from the LBC samples, 2 ml were used for HPV DNA detection and genotyping. The sample was centrifuged and the cell pellet was lysed according to instructions in the Total Nucleic Acid Isolation kit. DNA was extracted using the MagNA Pure LC Robot and analyzed with the Linear Array HPV Detection and Genotyping Test (LA) according to manufacturer's instructions (all procedures by Roche®).

The method includes a PCR reaction of extracted HPV DNA using a pool of biotinylated primers that hybridize in the L1 region of the HPV genome, chemical denaturation of HPV DNA amplicons to single-stranded DNA, followed by hybridization with matching type-specific DNA probes immobilized on nylon strips, and detection by colorometric determination. The result is a pattern of blue lines on a nylon strip, which is visually read by comparing the pattern with a reference guide.

The 37 different HPV types included in the LA test were divided into three "risk categories": (1) HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, (2) pHR-HPV types 26, 53, 66, 68, 73, and 82, and (3) LR-HPV types or types of undetermined risk including 6, 11, 40, 42, 54, *55*, 61, *62*, 64, *67*, *69*, 70, *71*, 72, 81, *83*, *84*, IS39, and CP6108 (Munoz *et al*, 2003; Munoz *et al*, 2006). HPV types of undetermined risk are considered together with the LR-HPV types.

4.3.2 HPV detection using Hybrid Capture 2 (Paper IV)

After being used to smear cells on a glass slide for conventional cytology, the endocervical brush was inserted into a transport medium provided by the manufacturer (Digene Corporation). Specimens were tested using the Hybrid Capture II assay, performed according to the manufacturer's protocol, at the Department of Virology,

Karolinska University Hospital, Stockholm, Sweden. Briefly, DNA from cervical cytology 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 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 samples containing 1 pg of HPV DNA/microliter (about 5000 copies of the HPV genome) were designated as positive (Peyton *et al*, 1998).

4.4 Histology (Paper I, II, IV)

All histological specimen (cervical biopsies and cones) were evaluated within routine practice. The histopathological diagnoses were established according to the CIN classification (Richart, 1969; Richart & Barron, 1969) , see section 2.3.6.

4.5 Cost-effectiveness analysis of HPV triage (Paper IV)

Base case values (Table IV:1) used in the model were mainly based on data from the clinical trial by Andersson *et al* (Andersson *et al*, 2005b) and supplemented with data from published meta-analyses on the accuracy of HPV triage for detection of CIN2+ (Arbyn *et al*, 2004; Arbyn *et al*, 2009a; Arbyn *et al*, 2005; Arbyn *et al*, 2006).

We used recent cost data to reflect today's cost for follow-up within the Swedish healthcare system. Average unit costs for the different cost quantities (Table IV:2) were collected from local and national sources in Sweden. All costs were estimated in 2008 Swedish Kronor (SEK) based on Patient-level Clinical Costing (known as cost per patient (KPP) in Sweden), which is a method used for calculating the cost for each patient, stay, or visit. The method describes healthcare resource consumption from the perspective of diagnosis and is useful for decision-making at all levels of the Swedish healthcare sector.

Data on costs for a follow-up visit to physician for repeat cytology and immediate colposcopy with biopsy, respectively, are presented as the average cost per patient from three hospitals: Stockholm South General Hospital, Danderyd Hospital, and Karolinska University Hospital, Stockholm, Sweden. Wherever necessary, costs were converted to the 2008 SEK reference using the consumer price index for Sweden.

A decision tree based on the Markov model (using TreeAge Healthcare module software, 2007) was constructed to evaluate the cost-effectiveness of the three follow-up strategies described above. Calculations were carried out both considering ASCUS and LSIL as one group (ASCUS/LSIL) and separately. These cytological categories were considered together in: (1) women of all ages (23-60 years), (2) women in up to 30 years (<30 years) and (3) women aged 30 years and older (≥30 years). Figure IV:1

presents a simplified decision tree for ASCUS/LSIL (23-60 years). Costs (Table 3) for the different follow-up strategies represent average cost for the whole population and for each cytology and age category separately. For each follow-up strategy, prevalence of CIN2+ was used, along with sensitivity and specificity for detection of CIN2+, to estimate the number of CIN2+ detected for a simulated cohort of 1000 women in each cytology and age group.

Parameter[a]	Women 23–60 years	Women <30 years	Women ≥30 years
ASCUS/LSIL			
Prevalence, CIN2+	0.21	0.23	0.20
Prevalence, HR-HPV	0.66	0.81	0.54
Sensitivity for CIN2+			
Cytology	0.61	0.50	0.70
HPV triage	0.82	0.89	0.75
Specificity for CIN2+			
Cytology	0.56	0.52	0.59
HPV triage	0.39	0.22	0.52
ASCUS			
Prevalence, CIN2+	0.19	0.17	0.21
Prevalence, HR-HPV	0.44	0.67	0.32
Sensitivity for CIN2+			
Cytology	0.60	0.67	0.57
HPV triage	0.60	0.67	0.57
Specificity for CIN2+			
Cytology	0.69	0.60	0.74
HPV triage	0.60	0.33	0.74
LSIL			
Prevalence, CIN2+	0.22	0.25	0.20
Prevalence, HR-HPV	0.74	0.85	0.65
Sensitivity for CIN2+			
Cytology	0.61	0.47	0.77
HPV triage	0.89	0.93	0.85
Specificity for CIN2+			
Cytology	0.51	0.49	0.52
HPV triage	0.30	0.18	0.40

[a]Colposcopy with biopsy is referred to as gold standard.
Note: ASCUS/LSIL, 23–60 years ($n = 177$), <30 years ($n = 78$) and ≥30 years ($n = 99$); ASCUS, 23–60 years ($n = 52$), <30 years ($n = 18$) and ≥30 years ($n = 34$); LSIL, 23–60 years ($n = 125$), <30 years ($n = 60$) and ≥30 years ($n = 65$).

Table IV:1. Prevalence of CIN2+ and HR-HPV and diagnostic performance of follow-up strategies, based on results from the clinical trial by Andersson et al.

Parameter	Cost[a]
Pap smear	83[b]
HPV DNA test	859[c]
Office visit to midwife	604[d]
Office visit to physician	1,691[e]
Office visit to midwife for follow-up with HPV test	1,463[d,c]
Office visit to physician for follow-up with Pap smear	1,774[b,e]
Office visit to physician for follow-up using Colposcopy with biopsy	2,432[e]

[a]All costs were estimated in 2008 Swedish Kronor (SEK). If necessary, costs have been converted to year 2008 by using the consumer price index for Sweden.
[b]Cost for Pap smear from year 2009 performed at Karolinska University Hospital. Clinical cytological laboratory fee includes physician assessment for abnormal results and test costs.
[c]Cost for HPV DNA test from year 2010 performed at Karolinska University Hospital. HPV test laboratory fee includes physician assessment for abnormal results and test costs.
[d]Cost is from year 2004 were the follow-up procedure was performed by a midwife at Karolinska University Hospital.
[e]Patient-level Clinical Costing (KPP in Swedish) from Stockholm South General Hospital, Danderyd Hospital and Karolinska University Hospital, Stockholm, Sweden, during year 2008. Cytological and histopathological laboratory fees include physician assessment for abnormal results and test costs.

Table IV:2. Cost estimates in Swedish kronor (SEK).

Effectiveness is expressed as the number of CIN2+ cases detected by the different methods in each simulated cohort. Incremental effectiveness is the difference in number of CIN2+ cases detected (by a given follow-up method) per 1000 women in a given cytology and age category compared with the next less costly alternative. This report analyses the outcome of these different strategies within a time frame of one year and includes only the first round of follow-up results of abnormal smears showing ASCUS or LSIL.

The main base case outcome is represented by the incremental cost-effectiveness ratio (ICER) per detected CIN2+ case, from the perspective of the Swedish healthcare system and includes only direct medical costs. ICER is defined as the additional cost per CIN2+ case detected, relative to the next less costly follow-up strategy, where ICER= (Cost of Screening strategy A-Cost of screening strategy B)/ (Effect of Screening strategy A- Effect of Screening strategy B). The ICER for a strategy is computed relative to the next most effective option after eliminating strongly dominated strategies (i.e., strategies that are more costly and less effective than other options) and weakly dominated strategies that are ruled out by extended dominance (i.e. strategies whose costs and benefits are improved by a mixed strategy of two other alternatives) (Cantor, 1994). Costs for screening strategies were not discounted since the follow-up time is only one year.

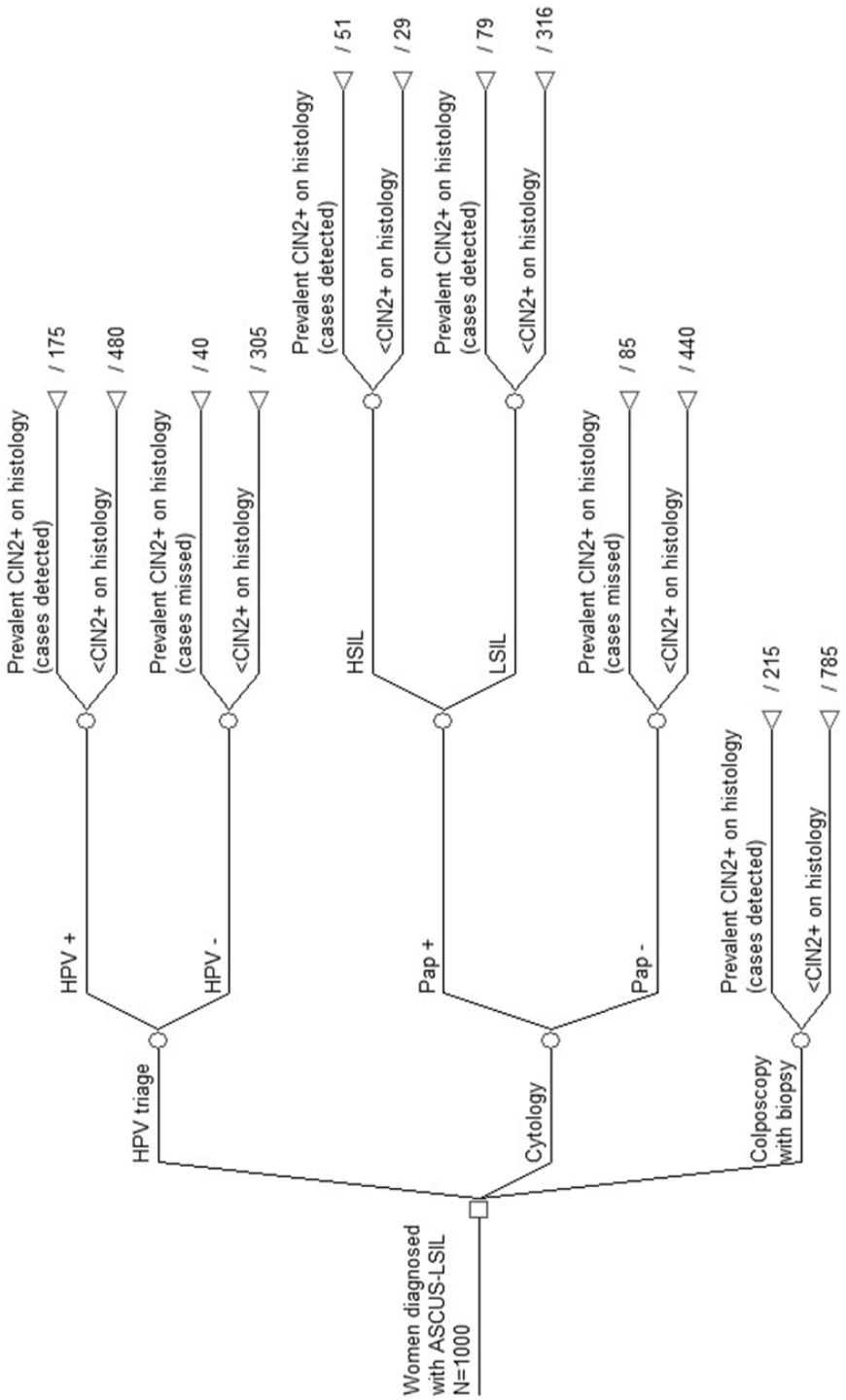


Figure IV:1. A simplified decision tree of three follow-up strategies showing 1000 women, irrespective of age, with initial cytology results showing ASCUS/LSIL, using data from the clinical trial by Andersson et al. The outcome is number of detected (or missed) CIN2+ cases per unit of cost of follow-up during one year.

One way sensitivity analysis was performed by varying the model values to examine when HPV triage becomes a cost-effective alternative (Table IV:4, section 5.4.2). Cost estimates were varied $\pm$100%. Ranges in HPV and CIN2+ prevalence, as well as ranges in sensitivity and specificity of the different strategies to detect CIN2+, were derived using data from published meta-analyses (Arbyn *et al*, 2004; Arbyn *et al*, 2009a; Arbyn *et al*, 2005; Arbyn *et al*, 2006). Among women <30 years and $\geq$30 years, prevalence, sensitivity, and specificity varied between 0-100%, since data for these parameters could not be obtained from the meta-analyses.

4.6 Statistical analyses

4.6.1 Paper I

We developed a multivariate logistic regression model to assess detection rates of histologically verified CIN and rates of abnormal cytology findings to compare LBC + HPV testing with CC. The model includes the following potential confounders: sampling center, age category (<35 years or ≥35 years), and time period (time period 1=Women screened day 0-224 from study start; time period 2=Women screened day 225 through December 31, 2006). Time period was included in the model to adjust for the possibility of a learning curve effect in regard to LBC. All explanatory variables except sampling center were binary. The interaction of time period x screening method was initially included, but since interaction was not significant it was excluded from the final analyses. The comparisons between methods are presented as odds ratios (OR) to detect CIN and cytological abnormalities, with 95% confidence intervals (95% CI). To evaluate the effect of confounding factors on a univariate model, screening method alone was used as a predictor to calculate unadjusted OR with 95% CI as well. A similar model, including age category and sampling center as potential confounders was used for sub-analyses when comparing CIN detection rates among the different LBC+ HPV testing screening cohorts (LBC1, LBC2 and LBC3) in relation to the control group (CC).

PPVs for LBC+ HPV testing and for CC in detecting CIN were calculated for all positive screening cases as a group (HR-HPV-negative cases of low-grade abnormalities were considered WNL), as well as for low-grade cytology and high-grade cytology separately. Cases for which histological follow-up results were not available were excluded from the analysis. To compare LBC+ HPV testing with CC, relative risks (RR) for detection were calculated with 95% CI.

The rate of abnormal cytological findings (both including and excluding HR-HPV-negative cases of minor cytological abnormalities) and CIN2+ detection rate were assessed over time at sampling center A and B, where the LBC+ HPV testing strategy was used over an extended period of time (September 2005-December 2007). The Jonckheere-Terpstra trend test was used to test significance of changes over time when appropriate; otherwise the Pearson χ^2 test was used. The null hypothesis of no difference was rejected at a significance level of $p \leq 0.05$.

SAS 9.2 (SAS Institute Inc., Cary, NC) was used to perform the logistic regressions, LogXact 7.0 (Cytel Inc., Cambridge, MA) was used for the Jonckheere-Terpstra test, and StatXact ((Cytel Inc., Cambridge, MA) was used for the Pearson χ^2 test.

4.6.2 Paper II

Diagnostic performance of HR-HPV detection to identify histopathologically verified CIN2+ was assessed by calculating sensitivity, specificity, PPV, and NPV with 95% CI (Newcombe, 1998). Proportions were compared using Chi-square statistics (exact tests). Means were compared using Student's *t*-test. The null hypothesis of no difference was rejected at a significance level of $p \leq 0.05$.

4.6.3 Paper III

Data were analyzed using STATISTICA 6.1 software (Statsoft Inc, Tulsa, OK, USA). Pearson's χ^2 and Yates corrected χ^2 (for $n<5$) tests were used to compare proportions, and Student's *t*-test was used to compare continuous variables between two groups. After plotting proportions of HR-HPV- and HPV-infected women and multiple infections, respectively, in each age group, a linear or polynomial fit was visually assessed to be the best model for logistic regression. We tested whether the proportion in each age group decreased linearly with age ($\log(p/1-p)=b_0-b_1x$) and whether this was significantly different from null. Then we added a second degree ($\log(p/1-p)=b_0-b_1x+b_2x^2$) to test whether the increase in women over a certain age was significantly different from null. We controlled for covariation between age and age^2 using centered age and centered age^2 in the model. The larger model, with a second degree, provided a better explanation as tested with Nagelkerke R^2. Therefore, we concluded that the quadratic function, $\log(p/1-p)=b_0-b_1x+b_2x^2$, could best describe the relationship between (1) prevalence of HR-HPV in LSIL cases and age, (2) multiple infections and age, and (3) HR/LR-HPV dominance and age. Logistic regressions were also carried out using age group and cytological diagnosis as categorical predictors, as well as HPV prevalence as the dependent variable. The null hypothesis of no difference was rejected at a significance level of $P<0.05$.

4.7 Ethical considerations

For study I, II, and III ethical permission was obtained from the local ethics committee. Informed consent was obtained from all study subjects. Study IV is mainly based on results from a previous clinical trial (Andersson *et al*, 2005c) for which ethical permission was obtained, as well as official cost data. Additional permission for cost-effectiveness analysis is not required.

5 RESULTS AND DISCUSSION

5.1 Paper I: Liquid-based cytology with supplementary HPV testing versus conventional cytology

5.1.1 Distribution of women by age and sampling center

Table I:1 presents data related to the distribution of women by age and sampling center.

Age data	CC (n=4261)	LBC+HPV test (n=6075)
Age range, years	22-60	22-65
Mean age, years	37.5	37.9
Median age, years	37	38
Distribution of sampling centers	CC (n=4261)	LBC+HPV test (n=6075)
A, % (n)	34% (1457)	24% (1471)
B, % (n)	17% (734)	19% (1182)
C, % (n)	15% (633)	10% (586)
D, % (n)	20% (847)	27% (1634)
E, % (n)	9% (400)	12% (699)
F, % (n)	4% (190)	8% (503)

Table I.1. Distribution of women by age and sampling center included in the main analyses.

5.1.2 Main analyses

Conclusive follow-up histological diagnoses were available for about 80% of all women referred for gynecological follow-up, with similar figures found among women screened with LBC+HPV testing and CC. Detection rates of histologically verified CIN1+, CIN2+, and CIN3+ were similar for both screening strategies (Table I:2). It should be noted that adjustment for potential confounders led to a somewhat lower OR for CIN detection - to the "disadvantage" of LBC+ HPV testing. The most important reason for this was the "time period" variable, which was a significant confounder for CIN1+, CIN2+, and CIN3+ detection. Interestingly, both strategies showed higher CIN detection rates for women included in time period 2 compared with time period 1. During time period 2 a smaller proportion of participants were screened with CC than with LBC+ HPV testing. The effect of "age category" was also a significant potential confounder. For the CIN2+ endpoint, sampling center was also a significant potential confounder.

CIN detection rates:			
Method	**CIN1+**	**CIN2+**	**CIN3+**
CC (%)	**2.35% (100/4261)**	**1.60% (68/4261)**	**0.99% (42/4261)**
LBC+HPV test (%)	**2.30% (140/6075)**	**1.56% (95/6075)**	**1.07% (65/6075)**
Comparison of CIN detection rates: LBC+HPV test vs. CC			
Unadjusted OR (95% CI)	**0.98 (0.76-1.27)**	**0.98 (0.72-1.34)**	**1.09 (0.74-1.61)**
Adjusted OR (95% CI)	**0.89 (0.68-1.18)**	**0.89 (0.64-1.25)**	**1.02 (0.67-1.54)**

Table I:2. Comparison of CIN detection rates: LBC+ HPV testing versus CC.

The rate of abnormal cytological findings was significantly higher for LBC + HPV testing if cases of minor cytological abnormalities among HR-HPV-negative women were regarded as abnormal (Table I:3), i.e. reflecting the situation using LBC without reflex HPV testing. Time period was a statistically significant potential confounder for detection rates of high-grade cytology, since there were increased rates of high-grade abnormalities during time period 2 compared with time period 1, for both methods.

Method /Odds ratio	**Abnormal cyt incl HR-HPV-neg**	**Abnormal cyt excl HR-HPV-neg**	**Low-grade cytology***	**High-grade cytology**
Abnormal cytology report rates:				
CC (%)	**3.75% (160/4261)**	**3.75% (160/4261)#**	**2.44% (104/4261)**	**1.31% (56/4261)**
LBC+HPV testing (%)	**4.84% (294/6075)**	**3.39% (206/6075)**	**2.07% (126/6075)**	**1.32% (80/6075)**
Comparisons of rates of abnormal cytology: LBC+HPV test vs. CC				
OR Unadjusted (95% CI)	**1.30 (1.07-1.59)**	**0.90 (0.73-1.11)**	**0.84 (0.65-1.10)**	**1.00 (0.71-1.41)**
OR Adjusted (95% CI)	**1.32 (1.07-1.63)**	**0.91 (0.73-1.13)**	**0.89 (0.67-1.17)**	**0.94 (0.65-1.35)**

- Cases of low-grade cytology in the LBC group are HR-HPV-pos. # No HPV testing with CC

Table I:3. Comparison of rate of abnormal cytological findings: LBC+ HPV testing versus CC.

The PPVs of the two screening strategies for detection of CIN1+, CIN2+, and CIN3+ were similar (Table I:4). When assessing low-grade and high-grade cytology separately, PPV of low-grade cytology is higher for LBC+ HPV testing than for CC (RR 1.43 (95% CI:0.76-2.67)), whereas in high-grade cytology, PPV for CIN2+ is slightly lower for LBC+HPV testing than for CC, with RR 0.91 (95% CI: 0.67-1.22).

Method/Relative risk:	CIN1+	CIN2+	CIN3+
Overall positive predictive value of abnormal cytology:			
CC, % (n)	79% (100/126)	54% (68/126)	33% (42/126)
LBC+HPV testing, % (n)	77% (140/182)	52% (95/182)	36% (65/182)
RR (95% CI)	0.97 (0.86-1.09)	0.97 (0.78-1.20)	1.07 (0.78-1.47)
Positive predictive value of low-grade cytology:			
CC, % (n)	68% (52/77)	35% (27/77)	16% (12/77)
LBC+HPV testing, % (n)	69% (74/108)	34% (37/108)	22% (24/108)
RR (95% CI)	1.01 (0.83-1.24)	0.98 (0.65-1.46)	1.43 (0.76-2.67)
Positive predictive value of high-grade cytology:			
CC, % (n)	98% (48/49)	84% (41/49)	61% (30/49)
LBC+HPV testing, % (n)	89% (66/74)	78% (58/74)	55% (41/74)
RR (95% CI)	0.91 (0.83-1.00)	0.94 (0.79-1.11)	0.91 (0.67-1.22)
NB: Only cases of abnormal cytology for which histopathological follow-up results were available were included in the analyses.			

Table I:4. Comparison of positive predictive values for detection of histologically confirmed CIN: LBC+ HPV testing versus CC.

5.1.3 Sub-analyses

Table I:5 presents comparisons between the different LBC+HPV testing cohorts and CC (described in Material and Methods) for detection of CIN. We found that detection of CIN2+ was initially lower with LBC+ HPV testing (LBC1 vs. CC). However, after

one year's experience and more than 4000 screened LBC samples, CIN2+ detection rates were higher using LBC+HPV testing compared with CC; the detection rate for CIN2+ in the LBC2 cohort was 2.13% versus 1.60% in the CC cohort.
Although the difference was proportionally large, it was not statistically significant (adjusted OR 1.36, 95% CI: 0.92-2.03). When LBC+ HPV testing came into use as a routine screening test on a small scale (LBC 3), CIN2+ detection rates were similar to CC detection rates in 2005-2006. Age category was a significant potential confounder.

Parameter	LBC1 vs. CC	LBC2 vs. CC	LBC3 vs. CC*
Proportion of CIN2+ detected using CC	1.60% (68/4261)	1.60% (68/4261)	1.60% (35/2191)
Proportion of CIN2+ detected using LBC+ HPV testing	1.28% (52/4059)	2.13% (43/2016)	1.57% (53/3383)
Unadjusted OR for CIN2+ detection (95% CI)	0.80 (0.56-1.15)	1.34 (0.91-1.98)	0.98 (0.64-1.51)
Adjusted OR for CIN2+ detection (95% CI)	0.86 (0.59-1.24)	1.36 (0.92-2.03)	1.15 (0.73-1.81)
*Only women screened at the two screening units A and B (as in LBC3) are included in the analysis.			

Table I:5. Screening cohort-specific sub-analyses of CIN2+ detection rates: LBC+ HPV testing versus CC.

When evaluating LBC+ HPV testing over time (at sampling centers A and B), we found that the detection rate of CIN2+ increased from 1.1% to about 1.60% during the first year, and remained at that level throughout the observed time period (Figure I:1). However, this increase is not statistically significant. The rate of abnormal cytological findings, including cases of minor cytological abnormalities in HR-HPV-negative women, changed significantly over time, with an initial increase followed by a marked reduction (Figure I:1). Consequently, the PPV for detection of histologically verified CIN2+ increased significantly (Figure I:2). A similar but more stable pattern was seen for the rate of abnormal cytological findings excluding cases of minor cytological abnormalities among HR-HPV-negative women.

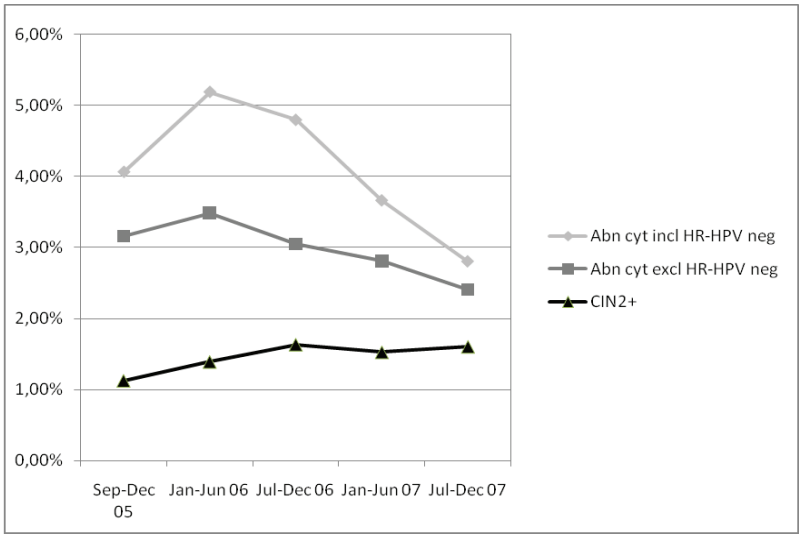


Figure I:1. Time trends in LBC+ HPV testing: rate of abnormal cytological findings and CIN2+ detection. Rate of abnormal cytological findings including HR-HPV-negative cases, p=0.01(χ^2). Rate of abnormal cytological findings excluding HR-HPV-negative cases, p=0.51 (χ^2). Proportion CIN2+ detected, p=0.26 (Jonckheere-Terpstra trend test, one-sided).

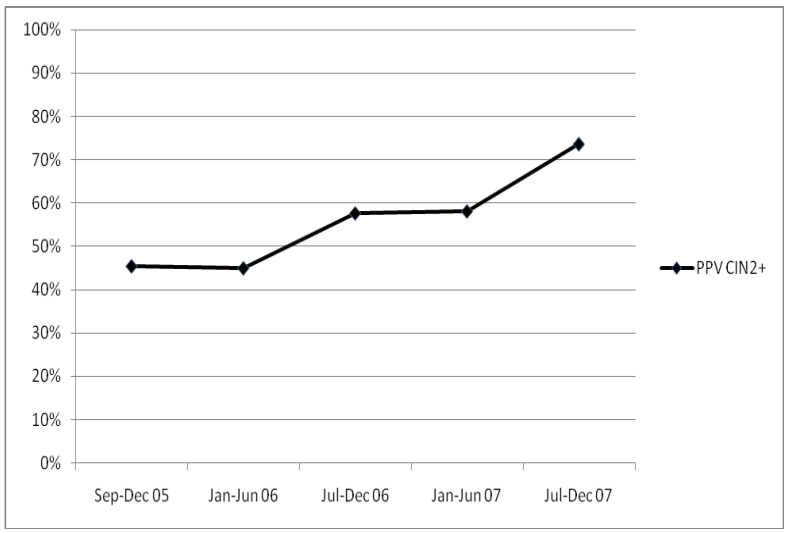


Figure I:2. Time trends in LBC+ HPV testing: positive predictive values for detection of histologically verified CIN2+. Positive predictive value for detection of CIN2+ increased significantly (p=0.005, Jonckheere-Terpstra trend test, one-sided). Cases of abnormal cytology without histopathological follow-up results have been excluded from calculations.

5.1.4 Discussion

This study reflects a dynamic process at a laboratory introducing LBC with supplementary HPV testing for screening samples showing ASCUS or LSIL. We noted that detection of precancerous lesions improved over time. The sub-analysis showed that initially, LBC+ HPV testing detected a smaller rate of histologically verified CIN2+ than did CC (Table I:5). In a subsequent cohort, the detection rate of CIN2+ was more than 30% higher compared with CC, although the difference was not statistically significant. Over the first year the CIN2+ detection rate increased by about 45% (from 1.1% to 1.6%) for LBC+ HPV testing, although the change was not statistically significant (Figure I:1).

Over time there was also a reduction in the rate of abnormal cytological findings, which led to a noteworthy improvement of the PPV for detecting CIN2+ over time, and with increasing experience (Figure I:2). These results indicate that with increased experience the LBC+ HPV testing strategy may lead to more targeted management of women with abnormal screening results. However, in this setting of high-quality, well-established conventional cytology screening, no significant differences between the two strategies were observed in the main analysis.

Before the LBC+HPV testing method was introduced on a small scale in routine testing, the rate of abnormal cytological findings was instead significantly higher for LBC than for CC, a finding made in several previous studies (Beerman *et al*, 2009; Halford *et al*; Hussein *et al*, 2005; Hutchinson *et al*, 1999; Ronco *et al*, 2007a; Strander *et al*, 2007a). A high rate of abnormal cytological findings without a simultaneous increase in detection of precancerous lesions would lead to unnecessary resource utilization and increase the cost of screening. Moreover, it would reduce the PPV for detection of CIN and lead to many unnecessary examinations, causing undue psychological and physical stress for women. Therefore we believe that LBC screening should be supplemented with high-quality reflex HPV testing.

Despite incorporation of reflex HPV testing for cases of minor cytological abnormalities in the LBC screening arm, our results show that each method is associated with a similar PPV for identifying CIN. One possible explanation may be that LBC yields both a higher rate of abnormal cytological findings and a higher rate of minor abnormalities compared with CC (Beerman *et al*, 2009; Davey *et al*, 2007; Halford *et al*; Hussein *et al*, 2005; Hutchinson *et al*, 1999; Ronco *et al*, 2007a; Strander *et al*, 2007a). This suggests that reflex HPV testing simply prevents a reduction of the PPV for detection of CIN in LBC+ HPV testing compared with CC, at least initially while personnel still have limited experience of LBC.

It should also be noted that this study was carried out in parallel with a study that examined HPV triage of minor cytological abnormalities using Hybrid Capture 2 (HC2, Digene Inc. Gaithersburg, MD) (Dillner *et al*) without gold standard verification in HC2-negative women. This probably led to a slight overestimate of the PPV of CC for detection of CIN, due to a negative selection of women at low risk of current or future

CIN (a total of eight cases lost to follow-up due to negative HC2 tests). Such selection is already incorporated in the LBC+ HPV testing strategy (two HC2-negative but Linear Array HR-HPV-positive cases lost to follow-up).

The current study shows no clear advantages to the newer screening method LBC + HPV testing over CC in detecting CIN, unlike the previous Swedish study by Strander *et al*. The reason for the discrepancy between our results, which stem from a similar setting, is unclear. However, both studies share one disadvantage: only women with positive screening findings were subjected to gold standard examination with colposcopy and biopsy, which introduces verification bias and prevents calculation of sensitivity, specificity, and negative predictive value. The reliability of these results depends on the assumption that CIN prevalence is equal between the study populations. This situation also applies to other studies, and may explain the heterogenous study results.

Moreover, the relative comparison of screening performance depends on the performance of the strategy used for comparison (i.e. CC), which differs between sites. Incorporating LBC screening with reflex HPV testing in cases of minor cytological abnormalities is not likely to have negatively affected detection of CIN2+, since the negative predictive value of HR-HPV detection was extremely high (Froberg *et al*, 2008).

Other findings are also worth discussing. Performance of LBC+ HPV testing seems to somewhat improve identification of the more advanced lesions (CIN3+). This weak tendency was also noted for PPVs for CIN. These small differences are far from statistically significant. Previous results obtained by others point in the same direction (Siebers *et al*, 2009). Additional studies would be needed to answer the question of whether the LBC + HPV testing strategy may improve identification of advanced lesions.

Experience may impact performance when using new methodology in the medical field. In this study, personnel at all levels (midwives, cytotechnologists, cytopathologists) had extensive experience of conventional cytology screening, while everyone had limited experience of LBC at the beginning of this study. One interesting finding was that performance improved over time for both screening methods. The explanation for this is probably multifactorial. One reason may be the increased focus and interest in gynecological cytology generated during this study. Moreover, using liquid-based cytology facilitates detection of more subtle changes since cells are better preserved, and it is easier to get an overview of the sample slide. It is possible that correct interpretation of these subtle changes was initially difficult. Greater vigilance in cytological interpretation seems to have influenced the performance for both strategies.

Previous large randomized controlled trials comparing LBC and CC in population-based screening found no support for learning curve effects (Ronco *et al*, 2007a; Siebers *et al*, 2009). An additional study used an expert panel to re-evaluate LBC slides

from the Ronco trial in which experience with LBC was initially relatively low (Confortini *et al*), without showing any improved diagnostic performance. However, the Siebers evaluation included only 25 false-negative cases.

In late 2006, HPV testing results were taken into account when making the final cytological diagnoses. The use of such supplementary virological information may be advantageous for the cytopathologist. One finding supporting this hypothesis is the marked increase in PPV to detect CIN2+ over time. Another factor that influences performance of the screening program is undoubtedly clinical follow-up of abnormal results. Over the course of this study, we noted a trend for clinicians to take more biopsies, which may have improved detection of CIN over time. Yet another issue to be taken into account is that CIN prevalence may fluctuate over time, thereby reducing the validity of using historic controls. This should be considered when interpreting sub-analyses results; changes have occurred over time, and comparisons of women screened during different time periods must be interpreted with caution.

In summary, this study shows no significant differences in screening performance between the use of liquid-based cytology supplemented with HR-HPV detection and conventional cytology. However, the study was conducted over a dynamic time period which saw implementation of new methodology, an increased focus on gynecological cytology, and improved expertise at all levels in the healthcare chain. We cannot exclude that true differences between methods may remain undetected.

5.2 Paper II: Human papillomavirus reflex testing

5.2.1 Histological findings

Table II:1 summarizes cytological findings in relation to histological diagnoses in the 112 cases of minor cytological abnormalities for which histopathological follow-up results were present.

	Histological diagnosis			
Cytology	**WNL (%)**	**CIN1 (%)**	**CIN2+ (%)**	**Total (%)**
ASCUS	23 (59)	11 (28)	5 (13)	39 (100)
LSIL	35 (48)	28 (38)	10 (14)	73 (100)
Total	58 (52)	39 (35)	15 (13)	112 (100)

Table II:1. Liquid-based cytology findings with histological follow-up diagnoses obtained within one year of screening in 112 women with minor cytological abnormalities.

5.2.2 HPV infection and histological findings

In all, 81% of all samples were HPV-positive. There was a significant proportion of multiple infections and overlap of infections with HPV types from one, two, or all three risk categories (HR-, pHR- and LR-HPV). The prevalence of HPV-infected samples increased with increasing degree of CIN (p=0.026). Sixty-three percent of the samples were HR-HPV-positive (Table I:2). In this cytologically selected material, the NPV for the LA HPV test to detect CIN2+ lesions was 100% (95% CI: 90-100%). As shown in Figure II.1, all 15 cases of CIN2+ were HR-HPV-positive, compared with 56% (22/39) of the CIN1 and 57% (33/58) of the WNL cases (p=0.019).

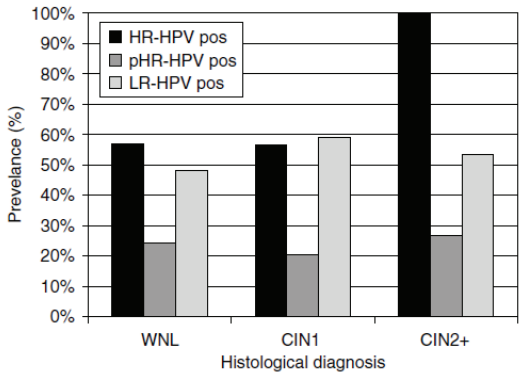


Figure II:1. Prevalence of HPV risk categories related to histological diagnosis in 112 cases of minor cytological abnormalities. Since infection by HPV types of different risk categories can overlap, the sum of the HR-, pHR- and LR-HPV prevalences in each histological diagnosis group can exceed 100%.

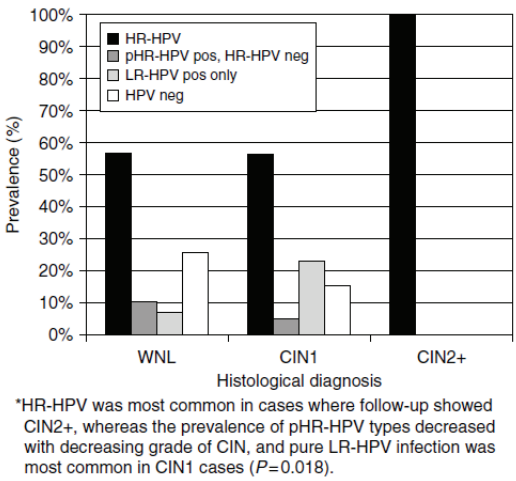


Figure II:2. Prevalence of HPV hierarchic risk categories related to histological diagnosis in 112 cases of minor cytological abnormalities.

Cytology classification	Probability of HR-HPV presence (%) (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
ASCUS+LSIL ($n = 112$)	63 (53–71)	100 (75–100)	43 (33–54)	21 (13–33)	100 (90–100)
ASCUS ($n = 39$)	46 (30–63)	100 (46–100)	62 (44–77)	28 (11–54)	100 (81–100)
LSIL ($n = 73$)	71 (59–81)	100 (66–100)	33 (22–46)	19 (10–33)	100 (81–100)
LSIL ⩾ 30 yrs ($n = 32$)	53 (35–70)	100 (40–100)	54 (34–72)	24 (8–50)	100 (75–100)
ASCUS+LSIL ⩾ 30 years ($n = 71$)	49 (37–61)	100 (63–100)	58 (45–70)	26 (13–44)	100 (88–100)

Table II:2. Performance of HR-HPV detection using Linear Array to detect histologically confirmed CIN2+ in cases of ASCUS and LSIL, with respect to sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with 95% confidence intervals (CI).

5.2.3 Distribution of HPV genotypes and histological findings

To detect all cases of CIN2+, the assay had to accurately detect at least HPV types 16, 18, 31, 52, and 58. HPV 45 was detected as frequently as HPV 52 and 58 among the CIN2+ cases (13% or 2/15), but never as a single HR-HPV infection. In this limited material, we were able to demonstrate a significant correlation between HPV types 16 (p=0.0045), 18 (p=0.039), and 31 (p=0.006) with high-grade cervical lesions. Presence of co-infection with other HPV types has not been taken into consideration when performing the calculations (Figure II:3).

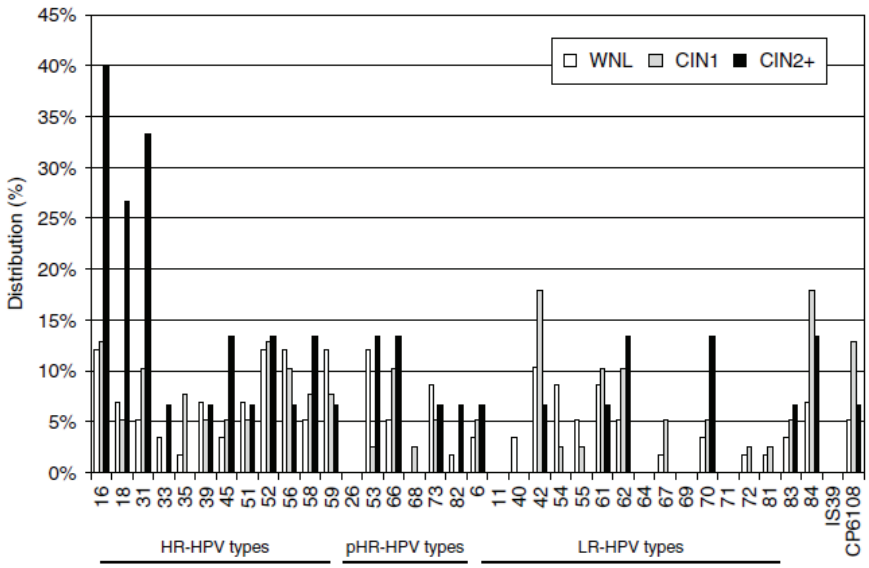


Figure II:3. Relative distribution of HR-, pHR- and LR-HPV genotypes in liquid-based cytology samples showing minor cytological abnormalities, related to histological diagnosis: WNL (n=58) (light grey bars), CIN1 (n=39) (dark grey bars) and CIN2+ (n=15) (black bars).

Among patients with minor abnormalities in their screening smears and subsequent histological findings showing CIN2+, HPV types 16, 31 and 18 clearly stand out as the most common, present in single or multiple infections in 40% (6/15), 33% (5/15), and 27% (4/15) of cases, respectively. Altogether, they were present in 73% (11/15) of the samples. Out of the 15 CIN2+ cases, 53% (8/15) were infected with HPV 16 and/or HPV 18; however, only three of these women were not infected with additional HPV types.

5.2.4 Multiple infections

In all, 54% had multiple HPV infections, and of 91 HPV-infected individuals, 67% were infected by more than one HPV type. Multiple HPV infection was slightly more common in cases of histological CIN2+ than in CIN1 and WNL cases (73, 64, and 67%, respectively). Among the HR-HPV-positive samples, 41% (29/70) were infected by more than one HR-HPV type. Among HR-HPV-positives, multiple HR-HPV infections were slightly more common in CIN2+ (Figure II:4) than in CIN1 and WNL cases. These weak trends may reflect that multiple HPV infection increases the probability of harboring a highly oncogenic HPV type.

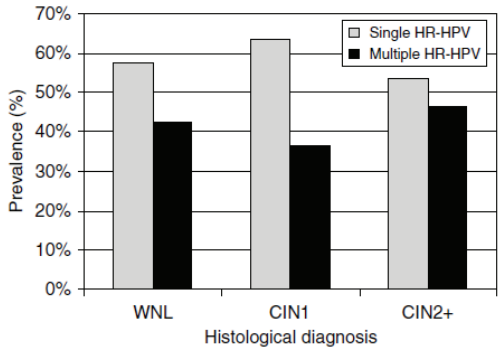


Figure II:4. Prevalence of single vs. multiple HR-HPV infections in HR-HPV-positive cases related to histological diagnosis.

5.2.5 Discussion

Reporting rates in the Scandinavian screening programs differ from those in the Anglo-Saxon world. Scandinavian cytologists tend to rate the samples as "within normal limits" more frequently than British or American cytologists (abnormal smear rate: 3-5% vs. 10-15%), discounting subtle changes in deference to achieving cytological certainty (Scott R et al, 2002). In spite of these differences, our results suggest that the value of HR-HPV detection in cases of minor cytological abnormalities is high even in a Swedish context.

Among cases of minor cytological abnormalities detected using LBC, HR-HPV detection using LA has a 100% (95% CI: 90-100%) negative predictive value for high-grade cervical dysplasia during a maximum follow-up time of 1 year (Table II:2). This is similar to findings in a large-scale prospective study including cases of ASCUS where carcinogenic HPV detection using the same HPV test showed an excellent NPV (95.67%; 95% CI: 94.22-96.85%) for 2-year cumulative CIN2+ (Castle et al, 2008). In the present study we used a different definition for carcinogenic or high-risk HPV, classifying HPV 66 and 68 as pHR-HPV types, which probably only has a marginal effect on performance of the HPV test. Our results suggest that the pHR-HPV group is of little or no importance in a screening context according to the IARC classification of HPV types by carcinogenicity (Bouvard *et al*, 2009).

In the LSIL group, HR-HPV prevalence is high, about 80-85%, among women younger than 30 years, and drops to about 50% among women aged 30 years or older. For this reason, specificity of the HPV detection test can be improved by setting an age limit of 30 years for HPV "reflex" testing in cases of LSIL. An age-limit at 35 years in cases of LSIL has been suggested (Ronco et al, 2007), yielding a somewhat higher specificity for histologically confirmed CIN2+ (using Hybrid Capture 2 for HPV detection), compared with our findings for women aged 30-59 years. In cases of ASCUS, where HR-HPV prevalence is considerably lower (Brismar-Wendel *et al*, 2009), the HR-HPV

prevalence was not significantly higher among women under the age of 30 years than among older women, for which reason the diagnostic value of HPV testing in this group is independent of age.

However, in a recent study on HPV triage against the background of conventional cytology (CC) screening, HR-HPV prevalence was high even among young women with ASCUS (Dillner *et al*). This difference may be attributable to differences in cytological evaluation between LBC and CC. In the study by Dillner et al, Hybrid Capture 2 was used for HPV triage, whereas we used the LA HPV detection and genotyping method. Since LA is a more sensitive assay than HC2, our use of a different test is not likely to explain this difference among younger women.

In this study, the HPV triage model using the LA HPV test for all cases of ASCUS and for cases of LSIL in women ≥30 years, could reduce the need for extensive follow-up investigations by about 50%, while retaining good patient safety. In such cases, cytological evaluation and virological analysis could be performed on the same material ("reflex testing"). Because there is only one sampling event, sampling error would be reduced. Screening program costs would also be reduced, because both analyses can be performed without recalling the patient. The decision of whether to set an age-limit for HPV "reflex" testing in LSIL cases should be based on health-economic calculations.

According to our results, only the HR-HPV group had a statistically significant correlation with CIN2+, for which HR-HPV types 16, 18 and 31 were the most important. In this small material, in order to identify all cases of high-grade cervical lesions, the HPV test had to accurately detect HPV 16, 18, 31, 52, and 58. These HPV types are represented among the main worldwide oncogenic HPV types (HPV 16, 18, 31, 33, 35, 45, 52, and 58), which account for about 91% of all cervical cancer cases worldwide (de Sanjose *et al*; Smith *et al*, 2007). However, since a high negative predictive value is necessary in a "reflex" screening situation, all HR-HPV types need to be accurately detected.

The main value of introducing LBC combined with HPV “reflex” testing is probably an improved ability to identify women with an increased risk of developing pre-malignant and malignant cervical lesions, without increasing the abnormal cytology reporting rate. Unnecessary investigations and unnecessary psychological stress could be avoided, and resources could be used for more accurately directed follow-up of women with abnormal cytological findings.

5.3 Paper III: Age-specific prevalence of HPV genotypes in ASCUS and LSIL

5.3.1 HPV prevalence

In all, 82% were positive for one or more of the 37 included HPV types. HPV prevalence ranged from 92% in the 20-29 year age group to 62% in women aged 40-45 years. Prevalence was 67% in women over 50 years. Age-specific prevalence for HPV (37 types) and HR-HPV (12 or 13 types) was higher in younger age groups and there was a linear decline with age ($p<0.001$, Table III.1). HPV was more common in women with LSIL than with ASCUS (93% vs. 63%, $p<0.001$). HPV prevalence was significantly dependent on age group (Figure III:1). However, there is an apparent tendency for increasing discrepancy with lower, and especially higher age.

HR-HPV (12 types) was found in 71% of all women with LSIL and 49% of women with ASCUS ($p=0.001$). These figures were only minimally influenced if HPV 68 was considered to belong in the HR-HPV group. Figure 1 shows that HR-HPV prevalence was age-dependent in women with LSIL, decreasing with age until the age of 50 years ($p=0.19$ for age^2), after which a small, statistically insignificant increase was found. In ASCUS cases, a linear correlation between HR-HPV and age was found. A statistically significant age-specific difference in HR-HPV between ASCUS and LSIL cases was only seen in the youngest women, aged 20-24 years (Table III:1)

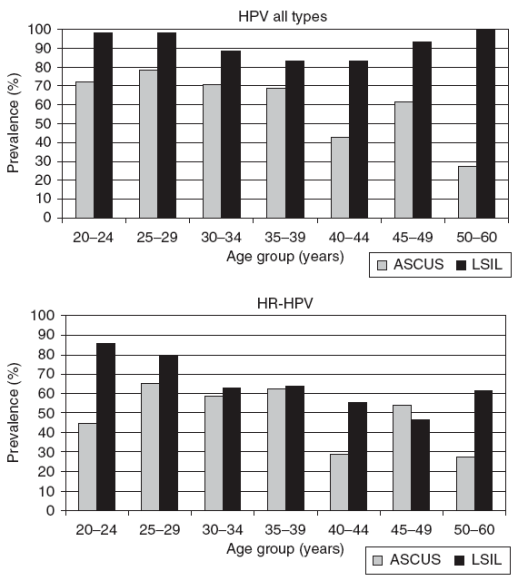


Figure III:1. Age-specific prevalence of human papillomavirus (HPV) (all types) and HR-HPV infection in women with ASCUS and LSIL. Human papillomavirus all types P(age group × cytological diagnosis)=0.10; LSIL P(age)=0.20, P(age group)=0.03, P(age^2)=0.003; ASCUS P(age)=0.001, P(age group)=0.04, P(age^2)=0.34; HR-HPV P(age group × cytological diagnosis)=0.15; LSIL P(age)=0.001, P(age group)=0.01, P(age^2)=0.19; ASCUS P(age)=0.04, P(age group)=0.11, P(age^2)=0.16; all P-values calculated by logistic regression.

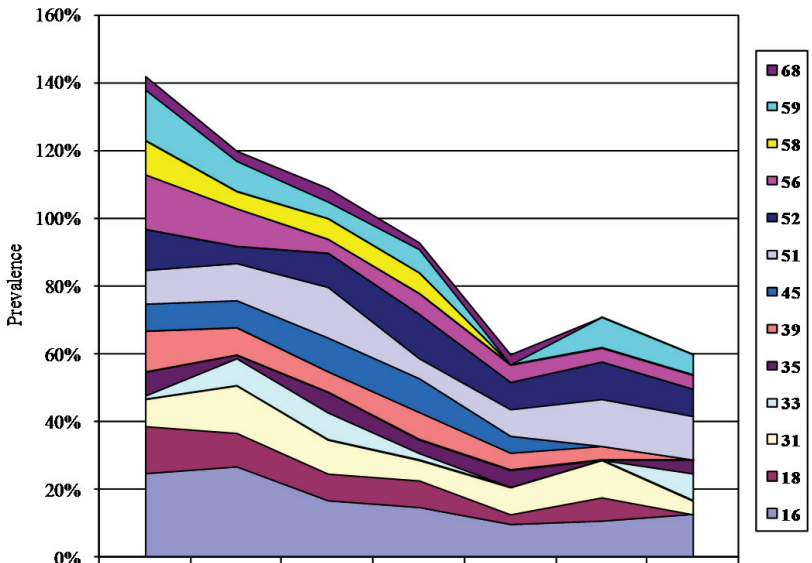
Age group (years)	Total number of women		HR-HPV			HR-HPV and HPV68			Multiple HR-HPV		
	Cytology		Cytology			Cytology			Cytology		
	ASCUS	LSIL	ASCUS No. (%)[a]	LSIL N (%)[a]	$P(\chi^2)$	ASCUS No. (%)[a]	LSIL No. (%)[a]	$P(\chi^2)$	ASCUS No. (%)[b]	LSIL No. (%)[b]	$P(\chi^2)$
20–24	18	55	8 (44)	47 (85)	<0.001	9 (50)	48 (87)	<0.001	5 (63)	23 (49)	0.74
25–29	23	50	15 (65)	40 (80)	0.17	15 (65)	40 (78)	0.17	7 (47)	18 (45)	0.91
30–34	17	35	10 (59)	22 (63)	0.78	10 (59)	22 (63)	0.78	4 (40)	9 (41)	0.73
35–39	16	36	10 (63)	23 (64)	0.92	10 (63)	23 (64)	0.92	2 (20)	8 (35)	0.66
40–44	21	18	6 (29)	10 (56)	0.09	6 (29)	10 (56)	0.09	0 (0)	3 (30)	0.41
45–49	13	15	7 (54)	7 (47)	0.70	7 (54)	7 (47)	0.70	2 (29)	1 (14)	1.0
50–60	11	13	3 (27)	8 (62)	0.20	3 (27)	8 (62)	0.20	2 (67)	1 (13)	0.30
All[c]	119	222	59 (49)	158 (71)	<0.001	60 (50)	159 (71)	<0.001	22 (38)	63 (40)	0.70

ASCUS = atypical squamous cells of undetermined significance; HR-HPV = high-risk human papillomavirus; LSIL = low-grade squamous intraepithelial lesion. [a]Percentage of total number of women in the age group. [b]Percentage of HR-HPV positive women in the age group. [c]Two women are missing; one ASCUS case with HPV42 and one LSIL case with HPV52.

Table III:1 Age-specific HR-HPV and multiple HR-HPV infections (in HR-HPV-positive women) in the study population.

5.3.2 HPV genotype distribution

Figure III:2 shows the prevalence of HR-HPV genotypes (including pHR-HPV 68) in each age group in all women. HPV 16 was the most common HR-HPV type and was found in 23% of the HPV-positive women, ranging from 30% (20/67) in women aged 20-29 years to 14% (3/22) in women aged 45-49 years (p=0.22). HPV 18 was found in only 10% of all HPV-positive women. HPV 51 was equally or more common than HPV 16 in women over 45 years of age. Among women with ASCUS, the most prevalent HR-HPV type was HPV 16 (16%), followed by HPV 51 and 52 (10%). Among women with LSIL, HPV 16 was found in 21%, followed by HPV 56 (11%).



	20-24	25-29	30-34	35-39	40-44	45-49	50-60
68	4%	3%	4%	2%	3%	0%	0%
59	15%	9%	5%	7%	0%	9%	6%
58	10%	5%	6%	6%	0%	0%	0%
56	16%	11%	4%	6%	5%	4%	4%
52	12%	5%	10%	13%	8%	11%	8%
51	10%	11%	15%	6%	8%	14%	13%
45	8%	8%	10%	10%	5%	0%	0%
39	12%	8%	6%	8%	5%	4%	0%
35	7%	1%	6%	4%	5%	0%	4%
33	1%	8%	8%	2%	0%	0%	8%
31	8%	14%	10%	6%	8%	11%	4%
18	14%	10%	8%	8%	3%	7%	0%
16	25%	27%	17%	15%	10%	11%	13%

Age group

Figure III:2. Age-specific distribution of the HR-HPV types including pHR-HPV 68. The total exceeds 100% since many women are infected with more than one HR-HPV genotype.

Table III:2 outlines women infected with HPV 16, 18, clade 9 HPV types, and clade 7 HPV types to elucidate the preventive potential of current prophylactic HPV vaccines against minor cytological abnormalities. As shown in Figure III.4, we found a shift in predominant HPV genotypes with age. The proportion of LR-HPV types increased with age, following a quadratic function (p=0.001).

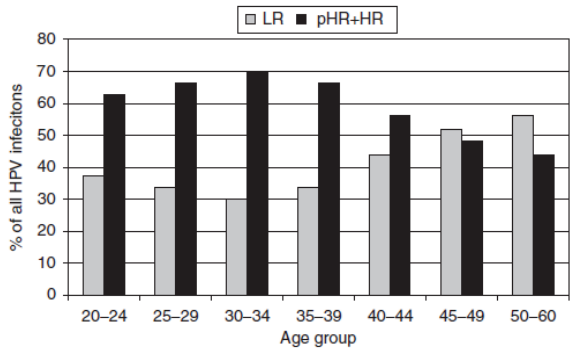


Figure III:4. Age-dependent shift in LR vs pHR+HR frequency (% of all HPV infections). LR and pHR+HR P(age^2)<0.001 (logistic regression). LR=low-risk HPV, pHR=probable high-risk HPV, and HR=high-risk HPV.

	Diagnostic group			
	All	ASCUS	LSIL	
HPV types	No. (%) (*n* = 343)	No. (%) (*n* = 120)	No. (%) (*n* = 223)	*P*
HPV+ (any type)	282 (82)	75 (63)	207 (93)	<0.001
HR-HPV+	217 (63)	59 (49)	158 (70)	<0.001
HPV16+	65 (19)	19 (16)	46 (21)	0.28
HPV18+	28 (8)	8 (7)	20 (9)	0.46
HPV16/18+	89 (26)	26 (22)	63 (28)	0.18
Clade 9+	154 (45)	44 (37)	110 (49)	0.02
Clade 7+	108 (31)	31 (26)	77 (35)	0.10
Clade 7/9+	206 (60)	58 (48)	148 (66)	0.001

ASCUS = atypical squamous cells of undetermined significance; HPV = human papillomavirus; LSIL = low-grade squamous intraepithelial lesion. *P*-values indicate differences between ASCUS and LSIL cases. Clade 7: HPV18, 39, 45, 59, 68, 70. Clade 9: HPV16, 31, 33, 35, 52, 58, 67.

Table III:2. Preventive potential of current HPV vaccines, ASCUS and LSIL.

5.3.3 Multiple infections

Multiple infections were common *(Figure III:5)*. On average, there were 2.5 genotypes per HPV-positive sample. In one LSIL sample, nine different genotypes were found. In all, 54% (185/343) of all women carried more than one HPV type. Taking only HPV-positive samples into account, multiple infections were equally common in ASCUS and LSIL (65% and 66% respectively). The age-specific prevalence of multiple infections followed a quadratic function in LSIL cases (p=0.001, Figure III:5).

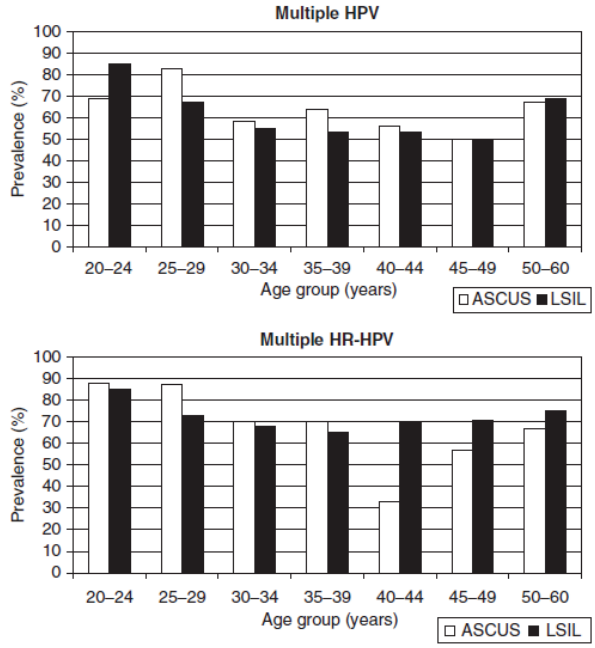


Figure III:5. Age-specific prevalence of multiple infections. Multiple HPV P(age group)=0.08, P(cytological diagnosis)=0.63, P(age group × cytological diagnosis)=0.73, LSIL P(age^2)=0.001, turning point 41.6 years, ASCUS P(age^2)=0.76. Multiple HR-HPV P(age group)=0.04, P(cytological diagnosis)=0.71, P(age group × cytological diagnosis)=0.24, LSIL P(age^2)=0.04, turning point 38.6 years, ASCUS P(age group)=0.27, P(age^2)=0.34. All P-values calculated by logistic regression.

A similar pattern was seen for multiple HR-HPV infections among the HR-HPV-positive LSIL cases (p =0.04). Among HR-HPV-positive samples, 39% (85/217) were infected by more than one HR-HPV type, and similar results were found for ASCUS and LSIL.

In all, there were 710 individual infections. Co-infection was equally common among LR-HPV genotypes (92%, 243 out of 268) and pHR-HPV genotypes (91%, 87/101). Individuals infected with HR-HPV were somewhat less likely to be infected with a second HPV type (83%, 283/341, p=0.06). The HR-HPV types that were most commonly seen in co-infection with HPV 16 (clade/species 9) were HPV 59 (46%), 45 (38%), and 39 (28%), belonging to HPV clade/species 7. Interestingly, HPV 18 did not occur with either HPV 39 or HPV 45 ($p \leq 0.01$). The high-risk types most commonly seen in co-infection with HPV 18 were HPV 56 (17%), 33(14%), and 51 (14%), which did not differ from frequencies of HPV 16 infection. HPV 16 infected women were more often co-infected with clade 7 HPV types than with similar clade 9 types (p=0.06). HPV 18-infected women carried genotypes from clade 9 in 43% of cases, compared with similar clade 7 genotypes in 11% (p=0.02).

5.3.4 Discussion

We found that HPV prevalence in women with screening results showing minor cytological abnormalities varied with age and there was a slight but non-significant increase in HPV prevalence in women over 45 years. Others have found similar age-related patterns and attributed this slight increase to menopausal hormonal and immunological changes that may facilitate HPV DNA detection or reactivate latent infections from exposures earlier in life (reviewed by (Bosch *et al*, 2008)). Such findings may also be related to sexual behavior and thus to increased exposure, as many women in this age group have divorced and remarried (Stevenson and Wolfers, 2007). Our finding of an increasing proportion of low-risk types in post-menopausal women was also observed by (Castle *et al*, 2006). This observation, including the increase in multiple infections among women over 50 years, might also reflect changes in hormonal or immunological status occurring at menopause. Some evidence suggests a hormonal effect on oncogene expression, at least for HPV16 and 18, through action on progesterone/glucocorticoid-responsive elements in the long control region of the HPV genome (Cole & Danos, 1987; Gloss *et al*, 1989). However, more recent research has called such a mechanism into question (Ruutu *et al*, 2006), and would instead suggest hormonal effects impairing apoptosis of infected cells. Oral contraceptives and high parity are known risk factors for cervical cancer (Munoz *et al*, 2002). One common characteristic of these factors and menopause is a state of anovulation. To our knowledge, no studies have explored whether other ovulatory hormones such as gonadotropins, inhibin, or prostaglandins could influence HPV-induced carcinogenesis.

The average prevalence of HR-HPV was higher in LSIL (71%) than in ASCUS cases (49%), which is consistent with a previous meta-analysis (Arbyn *et al*, 2009a). However, the LSIL cases had a significantly lower rate of HR-HPV than the rate reported by the ASCUS-LSIL-Triage-Study (ALTS) Group (82.9%) (2000). The ALTS group concluded that triage HPV testing of LSIL had limited potential for clinical decision making. The difference in HR-HPV prevalence might be due to different criteria for defining ASCUS and LSIL, and also to a difference in the mean age of the populations (24.9 years in ALTS and 33.6 years in this study). We used the LA assay covering 37 HPV types and defined 12 HPV types as high risk. ALTS used Hybrid Capture II to define the same 12 HR-HPV types and HPV68 as high risk. This approach should not introduce any major difference, as HPV68 without co-infection with another HR-HPV was found only in one case of ASCUS and in one case of LSIL in our material.

Age-dependent HPV prevalence in women without cytological abnormalities is a known phenomenon (Bosch *et al*, 2008; Castle *et al*, 2006; de Sanjose *et al*, 2007). Age-dependent prevalence in LSIL has been observed and discussed by Ronco *et al,* (Ronco *et al*, 2007b), although they only compared women who were older or younger than 35 years. High-risk human papillomavirus prevalence was age-dependent in our LSIL cases, but more stable in ASCUS cases. When using 5-year

intervals for age grouping, the only significant difference in HR-HPV prevalence between ASCUS and LSIL was found within the youngest (20–24 years) age group. In LSIL, the largest drop in HR-HPV prevalence was after the age of 30 years (83% in women <30 *vs.* 60% in women ≥30, $P<0.001$). These findings suggest that age 30, or even younger, is a suitable cut-off point for HR-HPV triage in LSIL, when consideration is given only to the prevalence argument. This is supported by the results from Paper II in which 48% of women over 30 years with LSIL and 60% of women with ASCUS were HR-HPV-negative and none of them had a histology result of CIN2+ (Froberg *et al*, 2008) and could therefore have avoided additional investigation.

One of the most noteworthy findings in our study was the high frequency of multiple infections. Similar results were previously reported by (Cuschieri *et al*, 2004) in a general screening population from Edinburgh using a linear array assay capable of identifying 27 types and similar liquid-based sampling. We found that the prevalence of multiple infections was consistent with a quadratic function, with increasing prevalence among women over the age of 50 years. One may speculate whether this finding is caused by re-infection, triggering of a latent infection, or if detection is favored by menopausal changes in the epithelium as mentioned above.

The observed elevated specific co-infection rates of HPV16 with certain other HPV types, such as HPV39, 45, and 59, may be because of differences in susceptibility to certain genotypes (all clade/species 7), as postulated by Liaw (Liaw *et al*, 2001). An international epidemiological study based on the IARC HPV prevalence surveys including > 13 000 individuals noted a tendency for clustering of HPV types with a high degree of similarity in the L1 region with high expected-to-observed ratios for closely homologous types, including HPV33/58, 18/45, 33/35, and 31/35 (Vaccarella *et al*). We found that HPV18-infected women were more likely to be co-infected with non-related clade/species 9 types than with similar clade/species 7 types, arguing against specific susceptibility. This may be a random finding, or an artifact caused by the relatively large contribution of HPV 16 and 18 among the species 9 and 7 HPV types detected.

Since 26% of all women were positive for HPV16 and/or 18 (Table II:2), and 60% were positive for any of the species 7 or 9 HPV types, one might estimate that HPV vaccination will decrease rates of low-grade abnormalities by about 26-60%. Although this estimation is uncertain due to the frequent multiple infections, it is similar to the estimation obtained from epidemiological studies by the IARC; in ASCUS 6-27% were positive for HPV 16 and/or 18 and the corresponding figure for LSIL was 16-32% (Clifford *et al*, 2006).

As many as about 75% of all these women could benefit from catch-up vaccination, provided full cross-protection can be assumed, as discussed by Castellsague (Castellsague *et al*, 2008). Our results show relative frequencies of individual HR-HPV types similar to those found during primary screening in a Swedish multicenter study comprising 5696 women aged 32–38 years (Swedescreen) (Naucler *et al*,

2007c). The Swedescreen group found that HPV16, 31, and 33 represented the highest population-attributable risk proportion in the development of CIN2+. Human papillomavirus type 18 was only the sixth most commonly identified HR-HPV type resulting in CIN2+.

Worldwide, HPV33 is associated with the third highest odds ratio (OR 373.5) for squamous-cell cervical cancer, behind HPV16 (OR 434.5) and HPV59 (OR 419.5) (Munoz *et al*, 2003). Considering that odds ratios vary for different HPV types, genotyping is needed to estimate risk in the individual case as well as to plan clinical follow-up and vaccination strategy. We think our data support the statement of Cuschieri *et al* (2004) that a broad spectrum test should be implemented until the true impact of the persistence of less common HR-HPV types in neoplastic progression is established.

5.4 Paper IV: Economic analysis of HPV triage

5.4.1 Cost-effectiveness of HPV triage

For ASCUS ≥30 years (Table IV:3), HPV triage is the least costly alternative, while Cytology is dominated by Colposcopy with biopsy. Colposcopy with biopsy detects 88 more CIN2+ lesions per 1000 women and is a cost-effective alternative with an ICER of SEK 2 056 per additional CIN2+ case detected, compared with HPV triage, assuming that society is willing to pay the additional cost. However, for all cytology and age categories except ASCUS ≥30 years, both HPV triage and Cytology are strongly dominated by Colposcopy with biopsy, i.e. Colposcopy with biopsy constitutes the least costly and most effective follow-up strategy.

For women older than 30 years, HPV triage is less costly and equally or more effective than Cytology as a follow-up strategy for ASCUS and LSIL (considered together and separately). In the categories which include women younger than 30 years, HPV triage is the most costly follow-up strategy, except for ASCUS 23-60 years, where HPV triage is SEK 124 less costly (SEK 2 662 vs. SEK 2 538) and equally effective compared with Cytology. For ASCUS/LSIL 23-60 years, HPV triage is more effective than Cytology, detecting 45 more CIN2+ cases per 1000 women, at a marginally higher cost of SEK 129 (SEK 3 057 vs. SEK 2928).

Comparing HPV triage to Colposcopy with biopsy, we found that for ASCUS/LSIL 23-60 years (Table 3), HPV triage is SEK 625 more costly (SEK 3 057 vs. SEK 2 432), while less effective, detecting 40 fewer CIN2+ cases per 1000 women. The largest difference in cost between these two strategies was seen for LSIL <30 years, where HPV triage was SEK 1 098 more costly (SEK 3 530 vs. SEK 2 432) and less effective, detecting 17 fewer cases of CIN2+ per 1000 women. The smallest difference in cost between the same strategies was seen for ASCUS 23-60 years, where HPV triage is SEK 106 more costly than Colposcopy with biopsy (SEK 2 538 vs. SEK 2 432) , detecting 77 fewer CIN2+ cases per 1000 women.

Follow-up strategy	Cost (SEK)	Incremental cost (SEK)	CIN2+ cases detected	Incremental effectiveness	Incremental cost-effectiveness ratio
ASCUS/LSIL					
23–60 years					
Colposcopy with biopsy	2,432		215		
Cytology	2,928	496	130	−85	Dominated
HPV triage	3,057	625	175	−40	Dominated
<30 years					
Colposcopy with biopsy	2,432		231		
Cytology	2,959	527	115	−115	Dominated
HPV triage	3,427	995	205	−26	Dominated
≥30 years					
Colposcopy with biopsy	2,432		202		
HPV triage	2,765	333	152	−51	Dominated
Cytology	2,904	472	141	−61	Dominated
ASCUS					
23–60 years					
Colposcopy with biopsy	2,432		192		
HPV triage	2,538	106	115	−77	Dominated
Cytology	2,662	230	115	−77	Dominated
<30 years					
Colposcopy with biopsy	2,432		167		
Cytology	2,856	424	112	−55	Dominated
HPV triage	3,085	653	111	−56	Dominated
≥30 years					
HPV triage	2,251		118		
Colposcopy with biopsy	2,432	181	206	88	SEK 2,056 /CIN2+ case
Cytology	2,562	130	118	−88	Dominated
LSIL					
23–60 years					
Colposcopy with biopsy	2,432		224		
Cytology	3,039	607	136	−88	Dominated
HPV triage	3,272	840	200	−24	Dominated
<30 years					
Colposcopy with biopsy	2,432		250		
Cytology	2,990	568	117	−133	Dominated
HPV triage	3,530	1,098	233	−17	Dominated
≥30 years					
Colposcopy with biopsy	2,432		200		
HPV triage	3,034	602	169	−31	Dominated
Cytology	3,084	652	154	−46	Dominated

Table IV.3. Cost-effectiveness results for follow-up strategies in different subgroups according to index cytology result (ASCUS/LSIL, ASCUS and LSIL) and age (all ages, <30 and >30 years). The follow-up strategies are listed in order of increasing cost. Incremental cost, CIN2+ cases found (Effectiveness) and ICER are calculated relative to the next less costly follow-up strategy. Incremental cost, CIN2+ cases Detected (Effectiveness) and Incremental Cost Effectiveness Ratio (C/E) are calculated relative to the next less costly follow-up strategy according to the following equation: ICER= (Cost of Screening strategy A-Cost of screening strategy B)/ (Effect of Screening strategy A- Effect of Screening strategy B). Dominated strategies are those with higher costs and lower clinical efficiency than other strategies.

a) The strategy is extended dominated.
b) Incremental cost-effectiveness ratios after eliminating strategies that are extended dominated

5.4.2 Sensitivity analysis

When changing costs for follow-up strategies, the model results were most sensitive to change in HPV test costs (Table IV:4). Most importantly, considering ASCUS and LSIL as one group, HPV triage is the least costly and next most effective follow-up strategy when HPV test cost is below SEK 231 for women 23-60 years and below SEK 522 for women ≥30 years, while Cytology is a dominated alternative. For ASCUS, management with HPV triage is the least costly alternative when the HPV test cost is below SEK 749 for women 23-60 years, and below SEK 1 035 for women ≥30 years. For ASCUS <30 years, HPV triage is no longer a dominated alternative if HPV test cost is below SEK 200. For LSIL ≥30 years, HPV triage is no longer a dominated alternative if HPV test cost is below SEK 252 for women.

Moreover, in ASCUS/LSIL and ASCUS, the model results were sensitive to changes in costs for visit to midwife and physician, as well as changes in costs for visit to physician for colposcopy with biopsy (Table IV:4, see the following page).

5.4.3 Discussion

A high sensitivity and negative predictive value to detect high-grade cervical lesions are required for HPV triage to be considered an attractive alternative for follow-up of ASCUS and LSIL. It should be noted that in the clinical trial upon which this analysis is based (Andersson *et al*, 2005a), the performance of HPV triage was low compared with other studies on the same topic (Arbyn *et al*, 2006; Cuzick *et al*, 2008).

This cost-effectiveness analysis shows that an additional 85 CIN2+ cases would be detected per 1000 women with ASCUS or LSIL going through secondary screening per year, if immediate colposcopy with biopsy were offered to all these women, instead of repeat cytology. Using the CIN2+ cases detected in this study, the exclusive use of immediate colposcopy with biopsy for secondary screening in Sweden translates into 1822 additional CIN2+ cases detected, compared with exclusive use of repeat cytology. Compared with HPV triage, immediate colposcopy with biopsy would detect 858 additional CIN2+ cases. The choice of alternative follow-up strategy is of great importance since an estimated 5-30% of missed CIN2+ cases could progress to invasive cancer (McCredie *et al*, 2008; Ostor, 1993).

According to the results from our study, HPV triage is the least costly follow-up strategy for ASCUS ≥30 years compared with immediate colposcopy with biopsy and repeat cytology, although it is less effective than immediate colposcopy with biopsy. In the sensitivity analysis, the model results were most sensitive to changes in HPV test costs. Lower costs for HPV testing are needed in order for HPV triage to be a cost-effective follow-up strategy compared with immediate colposcopy with biopsy for women <30 years with LSIL. Additional support for this idea was presented in a British cost-effectiveness study showing that HPV triage would be cost-neutral at a price of £34.37, and cost-saving below that level, compared with colposcopy (Guyot *et al*, 2003).

Parameter	Base case values	Ranges	23–60 years	<30 years	≥30 years
ASCUS/LSIL					
Cost of HPV test	SEK 859	SEK 0–1,718	231[a]	NS	522[a]
Cost of Pap test	SEK 83	SEK 0–166	NS	NS	NS
Cost of visit to midwife	SEK 604	SEK 0–1,208	NS	NS	265[a]
Cost of visit to physician	SEK 1,691	SEK 0–3,382	NS	NS	NS
Cost of visit to physician for follow-up using colposcopy with biopsy	SEK 2,432	SEK 0–4,864	NS	NS	3,161[b]
Prevalence of CIN2+	0.22/0.25/0.20	0.05–0.47d/0–1	NS	NS	NS
Prevalence of CIN2+	0.21/0.23/0.20	0.07–0.40[d]/0–1	NS	NS	NS
Cytology sensitivity	0.61/0.50/0.70	0–1	NS	NS	NS
Cytology specificity	0.56/0.52/0.59	0–1	NS	NS	NS
HPV triage sensitivity	0.82/0.89/0.75	0.87–0.97[d]/0–1	NS	NS	NS
HPV triage specificity	0.39/0.22/0.52	0.39–0.65[d]/0–1	NS	NS	NS
ASCUS					
Cost of HPV test	SEK 859	SEK 0–1,718	749[a]	200[a]	1,035[a]
Cost of Pap test	SEK 83	SEK 0–166	NS	NS	NS
Cost of visit to midwife	SEK 604	SEK 0–1,208	495[a]	NS	779[a]
Cost of visit to physician	SEK 1,691	SEK 0–3,382	NS	NS	1,386[b]
Cost of visit to physician for follow-up using colposcopy with biopsy	SEK 2,432	SEK 0–4,864	2,641[b]–4,046[a]	NS	2,164[b]
Prevalence of CIN2+	0.19/0.17/0.21	0.04–0.37[d]/0–1	NS	NS	NS
Cytology sensitivity	0.60/0.67/0.57	0.60–0.85[e]/0–1	NS	NS	NS
Cytology specificity	0.69/0.60/0.74	0.45–0.72[e]/0–1	NS	NS	NS
HPV triage sensitivity	0.60/0.67/0.57	0.60–1.00[e]/0–1	NS	NS	NS
HPV triage specificity	0.60/0.33/0.74	0.37–0.80[e]/0–1	NS	NS	NS
LSIL					
Cost of HPV test	SEK 859	SEK 0–1,718	17[a]	NS	252[a]
Cost of Pap test	SEK 83	SEK 0–166	NS	NS	NS
Cost of visit to midwife	SEK 604	SEK 0–1,208	NS	NS	NS
Cost of visit to physician	SEK 1,691	SEK 0–3,382	NS	NS	NS
Cost of visit to physician for follow-up using colposcopy with biopsy	SEK 2,432	SEK 0–4,864	NS	NS	NS
Prevalence of CIN2+	0.22/0.25/0.20	0.05–0.47[d]/0–1	NS	NS	NS
Cytology sensitivity	0.61/0.47/0.77	0.33–1.0[d]/0–1	NS	NS	NS
Cytology specificity	0.51/0.49/0.52	0.23–0.69[d]/0–1	NS	NS	NS
HPV triage sensitivity	0.89/0.93/0.85	0.89–1.0[e]/0–1	NS	NS	NS
HPV triage specificity	0.30/0.18/0.40	0.19–0.44[e]/0–1	NS	NS	NS

Table IV.4. Sensitivity analysis results. The table presents factors which influence cost-effectiveness of HPV-triage for ASCUS/LSIL, ASCUS and LSIL in different age groups.

a. Value below which HPV-triage is a cost-effective follow-up strategy.
b. Value above which HPV-triage is a cost-effective follow-up strategy.
c. Arbyn et al, 2004
d. Arbyn et al, 2005
e. Arbyn et al, 2006
NS: Cost-effectiveness of HPV-triage is not sensitive to changes in these values.

In today's cost situation however, HPV triage is dominated by immediate colposcopy and biopsy in all groups except ASCUS ≥30 years, and immediate colposcopy with biopsy provides a cost-effective alternative for follow-up of minor cytological abnormalities in all age groups. In the present analysis, changing the age limit for HPV triage from 30 to 35 years (data not shown) does not change this conclusion.

Compared with repeat cytology, HPV triage is a less costly and equally or more effective follow-up alternative for women ≥30 years. Repeat cytology is the overall least effective follow-up method and a dominated strategy for follow-up of ASCUS and LSIL in all age groups. Our results indicate that HPV triage is a preferred option to repeat cytology for follow-up of minor cytological abnormalities among women ≥30 years.

If liquid-based cytology were used in primary screening, HPV triage would become substantially less expensive since leftover sample after cytology slide preparation could easily be used for HPV testing (so-called HPV "reflex" testing), thereby avoiding costs for an additional physician visit. It is noteworthy that performance between liquid-based and conventional cytology may differ, which may influence cost-effectiveness of follow-up methods.

Use of an age-limit for HPV triage has previously been discussed. In the current study, all HR-HPV-positive women were referred for colposcopy with biopsy. The ASCUS-LSIL Triage Study (ALTS) investigated whether restricting HPV triage to women ≥29 years would also enable effective HPV triage in cases of LSIL. Results showed that although HPV test positivity rate was somewhat reduced in the older age group (74% vs. 88% in women aged 23-28 years), sensitivity of HPV triage for detection of CIN3 was also reduced (83% in the older vs. 98% in the younger age group), which is why HPV triage was not considered to be effective for women with LSIL regardless of age. For ASCUS, a small reduction in HPV test sensitivity for detection of CIN3+ with higher age was seen (Sherman *et al*, 2002). These figures are similar to those found in the clinical trial by Andersson *et al* upon which this cost-effectiveness analysis is based (Andersson *et al*, 2005a; Ostensson *et al*). In the HPV triage trial by Ronco *et al (Ronco et al, 2007b)*, performance of Hybrid Capture 2 was also high in women >35 years. In contrast to previous studies, HPV test performance was low for triage of ASCUS, with somewhat better sensitivity for detection of CIN2+ in the younger age group. This phenomenon may be related to the sensitivity of the HPV detection method used and/or sampling technique.

The cost-effectiveness analysis is based on a split sample trial where HPV tests were evaluated in a routine clinical setting, and HPV test results were evaluated in a blinded fashion in regard to follow-up cytological and histological diagnoses. The strength of this approach is that it minimizes observer bias and confounding from other variables that may affect the results. Some limitations should however be pointed out. The 177 patients in the clinical trial represent a limited number of patients and the results should therefore be interpreted with caution. The follow-up time in the clinical study is limited

to only one year, which implies that costs and health outcomes after the cessation of the clinical study is not available. To improve decision-making on societal efficiency of healthcare resource allocation, economic evaluations should ideally be based on a societal perspective. This also implies inclusion of costs outside the healthcare sector (e.g. indirect costs). Ideally quality adjusted life years (QALYs) should also be used as the outcome measure.

Today, significant costs are associated with the unnecessary follow-up of women with minor risk of developing cervical cancer (Naucler *et al*, 2007c). Since a majority of women with ASCUS or LSIL have normal findings or low-grade lesions with a high rate of spontaneous resolution, the use of HPV triage could be considered a less aggressive follow-up strategy. Development of high-throughput HPV DNA detection of high quality is rapid. Efficient triage, using highly sensitive HPV DNA testing at lower cost, would reduce the financial burden as well as the negative psychological and physical effects associated with abnormal screening results and colposcopic examination.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

Paper I:
When comparing LBC with supplementary HPV testing with conventional cytology in population-based screening, we could not demonstrate any significant differences in relative sensitivity for detection of CIN2+ or CIN3+, or any improvement in PPV for such lesions. However, it is noteworthy that the performance of both methods improved significantly over time, indicating that dynamic changes occurred during the study period, leading to improved cytological evaluation. The introduction of LBC may have resulted in enhanced detection of subtle cellular changes and an increased vigilance in cytological interpretation.

The sub-analyses showed that during the first fifteen months, total rate of abnormal cytological findings increased due to a high proportion of minor abnormalities, and detection of CIN2+ also gradually increased. When LBC sampling continued in small-scale routine during 2007, abnormal cytology report rates decreased significantly, to a level below that usually seen for CC. Although CIN2+ detection rates remained high, the decreased rate of low-grade cytology suggests a shift from overdiagnosis to overconfidence. The detection of high-grade precancerous lesions could be improved by a lower threshold to test for HR-HPV when nonspecific subtle cytological changes are encountered. This is in fact the value of HPV reflex testing – it allows for uncertainty and humility when confirming a preliminary diagnosis of ASCUS or LSIL. Monitoring the rate of abnormal cytology is highly important for maintaining a reasonable level of cytological evaluation.

To determine whether one of these screening strategies is superior to the other in the Swedish healthcare arena, additional evaluations need to be conducted in a setting where the staff is highly experienced in using both methods, and using a study design that includes gold standard verification of all cases. A prospective evaluation covering a second screening cycle is needed to answer whether either of these methods is superior when it comes to lowering the rate of CIN3+ (Arbyn *et al*). Improved knowledge about possible differences in performance between these two methods will also be important if future screening should change to include primary HPV testing, since adjunct cytology testing is likely to be needed.

Paper II, and III:
The NPV of HR-HPV detection for histologically confirmed high-grade lesions in cases of minor cytologicalabnormalities was 100%. An age limit for HPV reflex testing may be motivated in cases of LSIL. By using HPV "reflex" testing, additional extensive workup can safely be avoided in about 50% of all cases of ASCUS and LSIL among women ≥30 years. The prevalence of HR-HPV is similar among women with ASCUS and LSIL after the age of 30 years, supporting an age limit of 30 years for triage testing in LSIL cases and no age limit in ASCUS cases. Such a strategy can improve the effectiveness of the screening program, and reduce risks of overdiagnosis and overtreatment.

HPV triage/reflex testing may also lead to an improved protective effect by improving follow-up of HR-HPV-positive women. In cases where histology findings were WNL or CIN1, more than 50% were HR-HPV-positive. These women need to be followed-up, given their elevated risk for developing a high-grade cervical lesion. Repeated HPV genotyping allows for diagnosis of persistent HPV infection and for risk stratification of women with cytological abnormalities, which might be useful in clinical management (Naucler et al, 2007; Wheeler et al, 2006; Bulkmans et al, 2007).

Our results from Paper I showed that LBC yielded a higher rate of minor cytological abnormalities initially, which might explain the lower prevalence of HR-HPV in young women with screening results showing ASCUS, compared with other recent Swedish data in a conventional cytology screening setting (Dillner *et al*). At the same time, our data are consistent with many previous HPV triage trials, showing that HPV triage is worthwhile in ASCUS irrespective of age.

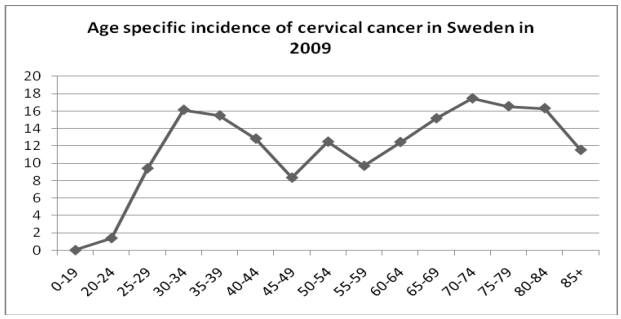
Paper III showed that multiple infections and LR/HR-HPV dominance are age-dependent. Genotyping in longitudinal design is needed to elucidate the importance of multiple infections in cancer progression and in cross-protection from vaccination. There is evidence that HPV types 16, 18, and 45 in particular are linked to high carcinogenicity, since they seem to lead to cancer at least 5 years earlier than do other HR-HV types (de Sanjose *et al*). Infections with these HPV types demand special consideration in follow-up and treatment of women with abnormal screening results.

Paper IV:
HPV tests used in HPV triage/reflex testing require high sensitivity and NPV for detection of CIN2+ and in particular CIN3+; otherwise they would reduce sensitivity of screening. HPV triage was the least costly alternative among women with ASCUS $\geq$ 30 years, although less effective than immediate colposcopy with biopsy. In the current setting of conventional cytology screening, HPV triage is not cost-effective, whereas immediate colposcopy with biopsy is the cost-effective alternative. HPV triage could potentially become a cost-effective management option, if HPV testing were performed as a “reflex” test, thereby avoiding an extra medical visit and bringing costs down below the cut-off, and if costs for HPV tests were lower. Our results motivate a discussion about what requirements should be fulfilled by HPV tests which are used for triage of ASCUS and LSIL, from a patient safety and cost-effectiveness perspective. They also provide policy makers with important information about issues regarding costs and effect to be consideredwhen including HPV testing in current follow-up strategies.

Future evaluations of alternative screening strategies require assessment of costs and QALYs from a lifelong perspective. Since the time frame to develop cervical cancer is long, there is uncertainty related to rate of HPV infection persistence, development of future CIN, and the rate of progression and regression of present CIN, which should also be considered.

General considerations

WHO recommends that cervical screening begin at age 25 -30 years, and stop at age 65 years, provided that previous smears have been normal (2006). Given the situation in Sweden today, with a first peak in incidence of cervical cancer in the age group 25-49 years (Figure 6.1 a and b), postponing screening start until age 25 or later seems risky. Taking into account the natural course of the disease, screening start must precede this first peak.



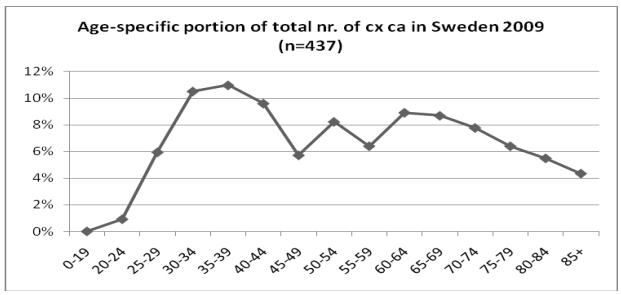


Figure 6.1 a and b. Age-specific incidence of cervical cancer (number of women per 100 000 (a) and the age-specific portion of the total number of cervical cancer cases in percent (n=427) (b) in Sweden, 2009. Adapted from (Socialstyrelsen, 2010).

The second major incidence peak, women aged 60-85 years, clearly shows that older women comprise a risk group with poor protection against cervical cancer. Extending the screening program until at least age 65 years as recommended by WHO and complementing or replacing cytology screening with HPV testing in older women may be a viable pathway. An interesting and potentially highly cost-effective alternative is HPV-self-testing for older women. Swedish studies on such possible solutions are underway.

High inclusion rates by organized population-based screening programs are crucial for screening efficacy. For various reasons, about 25% of all Swedish women do not participate in screening. Introduction of self-HPV testing may be an additional screening modality to help improve screening coverage.

In summary, given the lessons learned from the studies included in this thesis and literature in the field, I believe screening could be improved by the following strategy:

While using the same screening age reference range as today (23-60 years), women up to 30 or 35 years should be screened with cytology using reflex HPV testing/ HPV triage for ASCUS. For women over 30 or 35 years, primary HPV screening with cytology triage should be used, or alternatively, a combination of primary HPV testing and cytology. Women screened with HPV testing and cytology should be referred directly for colposcopy if HPV-positive and cytology-positive, using ASCUS as a cut-off. If more pronounced cytological abnormalities are found, women should be directedly referred to colposcopy regardless of HPV-test result. Women who are HPV-positive but cytology-negative should undergo repeated HPV testing after one year, and then be referred for colposcopy if persistently HPV-positive. Since several studies have shown that screening strategies based on primary HPV testing reduce the rate of CIN3+ in the following screening round, longer screening intervals are probably suitable.

Considering the effects of mass HPV-vaccination, and future changes in the spectrum of HPV types in the population, an age limit for primary HPV testing may become unnecessary. Using additional biomarkers, such as TERC, viral oncogene E6/E7 mRNA from the most important HR-HPV types, as well as p16 and L1, may improve specificity of screening in the likely scenario that the most carcinogenic HPV types will become proportionally less common. Future changes arising from the protective effects of prophylactic HPV vaccination will require monitoring of the cervical screening program to ensure that balance is achieved between the protective effects against cervical cancer and potential side effects.

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