Division of Obstetrics and Gynecology
Department of Clinical Science, Intervention and Technology
Karolinska Institutet, Stockholm, Sweden

HPV GENOTYPING AND POTENTIAL PROGRESSION MARKERS IN CERVICAL INTRAEPITHELIAL NEOPLASIA: CLINICAL AND DIAGNOSTIC IMPACT

Sophia Brismar Wendel

Karolinska Institutet

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To all women

ABSTRACT

The aim of this thesis is to identify clinically useful early molecular markers to predict progression to carcinoma in women with preinvasive lesions of the cervix, for the purpose of improving care of these women and enabling more individualized treatment.

In order to define the human papillomavirus (HPV) types in minor cytological abnormalities, 343 liquid-based cytology (LBC) samples with atypical squamous cells of uncertain significance (ASCUS) and with low-grade squamous intraepithelial lesions (LSIL) were genotyped using Linear Array. We found high-risk (HR-) HPV in 71% of LSIL and 49% of ASCUS cases (p<0.001). HR-HPV prevalence was similar in LSIL and ASCUS cases among women over 25 years. Younger (<25 years) and older (>50 years) women had higher prevalence of HR-HPV and multiple infections (p=0.01). HPV16 was found in 23% and HPV18 in 10% (p<0.001) of HPV-positive women. To test the utility of HPV genotyping in post-surgical monitoring, we genotyped cones of 90 women and follow-up samples after conization, and evaluated cytological results from two consecutive visits. Margin status and presence of CIN3+ in the cone were poor predictors of treatment outcome (sensitivity <50%). Presence of any probable HR/HR-HPV (18 types) predicted all residual high-grade SIL/ cervical intraepithelial neoplasia (CIN) 2 or worse with 73% specificity. Consideration of only 13 HR-HPV types showed equal sensitivity, but higher specificity (86%, p<0.01). True persistent infection detected high-grade residual disease with 60% sensitivity and 95% specificity, resulting in a positive predictive value (PPV) of 43%. We also assessed the utility of $p16^{INK4a}$ immunocytochemical detection of dysplastic cells in 118 samples from patients referred for further testing because of cytological abnormality. Intensity of $p16^{INK4a}$ staining correlated well with CIN grade, particularly when diagnosis was based on simultaneous routine cytology (p<0.001, Rho 0.70). Immunostaining for $p16^{INK4a}$ was feasible in clinical practice and helped to distinguish premalignant cells from reactive cells. To map local immune responses to HPV, we analyzed expression of several immune markers using real-time RT-PCR in cervical biopsies from 24 female volunteers who had been genotyped for HPV. No difference between the 11 HPV-positive and 13 HPV-negative women was found for mRNA expression of any of the immune markers. Surprisingly, levels of the B cell marker CD19 were elevated among women using hormonal contraception (p<0.05).

HPV genotyping revealed age-dependent patterns of HPV infections in LSIL and ASCUS cases. We propose triage HPV testing of LSIL after 30 years of age. Genotyping after conization substantially increased PPV, but with loss in sensitivity. General HR-HPV testing will identify all recurrent or residual high-grade CIN. Demonstration of $p16^{INK4a}$ accumulation in the cell nucleus is a simple way to enhance presence of dysplastic cells and to distinguish these from reactive atypia. HPV infection per se does not evoke local immune response as measured by semiquantitative RT-PCR. Hormonal contraception may influence B cell activity in the cervix. Further studies are needed to identify potential progression markers.

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. BRISMAR WENDEL S, Fröberg M, Hjerpe A, Andersson S*, Johansson B*. (*Equal contribution)
Age-specific prevalence of HPV genotypes in cervical cytology samples with equivocal or low-grade lesions.
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II. BRISMAR S, Johansson B, Börjesson M, Arbyn M, Andersson S.
Follow-up after treatment of cervical intraepithelial neoplasia by HPV-genotyping.
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III. Norman I*, BRISMAR S*, Zhu J, Gaberi V, Hagmar B, Hjerpe A, Andersson S. (*Equal contribution)
p16(INK4a) immunocytochemistry in liquid based cervical cytology: is it feasible for clinical use?
International Journal of Oncology 2007 Dec; 31(6): 1339-43.

IV. BRISMAR WENDEL, S, Kaldensjö T, Petersson P, Andersson S, Broliden K, Hirbod T.
Slumbering mucosal immune response in the cervix of human papillomavirus DNA-positive and negative women.
In manuscript.

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LIST OF ABBREVIATIONS

AGUS	Atypical glandular cells – uncertain significance
AIS	Adenocarcinoma in situ
APC	Antigen-presenting cell
ASC-H	Atypical squamous cells – HSIL cannot be ruled out
ASCUS	Atypical squamous cells – uncertain significance
CCL	Cysteine-cysteine chemokine ligand
CCR	Cysteine-cysteine chemokine receptor
CD	Cluster of differentiation
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CIS	Cancer in situ
C-LETZ	Contoured large excision of the transformation zone
DC	Dendritic cell
E	Early
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
HPV	Human papillomavirus
HR-HPV	High-risk HPV
HSIL	High-grade squamous intraepithelial lesion
Ig	Immunoglobulin
IL	Interleukin
L	Late
LCR	Long control region
LR-HPV	Low-risk HPV
LSIL	Low-grade squamous intraepithelial lesion
$P16^{INK4a}$	Protein 16 (inhibits cyclin-dependent kinase 4)
p53	Protein 53
PCR	Polymerase chain reaction
PD(-L)	Programmed cell death receptor (and ligand)
pHR-HPV	Probable high-risk HPV
pRb	Retinoblastoma protein
ORF	Open reading frame
RANTES	Regulated upon activation T cell normal expressed and secreted
RT	Reverse transcriptase
SCC	Squamous cell carcinoma
SIL	Squamous intraepithelial lesion
VLP	Virus-like particle

1 INTRODUCTION

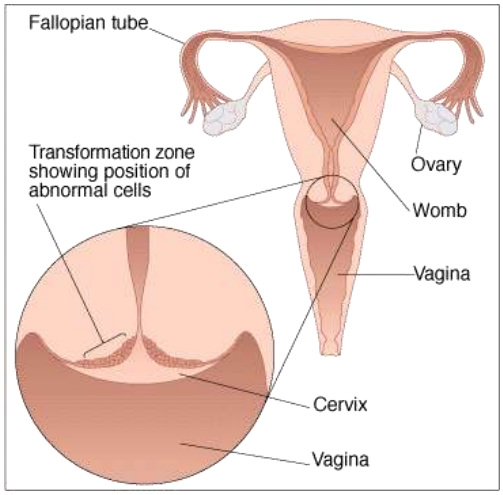
Human papillomavirus (HPV) is an ancient virus, which has always lived in harmonic symbiosis with its host, replicating and spreading, without intending to kill its provider. Nevertheless, HPV infection is a widely disseminated sexually transmitted infection that causes cutaneous and genital warts in millions of men and women every year, along with cervical and anal dysplasia, as well as cancer of the cervix, anus, skin, head, and neck. During the sexual revolution of the 1960s and 1970s, the stage in the Western world was set for an increase in the prevalence of HPV infection and subsequent clinical manifestations, but cytological screening kept this trend in check. In the post-HIV/AIDS-awareness era (at least in the mass media), young and even old people are again less wary of sexually transmitted diseases and interpret condom campaigns as encouragement to engage in more (unsafe) sex, cheered on by glossy magazine articles with advice on "how to become a sex god/goddess". Development of HPV vaccine has been timely, helping to decelerate this epidemic and to increase public awareness about the contagious nature of underlying causes of cervical dysplasia and cancer. However, vaccination will prevent only about 70% of cervical cancers, assuming that the spectrum of HPV types causing cancer today remains unchanged over the next 10-15 years. Before the long-term benefits of public vaccination against HPV can be observed and documented, cervical cancer screening must continue. Until such time that screening methods become perfect and vaccines totally eradicate the virus, we must strive to improve identification of women at risk for cervical cancer. This objective can be achieved through better understanding of HPV carcinogenesis and host immune responses to HPV.

2 BACKGROUND

2.1 THE CERVIX

The cervix uteri is the part of the uterus that protrudes into the vagina and is covered on the outside (ectocervix) by non-keratinized stratified (multi-layered) squamous epithelium, while the inside (endocervix) is lined by a single layer of columnar mucus-secreting epithelium (Burghardt et al, 1998). The columnar epithelium may extend outside the cervical canal and assume varying size and shape, forming an ectopy mostly seen in teens, gravidae and women taking hormonal contraceptives. The columnar epithelium exposed to the acidic, non-sterile environment of the vagina will transform into squamous epithelium through a process known as metaplasia, occurring in the transformation zone (**Figure 1**).

Figure 1. Cervix with transformation zone (adapted from www.cancerresearchuk.org).



The squamocolumnar junction will migrate into the cervical canal during the course of life and is commonly no longer visible after menopause (Autier *et al*, 1996). The tight squamous epithelium may obstruct mucus secretion and thereby cause formation of Nabothian cysts. For unclear reasons, the metaplastic transformation zone is a weak spot susceptible to infectious agents and malignant transformation, and it is where cervical cancer must arise. Underneath the epithelium is a layer of connective tissue and beneath this is smooth muscle. Components of the immune system reside in the epithelium and subepithelial layers and will be addressed later in the background section.

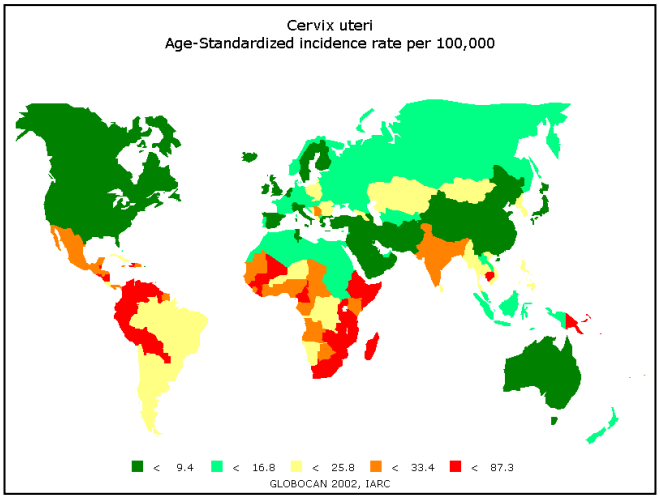
2.2 CARCINOMA OF THE CERVIX UTERI

2.2.1 Incidence and prevalence

Cervical cancer has two major forms: the predominant squamous cell carcinoma (SCC) that shares similarities with the stratified squamous epithelium, and the less common adenocarcinoma, which is thought to originate in the single columnar epithelium of the endocervix. Cervical cancer is the second most common cancer among women in the world, with an estimated 483 000 new cases and 274 000 deaths yearly (Parkin et al, 2005). In Sweden, as in many western countries, incidence and prevalence of SCC has decreased since the implementation of cytological screening (Bergstrom et al, 1999). In 2007, 128 women were diagnosed with cervical cancer in the Stockholm-Gotland County (age-standardized rate 13/100 000 women) (Hellborg, 2009). Globally, developing countries have the highest incidence mainly due to lack of screening and subsequent treatment (Figure 2). Even within the European Union, there is a striking difference in incidence and mortality between old and new member states. Finland has

the lowest incidence, with 54/1 000 000 women-years, while Lithuania has the highest with 220/1 000 000 women-years (Arbyn *et al*, 2007b).

Figure 2. Incidence of cervical cancer worldwide (from www-dep.iarc.fr).



2.2.2 Risk factors

In 2008, Harald zur Hausen received the Nobel Prize in Physiology or Medicine for his pioneering work more than 30 years ago, concerning the role of HPV in the development of cervical cancer (zur Hausen, 2009). Certain high-risk types of HPV (HR-HPV) have now been established as obligate etiologic agents for the development of cervical cancer, at least in SCC (Bosch *et al*, 2002; Munoz, 2000; Walboomers JM *et al*, 1999; zur Hausen, 1990). In the 1980s, studies revealed the presence of HPV DNA in 30-60% of cervical cancer cases, but as detection methods improved, an increasing percentage of cases were shown to contain HPV DNA (Bosch *et al*, 2002). Walboomers and colleagues decided to reanalyze HPV DNA-negative cases from a previous report using three different HPV PCR assays targeting different open reading frames, excluding inadequate specimens, and found that the worldwide HPV prevalence in cervical carcinoma is 99.7% (Walboomers JM *et al*, 1999). Thus, already 10 years ago, infection with certain oncogenic HPV types was established as a necessary cause of cervical cancer. The presence of HPV in virtually all cervical cancers implies the highest attributable fraction so far reported for a specific cause of any major human cancer.

HPV infection is a necessary but insufficient event preceding the development of cervical cancer, since only a fraction of HPV-positive women eventually are diagnosed with cancer. Adhering to the central dogma of the necessary presence of HPV, only HPV-positive cases should be included when assessing cofactors of malignant transformation (IARC, 2007). Therefore, early sexual debut, multiple partners, and non-use of condoms are now frequently considered to be merely surrogate markers for risk of HPV or other sexually transmitted infections (STI) (Bosch & de Sanjose, 2007; Castellsague *et al*, 2002; Hildesheim *et al*, 2001).

Co-infection with other STIs, such as Herpes Simplex type 2 (HSV2) and *Chlamydia Trachomatis* has inconsistently been associated with cervical cancer. A number of seroepidemiological studies have either found moderate or no association between

HSV2 antibodies and cervical cancer. The largest Nordic study conducted by Lehtinen and co-workers pooled data and specimens from three population-based cohorts of more than 500 000 women and found no association after adjusting for HPV-positivity and smoking (Lehtinen *et al*, 2002), while Smith and colleagues collected data and specimens from seven separate case-control investigations conducted in high-risk countries (such as Brazil, Colombia, and Thailand) and found an increased odds ratio (OR) of 2.2 (95% CI 1.4-3.4) in HPV DNA-positive subjects after adjusting for *C. Trachomatis* seropositivity (Smith *et al*, 2002). A few studies assessing the presence of HSV DNA by PCR in cervical neoplasia also present conflicting results (reviewed in (IARC, 2007)). As an example, Tran-Thanh and colleagues failed to detect any known HSV2 DNA sequences in 200 cervical cancer specimens compared with 244 normal specimens (Tran-Thanh *et al*, 2003).

The evidence for *C. Trachomatis* as a cofactor in cervical carcinogenesis is stronger. Serological proof of prior *C. Trachomatis* infection seems to double the risk of cervical cancer even after adjustment for age, oral contraceptive use, history of Pap smears, number of full-term pregnancies and HSV2 seropositivity (Koskela *et al*, 2000; Smith *et al*, 2004). Detection of pathogen DNA also points toward an increased risk of cervical cancer. In Sweden, Wallin and colleagues compared 118 cervical cancer cases with 118 controls and found that the relative risk for cervical cancer associated with *C. Trachomatis* DNA in the baseline smear, adjusted for concomitant HPV DNA positivity, was 17.1 (95% CI 2.6-infinity) (Wallin *et al*, 2002). They found no *C. Trachomatis* DNA in the cancer specimens. Golijow and colleagues repeated this study in a group of Argentinian women and found an increased prevalence of *C. Trachomatis* DNA in LSIL and HSIL samples compared with normal cytological samples, but not in invasive cervical carcinoma (Golijow *et al*, 2005). The molecular mechanisms by which local co-infections facilitate HPV carcinogenesis are not fully understood. Inflammation causing edema and increased permeability of the transformation zone may increase the susceptibility to HPV infection or facilitate early events in the malignant transformation.

HIV infection is undoubtedly associated with increased prevalence of HPV infection, HPV persistence, and risk of cervical dysplasia (Ahdieh *et al*, 2000; Cubie *et al*, 2000; Delmas *et al*, 2000; Lehtovirta *et al*, 2006; Mandelblatt *et al*, 1999; Minkoff *et al*, 1998; Palefsky *et al*, 1999; Sun *et al*, 1997). Invasive cervical carcinoma was classified as an AIDS-defining illness in 1993 by the United States Centers for Disease Control and Prevention after evidence became available of a higher prevalence of cervical squamous intraepithelial lesions (SIL) in HIV-positive immunosuppressed women (CDC, 1992), although the IARC concluded in 1996 that HIV was not associated with an increased prevalence of invasive cervical carcinoma (IARC, 1996). More recent studies argue for an increased risk of invasive SCC in HIV-positive women, at least in industrialized countries where endemic rates of cervical cancer are relatively low and where women have better access to care and longer survival after contracting HIV infection (reviewed in (de Vuyst *et al*, 2008)). The effect of HIV on HPV infection is still unclear, but immune suppression (low CD4 counts) increases the risk of HPV-related disease (Ahdieh *et al*, 2000; Delmas *et al*, 2000; Minkoff *et al*, 1998; Palefsky *et al*, 1999), as is similarly seen in women with impaired cell-mediated immunity due

to organ transplantation or aggressive immune therapy for other reasons (Grulich *et al*, 2007; Palefsky & Holly, 2003; Paternoster *et al*, 2008).

Tobacco smoking is known to covary with sexual behavior and it was only after 1990 that HPV status was taken in to account in epidemiological research (IARC, 2007). Since then, the majority of studies have identified smoking as an independent cofactor in the development of cervical cancer. The International Collaboration of Epidemiological Studies of Cervical Cancer brought together and combined individual data on 13,541 women with and 23,017 women without cervical carcinoma from 23 epidemiological studies, and concluded that current smokers had a significantly increased risk of cervical SCC compared with never smokers (RR 1.6, 95%CI 1.5-1.7) and that past smokers were at increased risk, but to a lesser extent (RR 1.1, 95%CI 1.01-1.3) (Appleby *et al*, 2006).

Medium (5-9 years) and long-term (10 or more years) use of combined oral contraceptives (OC, estrogen and progesterone) have been shown to increase the risk of invasive cervical cancer by 1.3-4 times among HPV-positive women in large pooled case-control studies (Appleby *et al*, 2007; Moreno *et al*, 2002; Smith *et al*, 2003), a view also supported by the IARC. In other studies, the use of OC was not associated with cervical cancer (Castle *et al*, 2002; Shapiro *et al*, 2003; Syrjanen *et al*, 2006). Syrjänen and co-workers in particular argue that sexual behavior differs among OC users, non-OC users, and non-users of contraception, and that the sexual behavior predisposes women to HR-HPV, high-grade CIN, and determines the outcome of cervical disease and HR-HPV infection (Syrjanen *et al*, 2006). Progestin-only contraception has not been associated with cervical cancer (Shapiro *et al*, 2003). Moodley and colleagues have summarized the pathogenetic effect of steroid contraceptive hormones in cervical cancer and emphasize that the upstream regulatory region of the HPV16 viral genome is thought to contain enhancer elements that are activated by steroid hormones and may increase expression of the E6 and E7 oncogenes (Moodley *et al*, 2003). However, estrogen and progesterone hormones had no significant effect on E6 or E7 expression in a study of HPV16 containing cell-lines (Ruutu *et al*, 2006). It was concluded that these hormones promoted cell proliferation and made the cells vulnerable to mutations during cell division by other pathways than via E6 and E7. Estrogen also had an anti-apoptotic effect, which resulted in a growth advantage of the cells infected with oncogenic HPV.

High parity has also been reported as an independent cofactor of cervical cancer. In the Guanacaste study of more than 10 000 women, the risk of HSIL/cancer increased with increasing number of live births in women positive for HR-HPV (Hildesheim *et al*, 2001). In a pooled analysis of HPV-positive women orchestrated by the IARC, Munoz and co-workers found an OR of 1.8 for cervical cancer in women with one or two full-term pregnancies compared with none, and OR 3.8 in women with 7 or more full-term pregnancies (Munoz *et al*, 2002). The literature provides no explanation for this phenomenon. One rather cynical explanation was given by a Thai study on the effect of early coitarche in a monogamous cohort: husbands were more prone to visit prostitutes in these relationships (Thomas *et al*, 2001) as well as in relationships within low socio-economic groups (de Sanjose *et al*, 1997). Young mothers and those with high parity are indeed known to have higher rates of HPV infection, which puts them at greater risk

for developing cervical cancer (Lorenzato *et al*, 2001). Similar to the use of OC, pregnancy involves elevated levels of sex hormones, anovulation, and cervical ectopy, which may increase the susceptibility to HPV infection, modulate the immune system, and increase cell turnover (Delvenne *et al*, 2007). Socioeconomic status depends on educational level and income, and is intimately related to high parity, smoking, and dietary factors, as well as lack of screening and resources to obtain adequate treatment, which is why the individual effect of this cofactor is difficult to pinpoint.

Hereditary factors also play a role in the development of cervical cancer. Register studies in Nordic and American countries have found a moderate increase of cervical cancer among women with relatives who have had cervical cancer, and most studies suggest that genes are responsible for 20-30% of cervical cancers (Couto & Hemminki, 2006; Czene *et al*, 2002; Hemminki & Chen, 2006; Magnusson *et al*, 2000; Zelmanowicz Ade *et al*, 2005). Variations in the human leukocyte antigen (HLA) class II genes and polymorphism of the p53 gene are the most frequently reported candidates to explain an inherited risk for cervical cancer (Andersson *et al*, 2001; de Araujo Souza *et al*, 2008; Gudleviciene *et al*, 2006; Hildesheim & Wang, 2002; Kohaar *et al*, 2009a; Madeleine *et al*, 2008), but other immune and DNA repair genes are also proposed (Wang *et al*, 2009).

2.3 PRECANCEROUS LESIONS OF THE CERVIX

2.3.1 Definitions

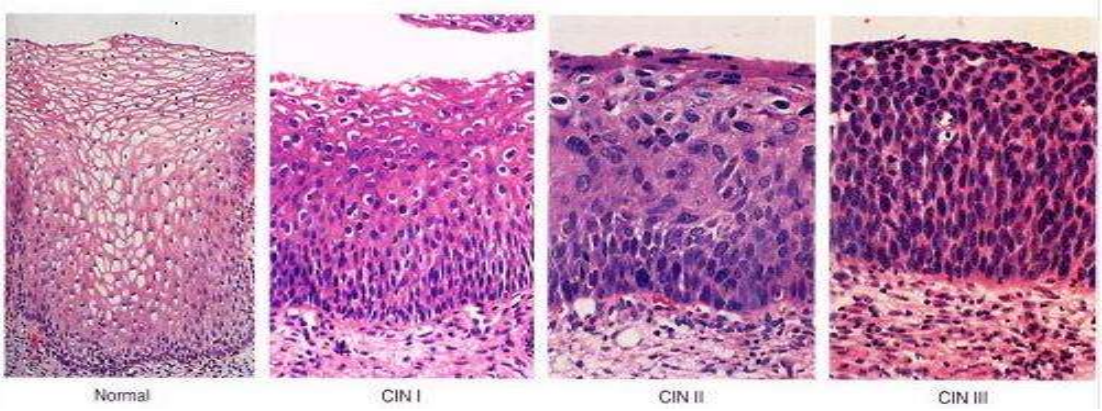
Precancerous lesions in histological samples of the cervix are classified based on severity into CIN1, 2, and 3, according to the system founded on the work of Richart (Richart, 1973). This system essentially paraphrases the traditional grouping into mild, moderate, and severe dysplasia. In cytological samples, two levels are defined according to the Bethesda system (Solomon *et al*, 2002): low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL). However, even the Bethesda system allows for doubt, using the terms "atypical squamous cells – uncertain significance" (ASC-US) and "atypical squamous cells – cannot exclude HSIL" (ASC-H), which replace the original "ASCUS" (**Figure 3**). According to the Swedish Society for Clinical Cytology, koilocytosis without nuclear atypia should be reported as non-pathologic and not as LSIL.

Figure 3. Classification of precancerous lesions of the cervix (from (Nanda *et al*, 2000)).

<table>
<tr><td>Classification system</td><td colspan="8">Cytology classification</td></tr>
<tr><td rowspan="2">The Bethesda System</td><td rowspan="4"></td><td rowspan="2">Infection Reactive repair</td><td rowspan="2">ASCUS</td><td colspan="5">Squamous Intraepithelial lesion (SIL)</td><td rowspan="4"></td></tr>
<tr><td colspan="2">LSIL</td><td colspan="3">HSIL</td></tr>
<tr><td rowspan="2">Richart</td><td colspan="2" rowspan="2"></td><td rowspan="2">Condy-loma</td><td colspan="4">CIN</td></tr>
<tr><td>Grade I</td><td>Grade II</td><td colspan="2">Grade III</td></tr>
<tr><td>Reagan (WHO)</td><td colspan="2">Atypia</td><td colspan="2">Mild dysplasia</td><td>Moderate dysplasia</td><td>Severe dysplasia</td><td>CIS</td><td>Invasive carcinoma</td></tr>
<tr><td>Papanicolaou</td><td>I</td><td colspan="2">II</td><td colspan="3">III</td><td colspan="2">IV</td><td>V</td></tr>
</table>

The hallmark of CIN or SIL is the proliferation of atypical squamous cells. Atypical cells are altered in size, shape, and polarity, with more mitoses and disturbed epithelial architecture. The nuclei are enlarged and hyperchromatic. In CIN1, atypical cells are present in the lower third of the epithelium and stratification is preserved. In CIN2, atypical cells are present in 2/3 of the epithelium, whereas in CIN3, the entire thickness of the epithelium is transformed, but there may still be some parakeratosis on the surface (**Figure 4**) (Burghardt *et al*, 1998).

Figure 4. Cervical precancer stages (image courtesy of Talaat S Tadros MD, Emory University School of Medicine, www.cancerquest.com).



2.3.2 Natural history

The now classical review by Andrew Östör from 1993 puts ”the presumption of CIN preceding cancer, being part of a continuum along progression to cancer, and ultimately almost always will proceed to cancer” and indeed “the raison d’être for the massive financial investment aimed at cancer prevention” at question (Ostor, 1993). His rather revolutionary conclusion, drawn from studies published between 1950 and 1990, is summarized in **Table 1**.

Table 1. Natural history of CIN (Ostor, 1993).

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

The major problems with this review are that not all the studies included were backed by histologically confirmed diagnoses and follow-up time was highly variable (0.5-10 years). In a large retrospective cohort study from Toronto, Canada, progression of mild dysplasia to moderate or worse was 11% within 2 years, 20% within 5 years, and 29% within 10 years. Corresponding numbers for progression of moderate dysplasia to severe or worse were 16%, 25%, and 32% (Holowaty *et al*, 1999). A study from National Women's Hospital in Auckland, New Zealand, where treatment of CIN3 was withheld from a substantial number of women between 1965 and 1974 as part of an unethical clinical study, is the most recently published on the subject (McCredie *et al*, 2008). In 1988, a judicial inquiry resulted in recorded follow-up of 1229 women who had been subjects in this study. A total of 1063 (86%) women had been diagnosed with

CIN3 at the hospital between 1955 and 1976. Up through December 31, 2000 in 143 women managed by punch or wedge biopsy alone, the cumulative incidence of invasive cancer of the cervix or vaginal vault was 31% (95%CI 23-42), while incidence was 50% (37-65) in a subset of 92 such women who had persistent disease after 24 months. However, cancer risk was only 0.7% (0.3-1.9) in 593 women, whose initial treatment was deemed adequate or probably adequate, and whose treatment for recurrent disease was conventional.

Pregnancy may be described as an immunomodulated state, which may impair the ability to locate and heal CIN. However, CIN1 associated with pregnancy had a better regression rate when comparing outcome after the post-partum period with a corresponding follow-up time in non-pregnant women (Serati *et al*, 2008). Pregnant women with CIN2+ seem to be at equal risk of persistence. In women with HIV, it is less clear whether the immune suppression results in lower regression rate of diagnosed CIN (Boardman *et al*, 2008; Ellerbrock *et al*, 2000; Massad *et al*, 2008). Rather, HIV may increase susceptibility to HPV infection and compromise clearance of HPV infection.

Since so few abnormal cases ultimately progress to any significant disease, there is ample reason to improve identification of women at risk for such progression. This thesis, among many other studies, is engaged in the quest to find useful progression predictors or markers.

2.3.3 Screening

2.3.3.1 Pap smear

An exfoliative cytological method for early detection of gynecological cancer was first published by Dr George Papanicolaou in 1941, hereafter referred to as the Pap smear (Papanicolaou & Traut, 1941). It was first proposed to detect uterine cancer, but soon proved to be useful in screening for precancerous stages of cervical carcinoma. Today, cells from the ecto- and endocervix are collected with a spatula and mascara-like brush, smeared on a glass slide, immediately fixed in 95% ethanol, and air-dried. Classical Papanicolaou staining involves five separate dyes applied in a three-step process: first, the nuclear stain haematoxylin is applied to the slide, then an orange counterstain (Orange G) which stains keratin, and last a turquoise (Eosin Azure) counterstain for staining several other components. Interpretation should be carried out by a cytotechnologist or pathologist.

Pap smears are carried out in systematic screening and on demand as part of the clinical gynecological examination (opportunistic screening). Systematic screening programs were implemented during the 1960s in Sweden and have proven effective in reducing the incidence of cervical SCC and mortality by around 35-75% (Bergstrom *et al*, 1999; Hemminki *et al*, 2002; Mahlck *et al*, 1994; Sigurdsson, 1999). However, Pap smears are not equally valuable in opportunistic screening, since the individual Pap smear has low sensitivity and limited specificity. In a pooled analysis of 109 studies published between 1991 and 2006, a conventional Pap smear showing HSIL+ had a sensitivity of 55.2% (95%CI 45.5-64.7) for detecting histologically confirmed CIN2+ (Arbyn *et al*, 2008b). In the event of any abnormality, Pap smears detects 88.2% (95% CI 80.2-93.2)

of all CIN2+ (Arbyn *et al*, 2008b). Specificity of Pap smears is between 71% and 97% depending on cut-off (ASCUS, LSIL or HSIL). Pariticipation in organized screening with Pap smears is still the most efficient way to detect dysplasia and more importantly, obtain treatment to prevent progression to cancer (Andrae *et al*, 2008).

2.3.3.2 Liquid-based cytology

An alternative method for detecting precancerous lesions is liquid-based cytology (LBC), where similarly collected cells are suspended in preservative, and subsequently spread on a glass slide in a thin layer. Two major types of LBC are commercially available: ThinPrep (Cytyc Corp, Marlborough, MA, USA) and Surepath (BD, Franklin Lakes, NJ, USA). Cytotechnologists and pathologists generally prefer this method because the uniform spread of epithelial cells in a thin layer facilitates microscopic interpretation. Several studies attest to higher sensitivity than found in conventional Pap smears (Bernstein *et al*, 2001; Strander *et al*, 2007a; Zhu *et al*, 2007), though others have questioned such findings (Arbyn *et al*, 2008b; Obwegeser & Brack, 2001). The U.S. Food and Drug Administration (FDA) approved the ThinPrep test in 1996 based on split-sample analysis (Limaye *et al*, 2003). In Sweden, until recently conventional Pap smear has been the method of choice but now many counties have switched or will soon switch to LBC.

Current screening guidelines in Stockholm County recommend triennial Pap smears between the ages of 23 and 50 (Ahlberg-Ranje *et al*, 2008). Subsequently one Pap smear is offered at 55 and one at 60 years. Any smear abnormality will be subject to further investigation with colposcopy and biopsies, a practice which is internationally supported (Kyrgiou *et al*, 2007). The sensitivity of colposcopic biopsies is dependent on lesion size and colposcopist training, and increases from 50-60% to 80-100% for detecting CIN2+ when two or more biopsies are taken (Gage *et al*, 2006; Jeronimo & Schiffman, 2006; Pretorius *et al*, 2006). Biopsies are subject to over- and underdiagnosis in half of the cases (Hopman *et al*, 1998). In addition, this procedure is both costly and cumbersome, why many advocate for HPV testing, at least for LSIL and ASCUS triage. A great advantage of LBC is the opportunity to easily perform such additional triage testing, such as HPV analysis or use of immunocytochemical technology to identify potential progression markers. HPV triage testing of ASCUS is strongly supported by a pooled analysis of 22 studies, which showed 93% sensitivity of HPV triage for detecting CIN2+ and 96% for detecting CIN3+ (Cuzick *et al*, 2008). Pooled specificity was 62% for an outcome of CIN2+ and 61% for CIN3+. Triage testing in LSIL is somewhat more questionable. HPV positivity is high especially among young women with LSIL (pooled estimate of 74%), rendering very high sensitivity (97%), but low specificity for detecting CIN2+ (30%) or CIN3+ (26%) (Arbyn *et al*, 2005; Cuzick *et al*, 2008). Most researchers therefore agree on setting an age limit for triage testing in LSIL, but whether this limit should be at age 30 or 35, or some other age, is under debate (Cuzick *et al*, 2003; Ronco *et al*, 2007).

2.3.3.3 HPV testing in primary screening

HPV testing for screening remains more controversial, despite results from meta-analyses and several recent large randomized controlled trials comparing HPV testing with cytology (Bulkmans *et al*, 2007; Mayrand *et al*, 2007; Naucler *et al*, 2009;

Naucler *et al*, 2007b; Ronco *et al*, 2008). In a comprehensive review of prospective studies published in 2006, Cuzick and colleagues concluded that HPV testing was substantially more sensitive for detecting CIN2+ than cytology (96.1% vs. 53.0%), but less specific (90.7% vs. 96.3%) (Cuzick *et al*, 2006). The sensitivity of HPV testing was similar in different areas of Europe and North America, whereas sensitivity of cytology was highly variable. HPV sensitivity was uniformly high at all ages, whereas sensitivity of cytology was much better in women over the age of 50 years (79.3% vs. 59.6% in women <50 years). The randomized trials published after 2006 present improved figures for sensitivity and specificity of HPV testing versus cytology in primary screening. For example, a large Canadian trial including more than 10 000 women aged 30-69 reported that sensitivity of HPV testing for CIN2+ was 95%, whereas smear sensitivity was 55%. Specificity was 94% for HPV testing and 97% for Pap smears (Mayrand *et al*, 2007). HPV testing in primary screening even decreases the incidence of CIN2+ at follow-up after 5 years, which may allow for longer screening intervals (Bulkmans *et al*, 2007; Naucler *et al*, 2007b). As expected, one trial reports no difference in sensitivity of HPV testing and cytology in detecting CIN3+ (Kotaniemi-Talonen *et al*, 2008).

2.3.4 Treatment options

2.3.4.1 Cryotherapy and laser vaporization

Since a small but significant portion of lesions graded CIN2+ will progress to invasive cancer of the cervix, treatment of women with such diagnoses is common practice and recommended in international guidelines (Wright *et al*, 2007). Destructive or ablative surgery is considered acceptable in younger women (with overt or presumed wish to become pregnant) with CIN2 or persistent CIN1. Cryotherapy and laser vaporization are the methods of choice. Both requires only local anesthesia and can be performed on an out-patient basis. For both procedures, the transformation zone is located by colposcopy and iodine staining. Cryotherapy induces tissue necrosis through intermittent hypothermia using carbon dioxide or nitrous oxide (-60 to -90 ºC), while laser vaporization uses laser energy that is absorbed by tissue water to cause destruction through heating. Both methods can be used to treat to a depth of 5 mm and are therefore not suitable for larger or more invasive lesions. Both treatment methods are comparable with results showing a recurrence rate of 3-23% (Hatch, 1995; Luciani *et al*, 2008; Martin-Hirsch *et al*, 1999; Persad *et al*, 2001). Laser vaporization is associated with more perioperative pain and bleeding, while cryotherapy is associated with more perioperative flushing, malodorous discharge, and difficulties performing adequate colposcopy at follow-up (Martin-Hirsch *et al*, 1999). The main drawback with ablative procedures is that no specimen is provided for histological diagnosis, which means follow-up for these patients must be well-organized.

2.3.4.2 Conization

Excisional therapy with various methods has the advantage of providing a specimen for histological examination, but the disadvantage of often requiring general anesthesia, as well as more education and training. Colposcopy and iodine staining are equally used for identification of the transformation zone, i.e. the area to be removed. The cut-out specimen is broad at the base, narrow at the tip, and resembles a cone in shape: hence the name conization. Cold knife (scalpel) conization was the only available

conservative treatment alternative to hysterectomy until the introduction of electrocautery in the 1970s, although most of the diathermy techniques did not enter common practice until the 1980s. Cold knife conization is not associated with thermal artifacts in the resection margins and therefore suitable when invasive or glandular disease is suspected (Martin-Hirsch *et al*, 1999; Wright *et al*, 2007). A 77-95% success rate has been reported for all conization methods as well as for destructive therapies (Martin-Hirsch *et al*, 1999; Mathevet *et al*, 2003). Other conization methods include laser conization and large loop excisional procedure (LEEP) or large loop excision of the transformation zone (L-LETZ or contoured LETZ, C-LETZ) of which the latter two are essentially synonymous, using an electrode of various size and shape to cut out the transformation zone at variable height and width. Laser conization involves special training and high capital cost, whereas LETZ procedures are cheaper and easier to learn. Cold knife conization as opposed to other methods has recently been documented to have worse adverse effects on subsequent pregnancies, such as a five-fold risk of extreme preterm delivery (before 30 weeks of gestation), and a three-fold risk of perinatal mortality, severe preterm delivery (before 34 weeks of gestation), and low birth weight of <2000g (Arbyn *et al*, 2008c). Nevertheless, all excisional methods are associated with increased risk (3-4 times) of preterm delivery and low birth weight: therefore, caution should be exercised when treating fertile women who may wish to become pregnant in the future (Albrechtsen *et al*, 2008; Kyrgiou *et al*, 2006; Sadler *et al*, 2004; Wright *et al*, 2007). Identification of those at risk for invasive disease is imperative to avoid unnecessary surgery.

2.3.5 Follow-up after treatment

2.3.5.1 Risk of recurrent CIN or cancer

Risk of residual, recurrent, or progressive disease after treatment for CIN has been estimated at about 5-15%, regardless of treatment method (Chirenje *et al*, 2001; Kalliala *et al*, 2007; Martin-Hirsch *et al*, 1999; Soutter *et al*, 1997). The increased risk of recurrent CIN persists for at least 25 years (Soutter *et al*, 2006; Strander *et al*, 2007b; van Hamont *et al*, 2006). These women must therefore continue with follow-up after treatment for a protracted period of time, which is costly and resource-demanding. Risk factors to help identify women at the highest risk of recurrence have been identified and discussed. Incomplete excision (especially with positive findings on endocervical curettage), lesion size and severity, as well as old or young age, may all predispose for residual or recurrent disease (Ghaem-Maghami *et al*, 2007; Johnson *et al*, 2003; Lu *et al*, 2006; Paraskevaidis *et al*, 2003; Park *et al*, 2007). Many of these parameters are only surrogate markers for persistent infection, which is why HPV testing has been proposed for use in follow-up.

2.3.5.2 Management alternatives

A number of follow-up protocols have been proposed, including cytology, colposcopy, a combination of the two, and HPV testing at a variety of intervals (Wright *et al*, 2007). The current practice in Stockholm County to follow-up women treated for CIN is to repeat cytological testing with Pap smear at 6, 12, and 24 months after surgery. Women with a CIN2+ lesion and free margins in the cone biopsy continue follow-up every other year. Women with a CIN1 lesion in the cone biopsy return to the three-year

cervical screening program after three consecutive normal smears. In the event of an abnormal smear, colposcopy, biopsy, and re-conization (if necessary) are performed. Thereafter, annual follow-up with cytology is recommended. The national guidelines do not yet include HPV testing before or after surgery, but the coming version will recommend this practice (available online at www.sfog.se). The European guidelines already contain this recommendation (Arbyn *et al*, 2008a) and it is also cost-effective (Coupe *et al*, 2007).

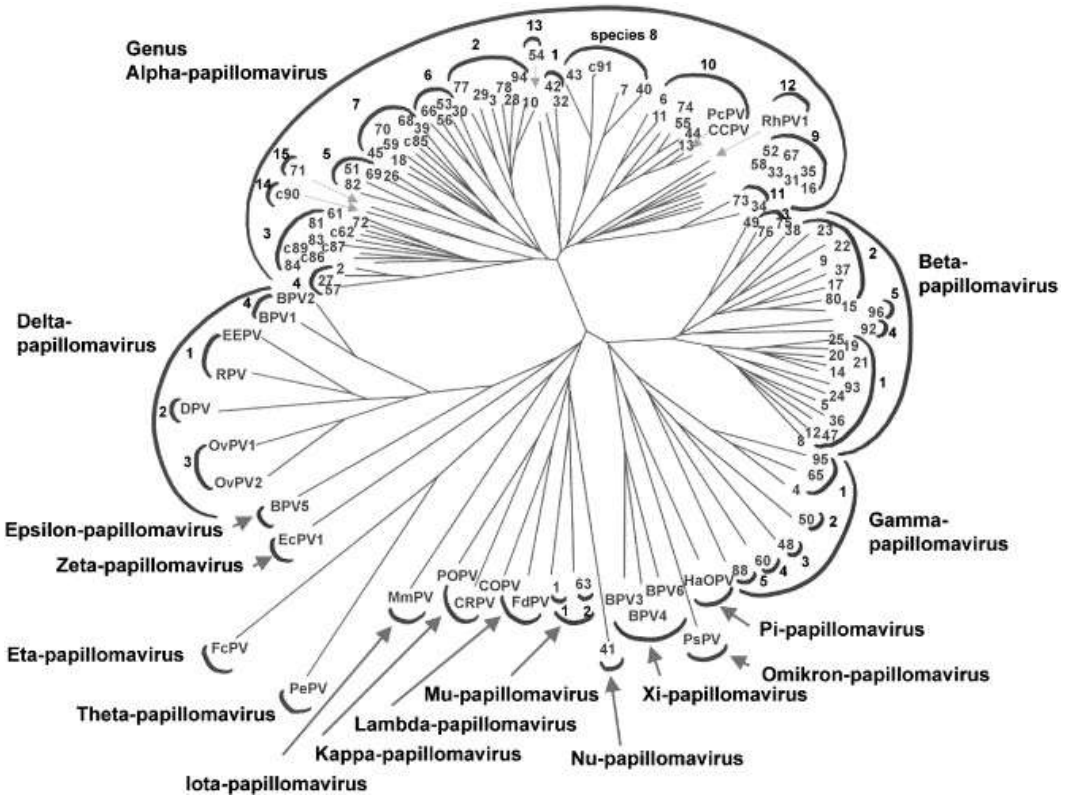
A systematic review by Paraskevaidis and colleagues of studies published 1985-2002, concerning HPV DNA testing in the follow-up period after conservative treatment for CIN, found that a positive HPV test had a sensitivity reaching 100% in several studies, whereas the specificity ranged from 44% to 95%. They concluded that a positive HPV test, even in the presence of normal cytology, may accurately detect a treatment failure at an early point (Paraskevaidis *et al*, 2004). Another meta-analysis of 11 studies from 1996 to 2003 by Zielinski and colleagues compared HR-HPV testing at follow-up with resection margins and cytology at follow-up. They found that HR-HPV testing had a NPV of 98%, resection margins 91%, and cytology 93%. HPV testing and cytology combined provided 96% sensitivity and 81% specificity, while improving NPV to 99%, even though the PPV was only 46% (Zielinski *et al*, 2004). The only randomized trial was published by a Dutch research group from Rotterdam this year, presenting equivalent figures and drawing the same conclusion about a feasible protocol: combined cytology and HR-HPV testing at 6, 12, and 24 months after treatment. Low-risk women may omit the 12-month visit, which results in cost reduction (Bais *et al*, 2009). A different research group from Ghent rebutted that cytology remains the cornerstone in follow-up and prudence is needed, since HPV testing only adds sensitivity when used in the first 6 months of follow-up (Aerssens *et al*, 2009). Most authors agree, however, that cytology and colposcopy may still be required in order to rule out self-limiting HPV-positive lesions and in cases where morphology and virology results are discrepant.

2.4 HUMAN PAPILLOMAVIRUS

2.4.1 Taxonomy and classification

Traditionally papillomaviruses were grouped with the polyomaviruses, including simian virus 40, in one taxonomic family, the *papovaviridae* (Bernard, 2006). Since 2004, they have instead been recognized as a unique family, the *papillomaviridae* (de Villiers *et al*, 2004). Phylogenetic studies suggest that papillomaviruses evolve together with their mammalian and bird host species, do not change host species, do not recombine, and have therefore been stable in their genomic organization for millions of years (Bernard, 2006). Thus HPV is specific to humans and in 2004 over 100 individual HPV types had been fully sequenced and completely described. A newly discovered HPV will be considered a new type if the L1 gene sequence is at least 10% dissimilar from other known types (de Villiers *et al*, 2004). The HPVs are grouped into phylogenetic families (genus) with a common tissue tropism; for example, genital HPVs are called alpha-papillomaviruses. Each family is subdivided into species (sometimes called clades), like the A9 species to which HPV16 belongs (de Villiers *et al*, 2004). Related genotypes are depicted in **Figure 5**.

Figure 5. Phylogenetic tree of HPV (de Villiers *et al*, 2004).



The genital HPVs are further divided into low-risk types (LR-HPV), probable high-risk types (pHR-HPV) and high-risk types (HR-HPV) according to risk for malignant transformation of the genital epithelium. A total of 15 HPV types are classified as HR types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82), three are classified as pHR types (26, 53, 66), and 12 are classified as LR types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108) (Munoz *et al*, 2003).

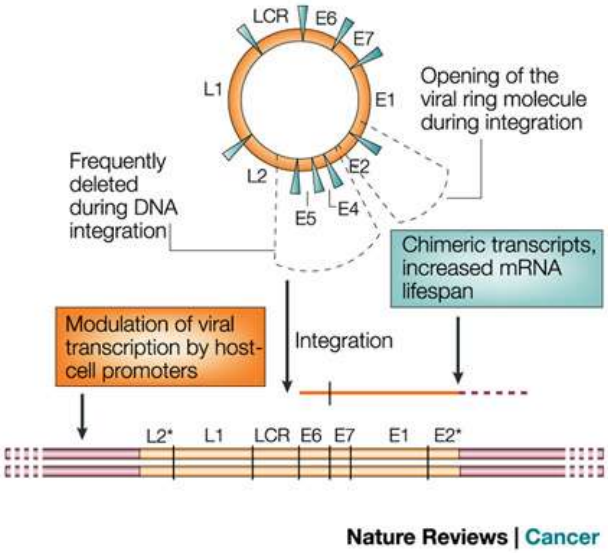
HPV16 and HPV18 are incriminated as causative agents in over 70% of cervical SCC and, together with other HR-HPV types, are found in 99.7% of all cervical carcinomas (Bosch *et al*, 1995; Munoz *et al*, 2003; Walboomers JM *et al*, 1999). Both are classified as human carcinogens (IARC, 2007). The LR types HPV6 and 11 are found in 90% of genital warts (zur Hausen, 2002).

2.4.2 Viral particle

HPV is a small non-enveloped virus with an icosahedral capsid of 50 to 55 nm in diameter. The capsid is composed of 360 copies of the L1 protein arranged in 72 pentamers and 12-72 copies of the L2 protein, believed to be attached to the center of the pentamer (Doorbar, 2006; Lowe *et al*, 2008). The viral genome, which may vary slightly in size depending on HPV type, contains double stranded DNA of 6800 to 8000 base pairs, and carries 8 or 9 open reading frames (ORF) (Doorbar, 2006; zur Hausen, 2002). An ORF is a sequence of bases that could potentially encode a protein. Theoretically double stranded DNA can be read in six different ways or reading frames (three on each strand), but in HPV all ORFs are read on one strand. The genome is episomal (a closed circle) in the capsid and in the infected host cell nucleus.

Linearization and integration of the viral genome into the host cell genome are important steps in malignant transformation (**Figure 6**) (zur Hausen, 2002).

Figure 6. The HPV genome (zur Hausen, 2002).



Eight ORF encoding proteins are known for alpha papillomaviruses: E6, E7, E1, E2, E4, E5, L1, and L2, where E and L signify early and late events in the viral life cycle. The episome also contains a long control region (LCR) with promoters and binding sites for E1 and E2 proteins (Doorbar, 2006). Proteins expressed by different HPV types have specific characteristics (e.g. affinity, conformation) and sometimes differ in function, which may explain why certain types, like HPV16, have particularly high carcinogenic potential. The proteins of HPV16 are described below.

2.4.2.1 Early proteins

The E1 and E2 proteins are important in viral genome replication. E1 is a viral DNA helicase, which unwinds the DNA helix before transcription and replication and also binds cellular DNA polymerase, which is necessary for replication. E2 has several functions both early and late in the viral life cycle. E2 modulates viral gene expression through binding sites in the LCR and co-operates with E1 to locate the origin of replication. E2 also anchors viral episomes to mitotic chromosomes during cell division to ensure correct segregation of viral episomes in stem cell and daughter cell. The E2 ORF is often disrupted during integration in the host cell genome, leading to uncontrolled expression of E6 and E7 (Doorbar, 2006; Munger, 2002; zur Hausen, 2002). E3 has not been found in HPV.

E4 is expressed in large quantities throughout the entire viral life cycle and probably has a pleiotropic role, mainly in the productive phase, and is therefore a rather “late” protein. E4 is transcribed with the E1 initiation codon, usually expressed E1^E4. It facilitates viral genome amplification and capsid protein expression. It also aids in virion assembly and release through interactions with keratin intermediate filaments in the cell skeleton causing collapse and reorganization of the keratin network into a fibrous clump (formation of koilocytes), and by cross-linking elements of the corneous cell envelope, thereby rendering the cell membrane fragile. E1^E4 *in vitro* can induce

premature apoptosis in differentiated keratinocytes, thus enabling release of newly-produced virions into the environment to infect other individuals. E1^E4 is often deleted when the viral DNA is integrated into the host cell genome, which may explain low viral loads in high-grade disease and cancer (Doorbar, 2006; Roberts, 2006).

E5 reduces cell surface expression of human leukocyte antigen (HLA) class I, which helps the virus evade the immune system (Ashrafi *et al*, 2005), disrupts gap junction communication between epithelial cells, and enhances activation of epidermal growth factor receptor leading to a cascade of events causing overexpression of proto-oncogenes and stimulating cell growth. Moreover, E5 can inhibit expression of tumor suppressor gene p21 and impair control of the cell cycle (Tsai & Chen, 2003).

One of the most studied HPV proteins is E6 (reviewed in (Howie *et al*, 2009)). E6 inactivates the p53 tumor suppressor gene product, which is a transcription factor activated by cellular damage that initiates pathways for DNA repair, cell cycle arrest, or apoptosis. Approximately half of human cancers harbor mutations in the p53 gene, illustrating the importance of this single protein. HR-HPV E6 protein binds to the core region of p53 effectively inducing its degradation in the proteasome, blocks the specific DNA binding site of p53, and may relocate p53 to the cytoplasm where it cannot exert its transcriptional function. In addition, E6 enhances cell growth through modulation of G-protein signaling and blocks both the extrinsic (via death receptors and caspases) and intrinsic (via mitochondrial pro-apoptotic Bak) pathways of p53-independent apoptosis. E6 also helps the virus evade innate immune responses by blocking the transcription of interferon-beta (IFNβ) and toll-like receptor 9 (TLR9), which both are needed for the activation of cytokine-mediated immunity. E6 induces genomic instability in several ways, thereby contributing to immortalization of the cell. It activates telomerase, which is an enzyme normally active only in stem cells and embryonic cells adding bases at the 3’ end of the chromosomes, prolonging the telomeres. Telomeres shed a small piece during each replication and cell division, and when telomeres become critically shortened cells are signaled to senesce, which is prevented by the actions of E6. Lastly, E6 – at least *in vitro* – disrupts cell-cell and cell-matrix contact, the cytoskeleton, and apicobasal polarity of the cell, allowing differentiated cells normally destined for desquamation to proliferate.

E6 and E7 acting in concert are the most important early proteins of HPV and are indispensable for hijacking the host cell machinery to enable viral reproduction. These two proteins are uniformly expressed in all cervical cancers, even though viral progenies are no longer being produced. HPV16 E6 and E7 alone are sufficient for malignant transformation of cells (at least in a mouse model) and necessary for maintenance of transformed cell lines, although they act synergistically to increase transforming activity (zur Hausen, 2002). The major effect of E7 is exerted by its interactions with the retinoblastoma susceptibility gene product, pRb. E7 binds to the pRb-E2F protein complex, releasing E2F and inducing proteasome degradation of pRb. E2F is actually a group of major transcription factors regulating G1 exit and S-phase progression; when E2F is dissociated from pRb, S-phase progression is activated in an uncontrolled manner. E7 also upregulates cyclins and cyclin-dependent kinases (CDK), which are promoters of the cell cycle (Martin *et al*, 1998; McLaughlin-Drubin & Münger, 2009). A physiological response to acceleration of cell cycling is to increase

CDK inhibitors, such as $p16^{INK4a}$. The kinase inhibitor $p16^{INK4a}$ prevents pRb phosphorylation, E2F liberation, and induction of cell division. Expression of $p16^{INK4a}$ is regulated by negative feedback from pRb, and the E7-induced degradation of pRb therefore results in overexpression of $p16^{INK4a}$ (Klaes *et al*, 2001; Mulvany *et al*, 2008; von Knebel Doeberitz, 2001). Liberated E2F also plays a role in epigenetic programming, functioning as a histone methyltranferase uncovering sequences for transcription. E7 also silences transcription repressors by inactivating deacetylases. Furthermore, E7 inhibits transforming growth factor beta (TGFβ), normally a potent inhibitor of epithelial cell growth, tumor necrosis factor alpha (TNFα), as well as interferon alpha and gamma (IFNα and IFNγ), all important mediators for immune defense specific to viruses (McLaughlin-Drubin & Münger, 2009). E7 also induces chromosomal instability in a multitude of ways, such as aneuploidy due to supernumerary centromeres and anaphase bridges (Duensing & Münger, 2004).

2.4.2.2 Late proteins

The L1 protein (55 kDa) is the major component of the viral capsid expressed only in the upper cell layers of the epithelium and preserved only in non-transformed cells. L1 plays a key role in viral entry into the epithelial cell (Day & Schiller, 2006). The L1 protein can self-assemble into virus-like particles (VLP) and is highly immunogenic (Kirnbauer *et al*, 1992), which has facilitated the development of HPV vaccines. L1 is highly variable and differs among HPV types, which may be the reason for limited natural cross-protection.

L2 is a larger protein (74 kDa), but nevetheless a minor component of the capsid. L2 facilitates viral entry into basal cells and enables movement of the endosome that contains viral DNA toward the cell nucleus. L2 is also essential for virion assembly *in vivo* (Pereira *et al*, 2009). It has immunogenic surface epitopes conserved across HPV types, and is thus a suitable candidate for a broad-spectrum second generation of HPV vaccines, although much less immunogenic (Lowe *et al*, 2008).

2.4.3 Life cycle

Infection by papillomaviruses is thought to occur through microwounds of the epithelium that expose cells in the basal layer to viral entry. Individual HPV virions interact with the epithelial cell surface primarily dependent on the L1 protein, since this alone can mediate cell entry, although further studies have shown that L2 facilitates this process *in vivo* (Day & Schiller, 2006). L1 binds to heparan sulfate proteoglycans (HSPG), which are widely expressed on the keratinocyte surface and are known receptors for other viruses and bacteria (Joyce *et al*, 1999). Initially alpha-6-integrin was proposed as the HPV receptor by Evander and co-workers (Evander *et al*, 1997), although it was subsequently shown that this receptor was not necessary for entry. A two-step process has been proposed, in which L1 binds HSPG non-specifically and may undergo conformational changes, thereby uncovering previously hidden L2 sequences (Day & Schiller, 2006; Pereira *et al*, 2009). Subsequently L2, or more likely L1, binds to a secondary receptor, possibly alpha-6-integrin, which is followed by internalization in a clathrin-coated vesicle (Day *et al*, 2003) and possible transfer to a caveolin vesicle (Bousarghin *et al*, 2003; Smith *et al*, 2008), where viral DNA is uncoated and released into the endoplasmatic reticulum, from which it proceeds to the

nucleus. Tissue tropism is not only defined by mechanisms of entry, since HPV can enter other cells than keratinocytes, such as immune cells. The Evander group recently found an association between cell tropism and surface net charge of the virion, which differ between HPV genus (Mistry *et al*, 2008).

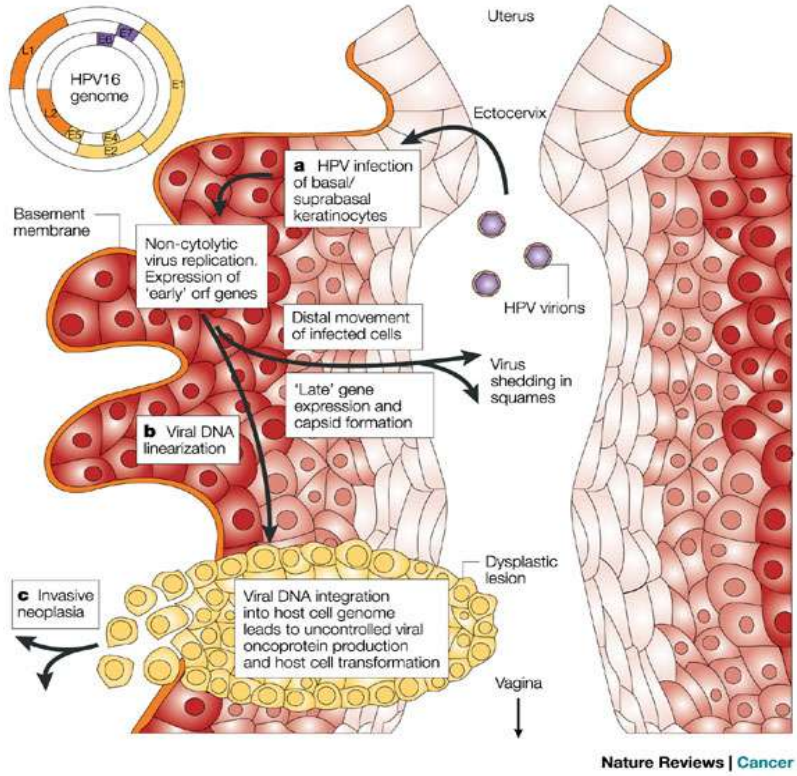
Replication takes place in differentiating epithelium (Longworth & Laimins, 2004). The mechanism by which HPV replication depends on epithelial differentiation remains elusive. HPV infects only basal cells or transit amplifying cells (product of division of stem cells) that pass through the early prophase of mitosis which is necessary for successful onset of transcription of the HPV genes (Pyeon *et al*, 2009). E2 transcription ensures equal distribution of viral copies in the divided cells, while E6 and E7 cooperate to inhibit the daughter cell from exiting the cell-division cycle and it remains poised in G_1. Once the infected cells reach the suprabasal layer, late viral proteins are then produced, and the genome undergoes one or two logs of amplification. Capsid proteins are recruited and virions are assembled through collaboration with cellular proteins in the host cell nucleus (Graham, 2006; Longworth & Laimins, 2004). Inhibitory RNA elements encoded in the L1 and L2 regions carefully regulate the expression of capsid proteins to take place only in the upper epithelial layer, which is beyond the reach of the host immune response (Graham, 2006). Epithelial cells laden with virions are sloughed off the surface and virions are probably released by a combination of "natural" cell disintegration and the effect of E1^E4 on the cell membrane.

2.4.4 The role of HPV in malignant transformation

Persistent infection with one of 12-13 HR-HPVs is the initial prerequisite for inducing near all cervical cancers (Munoz *et al*, 2003). Three viral proteins, E5, E6, and E7, have proliferative properties. However, only E6 and E7 are indispensable for malignant transformation and necessary for maintenance of transformed cell lines (Munger *et al*, 1989). The effects of E6 and E7 from certain HPV types (HPV16, 18 and 31 most extensively studied) on p53 and pRb are hallmarks of HPV carcinogenesis (zur Hausen, 2002). The deregulation of host cell replication with accumulation of mutations and genomic instability leads to stepwise transformation into a cell with malignant properties.

In high-grade CIN and cancer, expression of viral genes other than E6 and E7 is nullified, most likely due to integration of the viral genome into the host cell genome. The initial event in this process is disruption of the viral genome, typically at the E1/E2 ORF site, causing loss of important regulatory functions of these proteins; for example, expression of E6 and E7 accelerates (**Figure 7**) (Romanczuk & Howley, 1992). The viral genome is inserted at random and presumably at fragile sites in the host genome (Wentzensen *et al*, 2004). Loss of E1/E2 expression relates to grade of dysplasia (Cricca *et al*, 2009; Kalantari *et al*, 1998), as does the expression of E6 and E7, and the physical state of the viral genome (Andersson *et al*, 2006a; Hudelist *et al*, 2004; Kraus *et al*, 2006; Sathish *et al*, 2004).

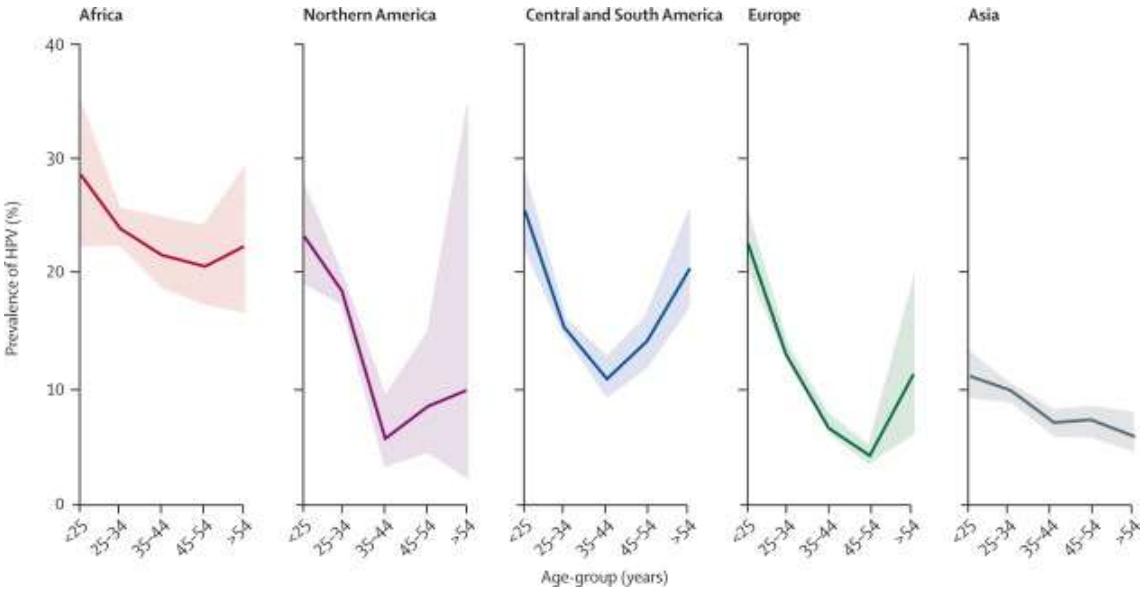
Figure 7. Events leading to malignant transformation (Tindle, 2002).



2.4.5 HPV epidemiology

HPV is one of the most common sexually transmitted infections in the world. Prevalence of HPV is highly dependent on geographic location and age group, but at any given time the average prevalence in women with normal cytology is estimated at 10% (**Figure 8**) (Bosch *et al*, 2008; de Sanjose *et al*, 2007).

Figure 8. Age-specific HPV prevalence (de Sanjose *et al*, 2007).



An IARC study on HPV prevalence in more than 15 000 women on four continents showed that age-standardized HPV prevalence varied nearly 20-fold among populations, from 1.4% in Spain to 26% in Nigeria (Clifford *et al*, 2005). Overall

prevalence of HR-HPV was 6.1% and LR-HPV (as LR infection alone) 2.5%. HPV16, at about 20%, was the most prevalent type and twice as common as the most prevalent LR type, HPV42, at about 9%. The increase in HPV prevalence among older women in Europe and America may be related to common sexual culture (divorce and new partners), genetic factors, or HPV variants.

The ARTISTIC trial group published data from a selected low-risk population in which over 24 000 British women underwent HPV testing in conjunction with cytologic screening. The most common genotype at all ages was HPV16 (overall prevalence 3.3%), followed by HPV types 52 (1.5%), 18 (1.3%), 31 (1.3%), 51 (1.2%), and 39 (1.1%). A marked decline was observed in the prevalence of HR-HPV with age, both overall and for each HPV type. In women under 30 years, 27% were HR-HPV positive, compared with an average of 6% for women 30 years or older (Sargent *et al*, 2008). In Sweden, the prevalence of HR-HPV for middle-aged women (32-38 years old) in the general screening program was reported to 7% and for HPV16 2.1% (Forslund *et al*, 2002). Among non-participating women who agreed to send a home-test swab to the laboratory, the prevalence of HR-HPV was 26% (Stenvall *et al*, 2007). The relative prevalence of LR-HPV compared with HR-HPV differs between continents and age groups, with increasing LR-HPV and decreasing HR-HPV prevalence among older women (Bosch *et al*, 2008; Franceschi *et al*, 2006; Herrero *et al*, 2005).

The incidence of HPV varies less with respect to oncogenic potential, age, and geographic location than does prevalence. The incidence for various types of HR-HPV was estimated at 9.3 per 1000 women-months and was similar for LR-HPV types (8.2/1000) among Hawaiian women aged 18-85 years (Goodman *et al*, 2008), while incidence of HR-HPV was 14.0 per 1000 women-months and 12.4 for LR-HPV among female university students in Montreal (Richardson *et al*, 2003). In the Ludwig-McGill cohort, which enrolled Brazilian women (mean age 33 years), the incidence was 6.1 for HR-HPV and 5.0 for LR-HPV infections per 1000 women-months (Trottier *et al*, 2008). For a cohort of Colombian women aged 18-85 years, the incidence of HR-HPV was significantly higher than in the other studies and higher than that of LR-HPV (5.0 vs. 2.0 cases/100 woman-years) (Munoz *et al*, 2004). HPV16 usually has the highest incidence, reported to be about 5 per 1000 women-months (Giuliano *et al*, 2002; Insinga *et al*, 2007; Richardson *et al*, 2003). In one frequently cited study by Syrjänen and colleagues, crude annual incidence was 7.0%, while the estimated life-time risk was as much as 79% in the Finnish females for contracting at least one HPV infection between the ages of 20 and 79 years (Syrjanen *et al*, 1990).

Prevalence is a function of incidence and duration: therefore differences in duration may explain differences in prevalence. Long duration also increases the probability of spreading infection and malignant transformation. Infection with HR-HPV is associated with a longer mean duration than infection with LR-HPV, reportedly 9-18 months compared with 4-10 months (Giuliano *et al*, 2002; Insinga *et al*, 2007; Munoz *et al*, 2004; Syrjanen *et al*, 2005; Trottier *et al*, 2008). HPV16 and co-infections with multiple types tend to have a longer duration than infections with a single type (Insinga *et al*, 2007; Trottier *et al*, 2008). Some studies indicate differences in transmissibility between HR and LR types, with a strong correlation between prevalence of HR-HPV

and number of sex partners, but none or only a little for LR-HPV (Herrero *et al*, 2005; Kjaer *et al*, 1997; Rousseau *et al*, 2000).

2.4.6 Methods of detection

2.4.6.1 Polymerase chain reaction (PCR)

The two currently relevant HPV detection methods are PCR and the Hybrid Capture (HC) test. PCR has very high analytic sensitivity and is able to detect as few as 10 copies of HPV genomic DNA in a few microliters of specimen. This method selectively targets a DNA sequence through a set of consensus (GP5+/6+, PGMY09/11) primers directed at a conserved region within the L1 gene, and is able to detect virtually all mucosal HPV types (Garland & Tabrizi, 2006; Iftner & Villa, 2003).

The PCR reaction usually involves 20-40 cycles of amplification. Initially the sample is heated to 95ºC to dissociate the DNA strands, after which the primers hybridize to a complementary single strand in the L1 region (annealing) and initiate polymerization via a heat-stable DNA polymerase. This process is repeated cyclically, yielding about one billion copies after 30 thermal cycles (a theoretic doubling of DNA in each cycle). The amplicons (products of amplification) have different lengths depending on the primer target, which can affect ability to distinguish between individual HPV types in subsequent analyses. After PCR, the amplicon can be used for genotyping by sequencing or hybridization with type-specific probes using dot blot, southern blot, microtiter ELISA, reverse line blot strip technique, or microchip assays (Garland & Tabrizi, 2006).

Commercially available PCR based detection systems include Amplicor and Linear Array (LA) by Roche, Basel, Switzerland. Amplicor allows detection of 13 HPV types in a cocktail (similar to HCII), although not able to perform genotyping. The Amplicor test has 93-95% sensitivity for detecting HSIL/CIN2+, which is equal to that of HCII (Mo *et al*, 2008; Monsonego *et al*, 2005).

LA allows detection and typing of 37 HPV types using a nylon strip covered with bands of immobilized type-specific oligonucleotides. The method is more sensitive than some other genotyping methods, but is not yet fully optimized for high-throughput analysis. At the Virology Department of Karolinska University Hospital, Huddinge, the LA assay, including PCR, takes two days to complete, running 24 to 48 samples in one assay.

LA correlates well with other HPV detection methods. In a study comparing LA with sequencing in a cohort of 102 HPV-positive women (by PCR), a concordant single genotype was found in 93 (91.2%) of them and only one sample was negative by LA, but positive by sequencing (Giuliani *et al*, 2006). Because most samples contain multiple HPV types, which cannot be resolved by sequencing, this method is not feasible for routine typing. Compared with line blot hybridization for genotyping in the large ASCUS LSIL triage study (ALTS), LA had higher sensitivity (about 90%) and lower specificity (about 50%) for 2-year cumulative CIN2+ or CIN3+ findings, although differences were small (Castle *et al*, 2008). LA has also been validated for use on archival specimens with satisfactory results (Woo *et al*, 2007).

Luminex-based HPV genotyping is a novel technique that combines PCR amplification with hybridization to fluorescence-labeled polystyrene bead microarrays. The detection limit for the various HPV types is above 500 plasmids and the technique shows excellent agreement with an established microarray chip (Oh *et al*, 2007).

2.4.6.2 Hybrid capture

HC is a high-throughput, semi-automated test approved by the FDA. HCII is a refinement of HCI, and permits detection of 13 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and 5 LR-HPV types (6, 11, 42, 43, 44) at a cut-off limit of 5 000 HPV genomes per reaction. The different probe cocktails of LR- and HR-HPV are used in separate reactions. Up to 176 samples can be processed by routine manual operation over a 5-6 hour period or 354 specimens by a robot in the same time. HCII uses long single-stranded RNA for hybridization with HPV DNA in the sample and for capture on microplates covered with immobilized antibodies specific for RNA-DNA hybrids. The method has a high median sensitivity of 94% for detecting CIN2+, although it does exhibit some cross-reactivity with other HPV types, including LR types, which lowers its specificity (Lörincz, 2006).

HCII and LA have been compared in large studies. In one by Monsonego and colleagues, 575 women referred for colposcopy because of abnormal Pap smears were analyzed with LA and HCII and the results compared for various histological outcomes. HCII and LA were 88.1% concordant. In detecting CIN2+ and CIN3+, LA was 5% and 6% more sensitive, but 9.5% and 8.7% less specific than HCII (Monsonego *et al*, 2008). In the ALTS trial more than 3000 paired LA and HCII results were compared for detection of HR-HPV and 2-year cumulative CIN3+ findings in cervical biopsies. They found that LA was more likely to give a positive test for carcinogenic HPV than HCII (55% versus 53%; p=0.001). For 2-year cumulative CIN3+, both methods had similar sensitivities (93%), and LA was marginally less specific than HCII (48% versus 51%). Both had similar NPV (99%), and LA had a slightly lower PPV than HCII (14.6% versus 15.1%) (Gravitt *et al*, 2008b).

In conclusion, HCII and LA are virtually equal in performance, although HCII is more suitable for screening and triage in large-scale laboratories, whereas LA is needed for genotyping in studies of true HPV persistence and evaluation of vaccination effects.

2.4.6.3 Other methods

DNA sequencing can be regarded as the gold standard for HPV genotyping. This can be done directly following the PCR reaction, or after cloning of amplified fragments (reviewed in (IARC, 2007). To detect HPV DNA in routinely processed and fixed tissue or smears, in situ hybridization can be used. Type-specific HPV DNA probes are applied to tissue sections or smears, allowing spatial localization of the genome. A quantitative method is provided by real-time PCR, detecting the release of fluorescence from the breakdown of a dual-labeled specific probe during each amplification cycle. One hypothesis claims that a large burden of viral infection, i.e. high copy numbers (viral load) is associated with higher risk for cervical cancer (Josefsson *et al*, 2000; van Duin *et al*, 2002; Ylitalo *et al*, 2000).

Infection with HPV can also be demonstrated by antibody detection. Serological assays have been greatly facilitated by the finding of self-assembled VLPs in the nuclei of cells infected with recombinant vaccinia or baculoviruses expressing L1 (Kirnbauer *et al*, 1992; Rose *et al*, 1993). VLPs are used as a coating antigen in enzyme-linked immunoabsorbent assays (ELISA), where they bind capsid-specific antibodies (patient serum), which are subsequently detected by anti-human antibodies linked to an enzyme generating color change or fluorescence when substrate is added. Serological detection of HPV infection lacks sensitivity since not all HPV infections generate an antibody response. An estimated 65-75% of all PCR-positive cases have detectable corresponding serum HPV antibodies (Kjellberg *et al*, 1999). In the footsteps of HPV vaccine development, serological techniques have been improved. For example, a multiplexed assay, using hybridization to fluorescence-labeled polystyrene beads (Luminex), has been developed that can accurately and with high sensitivity simultaneously quantitate neutralizing antibodies to HPV6, 11, 16, and 18 in only 50 µl of serum (Opalka *et al*, 2003).

2.5 MOLECULAR MARKERS OF CARCINOGENESIS

It is widely accepted that cervical cancer evolves through a series of precursor lesions and the main goal of cervical cancer screening programs is to identify women who would benefit from medical intervention, while sorting out women who would not benefit from such intervention. Therefore, such a screening test should have high sensitivity as well as high positive and negative predictive values to identify women with lesions that will not regress spontaneously. The test should target some semi-initial event during malignant transformation when the point of no return has been reached. Essentially two types of markers can be analyzed: viral markers and host cell markers.

2.5.1 Viral markers of carcinogenic activity

2.5.1.1 E6/E7 mRNA

The viral proteins E6 and E7 are not as easy to detect as their mRNA. Pretect Proofer (Norchip, Klokkarstua, Norway) is a commercially available test developed by a Norwegian company to measure E6 and E7 mRNA from HR-HPV16, 18, 31, 33, and 45 (Kraus *et al*, 2006; Molden *et al*, 2007). Expression of oncogenic mRNA measured as a positive Pretect Proofer test identifies 78-85% of women carrying LSIL/ASCUS lesions who are at risk of progressing to CIN2+ within 18-24 months, with 60-85% specificity (Molden *et al*, 2005b; Szarewski *et al*, 2008; Varnai *et al*, 2008). Thus E6/E7 mRNA detection is proposed to triage samples with ambiguous results on cervical cytological screening and has already been implemented in some parts of Norway (Molden *et al*, 2005a). However, since there are still questions remaining regarding sensitivity, as well as stability over time of the expression of these oncogenes, it is probably premature to suggest the use of this test for triage purpose.

2.5.1.2 HPV variants

Sequencing of the HPV16 genome has revealed numerous natural variants (Yamada *et al*, 1997). A variant is a subtype that differs from the consensus sequence in at least one amino acid. It has been proposed that certain variants of HPV16 and 18 are more

carcinogenic than others, since some have been observed with increasing frequency in cervical cancers (Andersson *et al*, 2000). The variants have been named according to geographic relatedness (European, African, Asian-American, etc) with greater risk (2-3 times) for development and recurrence of cervical lesions from non-European variants (Xi *et al*, 1997; Xi *et al*, 2007). Others have observed greater risk with European variants (Grodzki *et al*, 2006). Since there is no consistency in finding a limited number of variants suitable for screening, this approach probably will not come into large-scale use.

2.5.1.3 Integration status of the viral genome

Integration of the viral genome into the host genome is believed to be a random event that occurs during cellular repair of double stranded DNA breaks. Integration occurs at fragile sites in virtually all chromosomes and is unique to each precancerous lesion and tumor. This phenomenon has been observed in severe precursor lesions and cervical cancer with frequencies up to 90% (Cullen *et al*, 1991; Hudelist *et al*, 2004). However, episomal DNA has been observed in cancers and integrated DNA has been found in low-grade lesions, making integration a rather insensitive and unspecific marker, using regular non-uniform clinical samples (Kulmala *et al*, 2006; Sathish *et al*, 2004). Together with laser capture dissection of biopsies these types of assays might give more clear-cut results. Nevertheless, the integration site is still a unique fingerprint and has been found in all cells clonally related to the original lesion and can be used as a specific tumor marker for recurrence detection (Wentzensen & von Knebel Doeberitz, 2007).

2.5.1.4 Viral load

One potential problem with HPV testing is overdiagnosis due to low specificity; quantification of viral load might be an important novel screening tool-approach to optimize the performance of HPV testing (Gravitt *et al*, 2008a). High HPV16 viral load has been established as a risk factor for CIN (Gravitt *et al*, 2007; Hesselink *et al*, 2009; Josefsson *et al*, 2000; van Duin *et al*, 2002; Ylitalo *et al*, 2000) and invasive cervical cancer (Moberg *et al*, 2005). An association between high viral load and persistence of HPV infection has also been postulated (Munoz *et al*, 2009). The association between other common HR-HPV types, such as HPV18, and risk of SCC has still not been adequately studied. Given a positive relationship between viral load and persistent HPV, high viral load may possibly be useful as a disease marker for women at increased risk for cervical disease, but low viral load may also be useful in identifying women at low risk of developing invasive disease(Gravitt *et al*, 2008a). This would however not apply to high-grade CIN and cancer, in which viral progeny production is low.

2.5.2 Host cell markers

2.5.2.1 Chromosomal aberrations

Deregulated expression of E6 and E7 induces various chromosomal aberrations demonstrated in both precancerous lesions and cervical cancer. Aberrations are increasingly common with progressive disease and have been found in 19% of CIN1 and 90% of CIN3 lesions (Wentzensen & von Knebel Doeberitz, 2007). Aneuploidy is

seen with increasing frequency in higher grade lesions. Chromosomes lost 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 to high-grade CIN and 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 not yet been evaluated in the setting of large-scale screening, nor for triage of low-grade lesions.

2.5.2.2 Expression of cellular proteins

The effects of the viral oncoproteins E6 and E7 on host cell gene expression and protein synthesis has also been evaluated as possible progression markers. The HPV-Pathogen ISS study focused on systematically analyzing 13 different cellular proteins involved in regulation of apoptosis and proliferation in HPV-positive and negative women (Branca *et al*, 2004b). In a multivariate analysis, they concluded that in order to identify CIN2+, the best balance between sensitivity and specificity was obtained by combining the two most powerful predictors: vascular endothelial growth factor C (VEGF-C) and 67-kDa laminin receptor (LR67), providing 86% sensitivity, 93% specificity, 99% PPV, and 43% NPV. Many of the possible markers have not been evaluated for clinical use.

The most studied and most promising cellular biomarker is $p16^{INK4a}$ (von Knebel Doeberitz, 2001). As described above, $p16^{INK4a}$ is a CDK inhibitor, which is overexpressed in both CIN and cancer in response to the effects of E7 (Klaes *et al*, 2001). Immunostaining of histological samples with $p16^{INK4a}$ has been in use since the 1990s and was recently reviewed by Mulvany and colleagues (Mulvany *et al*, 2008). They specifically pointed out the fact that $p16^{INK4a}$ is a good marker to help diagnose CIN3, but is not specific for HR-HPV infection or exclusive for pathological tissue. The HPV-Pathogen ISS study group reported that the $p16^{INK4a}$ staining intensity showed linear variation with lesion grade, and that $p16^{INK4a}$ staining showed 100% specificity as an indicator of CIN, with 100% PPV. Furthermore, it showed 84% sensitivity for detecting HR-HPV, with 80% PPV. However, $p16^{INK4a}$ staining did not predict clearance or persistence of HR-HPV after treatment of CIN, nor survival of women with SCC (Branca *et al*, 2004a). This conclusion was contradicted by Wang and colleagues, who found that $p16^{INK4a}$-positive initial biopsies had a mean time to CIN3 or cancer of 64 months compared with 122 months in $p16^{INK4a}$-negative biopsies (Wang *et al*, 2004).

Using $p16^{INK4a}$ on cytology samples to help detect CIN2+ increases specificity up to 96% (Andersson *et al*, 2006a; Cuschieri & Wentzensen, 2008; Meyer *et al*, 2007; Murphy *et al*, 2003; Negri *et al*, 2006; Wentzensen *et al*, 2005; Yoshida *et al*, 2004), although in some cases with too much loss in sensitivity as well as increased costs (Carozzi & al, 2006; Duncan *et al*, 2008). A comparison of several triage tools, showed

that $p16^{INK4a}$ had the highest PPV at 53% (Szarewski *et al*, 2008). It may be particularly helpful in sorting out discrepancies between cytology and histology, as well as in improving correct diagnosis of ASCUS (Monsonego *et al*, 2007; Nieh *et al*, 2005). Few studies have evaluated $p16^{INK4a}$ staining in predicting outcome of CIN. One study analyzed 100 CIN1 cases, 50 CIN2+ cases, and 50 normal cases retrospectively with minimum follow-up of 5 years and found that the NPV for $p16^{INK4a}$ in predicting outcome of CIN1 was 96%, suggesting a role of $p16^{INK4a}$ in the assessment of CIN1 lesions (Hariri & Oster, 2007). Another group recently demonstrated that $p16^{INK4a}$ overexpression was associated with fourfold increased risk of recurrent CIN3/cancer (Anschau *et al*, 2009).

2.6 IMMUNE RESPONSES AND HPV

The immune system of the mucosa in the female genital tract is uniquely adapted to specialized functions including menstruation, fertilization, implantation, pregnancy, and parturition, while eliminating threatening sexually transmitted and environmental pathogens. Researchers have dedicated great effort to understanding the immunity of the female genital tract over the last decade in the wake of HIV vaccine failures. Research in this field has been redirected "back to basics" to better understand the mechanisms of infection and local immune responses in the vagina, cervix, and/or uterus (Broliden *et al*, 2009). Mucosal immunity can be divided into innate and adaptive responses.

2.6.1 Mucosal innate immunity

Innate immunity incorporates more rapid and primitive responses to pathogen challenge than the adaptive immune response, such as surface defense, cytokine elaboration, complement activation and phagocytic responses (Janeway & Medzhitov, 2002).

2.6.1.1 Surface defense

Surface defense in the cervix comprises a barrier of epithelial cells, interconnected with tight junctions. Epithelial cells secrete mucus, a viscoelastic gel mainly composed of glycoproteins, which physically protects the surface, excludes microorganisms, and provides a medium for effector molecules, such as natural antimicrobial peptides (Quayle, 2002). Antimicrobial peptides, such as defensins and cathelicidins (e.g. LL-37), as well as proteinase inhibitors, such as the secretory leukocyte proteinase inhibitor have been detected in human vaginal secretions and are known to disrupt the membrane of many microbes (Cole, 2006; Hein *et al*, 2002; Shaw *et al*, 2007; Valore *et al*, 2002). Alpha-defensins block HPV infection and seem to recruit dendritic cells to HPV-related lesions (Buck *et al*, 2006; Hubert *et al*, 2007). Beta-defensins are upregulated in HPV-associated anal lesions in men who have sex with men, although the biological significance remains to be clarified (Kreuter *et al*, 2009). Antimicrobial peptides and toll-like receptors (TLR) are the key mediators of the innate immune system (Wira *et al*, 2005).

TLRs are surface-bound pattern-recognition receptors mainly expressed on macrophages and dendritic cells that recognize non-self through pathogen-associated

molecular patterns (PAMP). PAMPs are unique to microbes; they are not produced by the host and invariant among microorganisms of a given class. The best known examples are the lipopolysaccharides (LPS) of gram-negative bacteria and peptidoglycans of gram-positive bacteria (Medzhitov & Janeway, 2002). Activation of TLRs induces a range of intracellular signals via nuclear factor kappa B (NFκB) dependent and independent pathways, to induce cytokine and chemokine responses. Epithelial cells express TLRs, taking active part in the innate defense (Quayle, 2002). The commensal vaginal flora also contributes to protection against pathogens by occupying potential receptors. Lactobacilli metabolize the glycogen released by epithelial cells to lactic acid, resulting in a low pH (3.5-5), and also produce antimicrobial hydrogen peroxide (Quayle, 2002).

2.6.1.2 Cytokines and chemokines

Cytokines are soluble molecules expressed by numerous immune cells and epithelial cells in a paracrine and autocrine fashion to regulate immune cell activities. The dogmatic view divides cytokines into proinflammatory cytokines, which stimulate cell-mediated responses or immune-inhibitory, tumor-permissive cytokines which mediate humoral immunity. Examples of proinflammatory cytokines include interleukin (IL) -2, IL-12, IL-17, and interferon gamma (IFNγ), while examples of antiinflammatory cytokines include IL-4, IL-10, and transforming growth factor beta (TGFβ) (Coffman *et al*, 1991; Mosmann, 1991). IFN-mediated responses are important in the clearance of HPV infections and associated lesions. This is illustrated by the drug Aldara (Imiquimod), which is an agonist of TLR7 that induces IFN secretion. It is effective against genital warts, as well as vaginal and vulvar intraepithelial neoplasia (Iavazzo *et al*, 2008; Moore *et al*, 2001). Other cytokines may also be important in clearing HPV, as will be described later. IL-17 is a cytokine produced mainly by T cells. It induces immune responses in neutrophils, fibroblasts, and epithelial cells, and promotes expression of anti-microbial genes for substances such as lipocalin, defensins, calgranulins, and mucins. Defensins in particular act as natural antibiotics and exhibit chemotactic activity (Gaffen, 2008). IL-17 may be important in the immune response against HPV, since the IL-17 receptor has been demonstrated in cervical epithelium (Ge & You, 2008).

Chemokines are soluble factors, chemoattractants that recruit cells to inflammation sites. One example is the proinflammatory cysteine-cysteine receptor ligand 5 (CCL5) also called RANTES (regulated upon activation T cell normal expressed and secreted), which binds to the CC receptor 5 (CCR5) on T cells, dendritic cells, and epithelial cells. HIV also uses this receptor to enter cells; consequently it is of great interest in HIV research. CCR5 is upregulated in the cervix in various inflammatory states (including cases with HPV) and can be affected by hormones (Patterson *et al*, 1998; Prakash *et al*, 2002).

2.6.1.3 Phagocytes – innate effectors and bridges to adaptive responses

The effector cells of innate immunity: macrophages, dendritic cells, natural killer (NK) cells and neutrophils have been reviewed by Wira and colleagues (Wira *et al*, 2005). **Macrophages** are professional phagocytes eliminating cellular debris and complement-bound pathogens. **Langerhan's cells** are tissue-specific dendritic cells with antigen-

presenting ability residing in both the cervical epithelium and lamina propria. Activation of TLRs on immature antigen-presenting cells (APC) in peripheral tissues plays a crucial role in initiation of adaptive responses. A cascade of intracellular signaling helps the cell to mature and express HLA class II and co-stimulatory molecules, and migrate to secondary lymphoid tissues, where naïve T cells are presented to the antigen, thereby activating and directing them to the infection site. **NK cells** have the ability to spontaneously kill tumor cells and secrete potent cytokines, such as IFNγ, that activate macrophages and mediate antibody-dependent cellular cytotoxicity. **Neutrophils** move along the blood vessels, sampling the walls for signs of infection or inflammation (upregulation of adhesion molecules), access the peripheral tissue in response to various chemokines, and kill pathogens through phagocytosis and release of intracellular granules containing microbicides and toxic oxidative compounds. They also produce cytokines and chemokines that activate and attract T cells, macrophages, and DCs. Lastly, neutrophils also facilitate wound healing and repair functions. Neutrophils are the most numerous leukocytes in cervical secretions. The distribution of cells throughout the female genital tract and expression of cytokines and chemokines varies, in a complex and sometimes contradictory way, with the hormonal cycle, providing selective immune activity at the time of fertilization.

2.6.2 Mucosal adaptive immunity

2.6.2.1 T lymphocytes – generals of adaptive responses

Adaptive immunity is an enhanced and somewhat delayed response to pathogens initially handled by innate responses. It is capable of a rapid response against previously encountered pathogens, when specific immunologic memory has developed. T cells play a central role in the two arms of adaptive immunity, cell-mediated and humoral responses, as reviewed by Stanley (Stanley, 2006b; Stanley *et al*, 2008). T cells recognize antigens that are presented in complex with HLA molecules on cell surfaces. Once activated, they exert cytotoxic effects or induce maturation of B cells. Two major subsets of T cells can be identified by the "cluster of differentiation" surface markers, CD4 and CD8. Brief, CD4+ T cells express the unique T cell receptor and recognize antigen presented by HLA class II, while CD8+ T cells are activated by HLA class I. HLA class I mainly displays endogenous proteins derived from intracellular synthesis and broken down in the proteasome. HLA class II presents exogenous antigen, which is broken down in the endosome for association into the HLA II complex in APCs.

HLA-DR, a subtype of HLA class II, is expressed on APCs and activated T cells. HLA-DR expression is increased in the cervix co-infected with HPV and HIV compared with uninfected controls (Behbahani *et al*, 2007). HLA-DR is composed of an α- and β-subunit. Polymorphism in the β-subunit gene has been identified as a risk factor for HPV infection and cervical carcinogenesis (Hildesheim *et al*, 1998; Kohaar *et al*, 2009b; Madeleine *et al*, 2008).

CD4+ and CD8+ T cells are found in the cervical mucosa with elevated counts in HPV-positive specimens, compared with normal uninfected tissue (Nicol *et al*, 2005). CD4+ T cells predominate in regressing warts and CIN1, both within the stroma and the epithelium (Coleman *et al*, 1994; Monnier-Benoit *et al*, 2006). CD8+ T cells dominate

in invasive cancers (Adurthi *et al*, 2008; Monnier-Benoit *et al*, 2006), although they are obviously not killing tumor cells effectively.

2.6.2.2 Th1 and Th2 lymphocytes and expression patterns

Activated CD4+ T cells secrete cytokines in two major patterns called Th1 and Th2, under the regulation of associated presenting DCs. By secreting IFNγ, Th1 cells create a milieu where cytotoxic CD8+ T cells can mature, and NK cells and macrophages are activated. This generates cell-mediated immune responses and consequently memory T cells (referred to above as proinflammatory). Th2 cells secrete IL-4, IL-13, and other cytokines to help naïve B lymphocytes mature into plasma cells or memory B cells. The plasma cells are responsible for the secretion of antibodies exerting humoral immune responses. A third category of T cells, known as regulatory T cells (T reg) with the phenotype CD4+ CD25+, expresses IL-10 and TGFβ. These cytokines down-regulate cell-mediated immune responses and thus prevent auto-immunity, but also allow proliferation and may even promote tumor growth (Stanley, 2006a).

Previous studies on the local expression of cytokines in healthy women and women with low-grade dysplasia are limited and somewhat contradictory. In two studies, IL-4, IL-10, IL-12, and IFNγ did not appear to correlate with ongoing HPV infection (Gravitt *et al*, 2003; Scott *et al*, 2006). El-Sherif and colleagues observed increased levels of IL-10 and decreased levels of IFNγ and TGFβ in HPV16+ CIN compared to normal HPV-negative tissue (El-Sherif *et al*, 2000; El-Sherif *et al*, 2001). Scott and colleagues recently found that elevated levels of IL-10 *and* IFNγ decreased the risk of developing CIN2+ (Scott *et al*, 2009). They had previously demonstrated a local Th1 response pattern in women who cleared their HPV infection (Scott *et al*, 1999). Not surprisingly, an inflammatory cervical condition is required to increase mRNA expression of Th1 cytokines (Gravitt *et al*, 2003; Patterson *et al*, 1998; Scott *et al*, 2006). In contrast, Th2 response cytokines, including IL-4 and IL-10, are increased in HIV-HPV co-infected women compared with HPV infected alone, or non-infected women, which may contribute to the inability to clear HPV infection in those infected with HIV (Behbahani *et al*, 2007; Crowley-Nowick *et al*, 2000; Nicol *et al*, 2005).

2.6.2.3 Regulatory T cells and immune exhaustion

Regulatory T cells have been implicated to regulate immune responses in chronic viral infections towards favoring of viral persistence. This is done through signals impeding further antiviral activity or by exhausting immune cells (Rouse & Suvas, 2007). Although immune exhaustion is believed to contribute to the persistence of viral infections, one must bear in mind that this is a physiological event that occurs to minimize collateral tissue damage. The programmed cell death receptor PD-1 is upregulated on activated T cells specific for chronic infections such as HIV, hepatitis B and C in humans, but not in T cells specific for non-persisting infections like vaccinia or influenza (Sharpe *et al*, 2007). PD-1 is thus a marker for T cell exhaustion. It is highly expressed in complex patterns with other inhibitory receptors such as the cytotoxic T-lymphocyte antigen 4 (CTLA-4) and lymphocyte-activation gene 3 **(**LAG-3) on exhausted T cells. Blockade of these receptors can reverse exhaustion and improve viral control (Blackburn *et al*, 2009). In monkeys with chronic simian immune deficiency virus (SIV) infection, PD-1 blockade enhanced T cell responses, as well as

memory B cell proliferation, decreased viral load, and prolonged survival (Velu *et al*, 2009). The relation between PD-1 and HPV has not been studied. PD-1 is also expressed on NK cells, B cells, and monocytes. Ligand 1 (PD-L1) is expressed on epithelial cells, which may imply that PD-1 is involved in regulating HPV persistence. Two studies on CTLA-4 provide clinical evidence supporting the importance of immune exhaustion. Polymorphism of the CTLA-4 gene, causing increased levels of the receptor, has been demonstrated in Taiwanese women with HPV16-associated cervical cancer (Su *et al*, 2007). Increased prevalence of circulating CTLA-4+ regulatory T cells has also been demonstrated in cervical cancer patients (Visser *et al*, 2007).

2.6.2.4 B lymphocytes and antibodies

Humoral immune responses are initiated by the T cell-Langerhan's cell complex in secondary lymphoid tissues, such as pelvic lymph nodes and lymphoid follicles within the subepithelial layers of the cervical mucosa, where B cells reside (Johansson *et al*, 1999). Some activated B cells differentiate into immunoglobulin (Ig) producing plasma cells and some into memory B cells.

B cells of most maturation stages are identified by a common surface molecule, CD19 (Anderson *et al*, 1984; Jackson *et al*, 2008). Plasma cells and memory B cells do not express CD19, but plasma cells can be identified by production of Ig and memory cells by CD27 (Agematsu *et al*, 2000; Jackson *et al*, 2008). Immunoglobulins are composed of two light and two heavy polypeptide chains. The light chain is either called lambda or kappa and the heavy chains defining the antibody isotype are called alpha (IgA), delta (IgD), gamma (IgG), epsilon (IgE), or mu (IgM). IgA is the major isotype in external secretions, locally produced and actively transported across the epithelial lining as a dimer adding a secretory component making secretory IgA (sIgA) less susceptible to degradation. The cervix is the main producer of sIgA in the genital tract and levels vary with the menstrual cycle. However, the IgG levels are actually higher than sIgA in cervical mucus. It is not clear whether this is transudated IgG from the circulation or locally produced (Russell & Mestecky, 2002).

CD27 is a type I glycoprotein involved in the transition of memory cells into plasma cells (Agematsu *et al*, 2000; Jackson *et al*, 2008). CD27 has not been studied in the genital mucosa. Knowledge of immunological memory in the genital mucosa is limited. The IL-7 receptor (IL-7R) has been identified on the subset of T cells that survive an acute viral infection and become memory CD8+ T cells or CD4+ T cells (Hand *et al*, 2007; Li *et al*, 2003; Schluns & Lefrancois, 2003). Neither the expression of IL-7R has been studied in the cervix.

2.6.3 Evasion mechanisms of HPV

Many viruses go to great lengths to subvert the immune response, for example herpes simplex virus 6 produces a decoy cytokine, or human cytomegalovirus which produces a protein that interferes with antigen presentation (Tindle, 2002). HPV has evolved to avoid immune recognition via a strict intraepithelial life cycle, without a bloodborne phase, and through minimal viral protein expression in the lower epithelial layers that are patrolled by macrophages and Langerhan's cells. Hiding within the keratinocyte

minimizes exposure to the immune system, although interferon (IFN)-mediated intracellular protection against viral invasion cannot be avoided in this way. As outlined above, HPV16 oncogenes E6 and E7 interfere with IFN signaling by blocking of the IFNβ promoter and the IFNα inducible genes. A preferred codon-usage mechanism minimizes levels of early proteins (E7 most studied) in the basal and parabasal keratinocytes (Tindle, 2002). The viral genes contain codons rarely used by human cells, limiting translation due to low availability of appropriate transfer RNA. E7 also escapes surface presentation by localizing to the host cell nucleus. Furthermore, when Langerhan's cells phagocytose E7, their maturation process is impeded and immature dendritic cells emit tolerogenic signals to T cells carrying E7-specific receptors. In addition, no cytolysis or virion release occurs until the infected squamous cell is sloughed off into the vaginal lumen.

2.6.3.1 Natural immunity

Innate and cell-mediated immunity are the major mechanisms by which humans clear and prevent HPV infection (IARC, 2007), as is supported by several lines of evidence:
1. Most HPV-infected tissues show signs of inflammation (dominated by Th1 cytokines and CD4+ T cells) at the time of regression.
2. Individuals who have genetic or acquired immune deficiencies are at increased risk for persistent HPV infection and subsequent development of cancer.
3. The use of immune modulatory drugs such as IFN and Imiquimod can promote regression of HPV lesions.
4. In animal models with inoculation of viral proteins, clearance is dependent on CD4+ and CD8+ T lymphocytes.

However, humoral immunity by neutralizing antibodies to HPV prevents infection and antibody measurements have become the flavor of the day since the introduction of HPV vaccines. Seroconversion against proteins of the HPV16 capsid is seen within a few months following acquisition of HPV16 DNA (Konya & Dillner, 2001). Antibodies are stable over time, related to life-time number of partners, and can be detected even after 15 years of follow-up (Dillner *et al*, 1996; Shah *et al*, 1997). Prevalence of HPV16 antibodies increased by 4% per partner (reaching 35% in women with more than 5 lifetime partners) (Dillner *et al*, 1996). IgG subclass 1 and IgA are the main antibodies directed against HPV. Antibodies are type-specific, though an IgM response has been demonstrated which may be cross-reactive among different HPV types (Sasagawa *et al*, 1996).

Seroprevalence of HPV antibodies varies over time and with geographic area (af Geijersstam *et al*, 1998), providing information on transmission patterns of HPV in the population. Seroepidemiology can be used as a tool to plan future vaccination programs (Ryding *et al*, 2008), and may also indicate trends in future HPV-related malignancies. Detection of serum antibodies (both IgG and IgA) against L1, E2, E6, and E7 of HPV16 and 18 can be correlated to incidence and prevalence of cervical cancer (Chua *et al*, 1996; Dillner *et al*, 1997; Naucler *et al*, 2007a; Olsen *et al*, 1996; Shah *et al*, 1997). Serum antibodies are indicative of lifetime exposure, while HPV DNA in the cervix is more indicative of recent exposure. It appears that the presence of serum antibodies reflects high-impact contact with the immune system, as would be the case

in progressive disease where the cell-mediated immune response has been insufficient to clear infection and heal the dysplastic lesion. A study looking at serum IgA response to whole native E2 protein (episomal, not disrupted by integration), showed elevation in CIN patients compared with normal control subjects, though no elevation in women with cervical cancer (Rocha-Zavaleta *et al*, 1997). Presence of locally sampled antibodies does not correlate to HPV16 clearance, while systemic IgG is associated with viral persistence and systemic IgA with viral clearance (Bontkes *et al*, 1999). Detection of type-specific IgA in cervical mucus is associated with a concomitant cervical HPV infection and local antibodies seem to correlate with degree of CIN (Dillner *et al*, 1989; Passmore *et al*, 2007; Wang *et al*, 1996). Local sIgA and IgG antibodies are more prevalent and abundant in HPV infected women with CIN (around 27%) compared with HPV infected women without visible pathology (IgA 13% and IgG 7%) (Rocha-Zavaleta *et al*, 2003). Which of these antibodies that is most important to exert natural or vaccine-induced immunity remains to be clarified.

2.7 HPV VACCINES

2.7.1 Prophylactic vaccines

2.7.1.1 Development of vaccines

The first HPV immunization study in humans was published in 1968, when Holinger tried to prevent relapse of laryngeal papillomatosis with an autogenous papilloma vaccine with some success (Holinger *et al*, 1968). Several efforts have been made since that time, but the real breakthrough in HPV vaccine research came in 1992, when Kirnbauer and colleagues demonstrated that papillomavirus self-assembles into VLPs when overexpressed in insect cells and that vaccination with these particles induces titers of serum neutralizing antibodies similar to those generated by native virions (Kirnbauer *et al*, 1992). Several studies in animal models (cow and rabbit) have shown effective vaccination with bovine and canine VLPs and the L1 pentamer, but not denatured L1 protein (reviewed in (Roden & Viscidi, 2006)). The immunogenic properties are dependent on conformational epitopes. L1-based vaccines were discovered to be type-specific, although VLPs of different genotypes with >85% similarity in the L1 gene showed weak cross-neutralization *in vitro* (e.g. HPV18 and 45, HPV6 and 11) (Roden *et al*, 1996; White *et al*, 1998). In 2001, when the first phase 1 trials on HPV11 and HPV16 specific vaccines had been conducted, vaccination with human VLPs in healthy volunteers were proven safe and generated high serum titers of specific antibodies (Evans *et al*, 2001; Harro *et al*, 2001). Subsequently, phase 2 and 3 trials have been conducted by two major pharmaceutical companies and two vaccines are now commercially available: Cervarix is a bivalent HPV16/18 L1 VLP vaccine developed by GlaxoSmithKline Biologicals (Rixensart, Belgium), and Gardasil is a quadrivalent HPV6/11/16/18 L1 VLP vaccine developed by Merck and Co. Inc (West Point, Pennsylvania, USA).

2.7.1.2 Mechanism of protection

Protective mechanisms are not fully understood. The most likely scenario involves a protective effect mediated by high titers of neutralizing serum antibodies elicited by VLPs (Mao *et al*, 2006). The need for neutralizing antibodies to be present at the site of infection to prevent infection remains controversial. In 2003, Nardelli-Haefliger and

colleagues showed that IgG serum titers were much higher than local sIgA antibody titers following vaccination (Nardelli-Haefliger *et al*, 2003). Moreover, they observed that most antibodies in cervical mucus seemed to be transudated IgG from the systemic pool and not locally secreted. HPV is thought to infect basal keratinocytes in genital mucosa and skin through microtrauma, which occurs even during voluntary sexual intercourse. This may increase exposure to systemic IgG and explain the excellent efficacy of the Gardasil vaccine against genital warts (Villa *et al*, 2005). Immunization with VLPs also induces an increased T cell response and a broad-spectrum cytokine response in blood, which is not seen in placebo recipients (Garcia-Pineres *et al*, 2007; Pinto *et al*, 2005; Pinto *et al*, 2003).

2.7.1.3 Efficacy, duration and need for booster

In 2002, the first results on protection against persistent HPV infection were published by Koutsky and co-workers (Koutsky *et al*, 2002) with a stunning 100% efficacy against persistent HPV16 infection (although not transient infection) in a controlled HPV16 vaccine trial of 2392 women. All 9 cases of HPV16-related CIN occurred in the placebo group and none in the vaccine group. Later on, several publications have shown similar efficacy against vaccine-related HPV types and their associated disease (Garcia-Pineres *et al*, 2007; Paavonen *et al*, 2007; Villa *et al*, 2005). The bivalent vaccine demonstrates cross-protection to a limited extent (Harper *et al*, 2006). The HPV-6/11/16/18 vaccine reduces risk of CIN2+ associated with non-vaccine types by 25-45% (Brown *et al*, 2009; Wheeler *et al*, 2009). Evidence even indicates 94-100% efficacy among women with ascertained infection with up to three vaccine HPV types (FUTUREII, 2007). A three-dose regimen of the quadrivalent HPV vaccine is highly efficacious and induces stable anti-HPV levels for at least 5 years. Vaccination also induces robust immune memory, since a booster dose at 5 years after initial immunization was shown to increase antibody titers more rapidly and to higher levels than those seen after the third initial dose (Olsson *et al*, 2007), suggesting that the effect of vaccination will be long-lasting. Immunological memory can also be awakened in women who already have vaccine-type antibodies at baseline, since these women demonstrate a 12-26-fold rise in titer after vaccination compared with women who are vaccine-type HPV naïve at baseline (Villa *et al*, 2006).

2.7.1.4 Implementation of HPV vaccine program

Many countries throughout the world have now implemented public vaccination programs which include preadolescent girls. Vaccination of boys and catch-up vaccination of women up to age 26 is under debate due to issues concerning cost-effectiveness, eventhough data support efficacy in women infected with up to three vaccine HPV types and rising incidence of other HPV-related tumors in men and women (Gillison, 2008; Giuliano *et al*, 2008; Hammarstedt *et al*, 2007; Palefsky, 2008; Robinson *et al*, 2009; Sturgis & Cinciripini, 2007). The Swedish National Board of Health and Welfare decided in December 2008, that HPV vaccination should be introduced in the national vaccination program, and administered to girls age 10-12 (Socialstyrelsen, 2008). However, it will take decades before the effects of HPV vaccination are visible at the population level. A vaccination program cannot replace cytological screening for at least 50 years, primarily because most women who are sexually active today have already been exposed to HPV and therefore will not

benefit from HPV vaccination. Secondly, current vaccines do not cover all oncogenic HPV types. Consequently, there is no doubt that cytological screening with Pap smears combined with HPV DNA testing should continue to be key tools for the prevention of cervical cancer even in the foreseeable future (Franco *et al*, 2006).

2.7.2 Therapeutic vaccines

The purpose of therapeutic vaccines is to eradicate already infected cells by inducing an effector response. Upregulation of HLA class I is needed to present endogenously produced viral proteins in order for cytotoxic T cells to recognize and destroy infected cells. Tumor cells are known to express E6 and E7, which provide an ideal target for the effector cells (Giles & Garland, 2006). The greatest challenge of therapeutic vaccine development is however, that E6 and E7 each have different ways of evading and impeding HLA class I upregulation in infected cells, making them "invisible" to the cytotoxic specific response generated by therapeutic vaccination.

Several strategies have been employed to create E6/E7 vaccines. Using synthetic peptides requires knowledge of the exact HLA-restricted epitope, which is too cumbersome in clinical practice, and has not been effective in clinical studies. Full-length proteins fused with other immunogenic proteins may elicit a stronger immune response than the E6/E7 protein alone. A randomized placebo-controlled study of a chimeric HPV16 L1-E7 VLP vaccine in 39 HPV16 mono-infected women with CIN2-3 showed high titers against L1 and low titers against E7 after 24 weeks and a trend for histological regression to CIN1 in vaccine recipients (39% vs. 25%, ns) (Kaufmann *et al*, 2007). The most promising results come from a study of local administration of a vaccinia vector vaccine composed of modified virus Ankara and HPV E2, which was injected weekly for 6 weeks into the uterus of 36 women with HPV-associated CIN1-3 (Corona Gutierrez 2004). Complete colposcopic regression of lesions was seen in 34 women. All women developed antibodies and a specific cytotoxic response against HPV transformed cells, and half the women cleared the infection. Other strategies include DNA vaccines in which plasmid E6/E7 DNA is administered by gene guns and DC-based vaccines using autologous dendritic cells loaded with E7 protein, though these approaches have not yet shown similar progress (Giles & Garland, 2006).

2.8 POSSIBLE SMALL INTERFERING RNA BASED THERAPY

The 2006 Nobel prize in medicine was awarded Andrew Fire and Craig Mello for their discovery of RNA interference (Fire *et al*, 1998). This natural cellular defense system can be used for specific post-transcriptional gene silencing. The concept has been used to target very specific sequences in the HPV18 E6 mRNA with complementary synthetic small interfering RNAs (siRNA) transfected into HeLa cell cultures (Butz *et al*, 2003), resulting in the reactivation of apoptosis, which could be a very powerful therapeutic technique. SiRNA has also been used to identify cellular target genes for E6/E7, and 648 genes were either up- or down-regulated after siRNA mediated destruction of E6/E7 mRNA (Kuner *et al*, 2007). The most challenging task ahead, is to convert the small therapeutic RNA molecules into drugs, which can be delivered intact to suitable target tissues.

3 AIMS

3.1 GENERAL AIM

The general aim of this thesis is to improve identification of women at risk for developing cervical cancer.

3.2 SPECIFIC AIMS

3.2.1 Paper I

To map the prevalence of HPV types in women of all ages who demonstrate ambiguous or low-grade lesions in cytology reports before implementing a public vaccination program.

3.2.2 Paper II

To assess the utility of HPV genotyping after treatment of high-grade cervical intraepithelial neoplasia and compare the prognostic value of HPV genotyping with conventional predictors.

3.2.3 Paper III

To assess the usefulness of a potential progression marker, $p16^{INK4a}$, in liquid-based cytology and correlate staining intensity with histologically confirmed diagnosis of precancerous lesions.

3.2.4 Paper IV

To map expression of molecules participating in mucosal immune responses among women with asymptomatic HPV infection and compare with uninfected controls as a pilot study of potential progression markers.

4 MATERIAL AND METHODS

4.1 STUDY SUBJECTS AND STUDY DESIGN

Prior to start of any study, ethical approval from the Regional Ethical Review Board in Stockholm, and written informed consent from the study subjects were obtained (ethical permit numbers 006-714-32 and 168/03 (I), 168/03 (II), 04-679/3 (III), 2009/74-32 and 2006/1357-31/4 (IV)).

4.1.1 Paper I

During 2005 and 2006, 6228 women participating in the population-based primary cervical screening program in Southern Stockholm were enrolled, as previously reported in detail (Froberg *et al*, 2008). Minor cytological abnormalities, meeting criteria for cytological classification as ASCUS and LSIL based on the Bethesda system were found in 343 (5.5%) women (Solomon *et al*, 2002), and these were eligible for the study. Koilocytosis without signs of dysplasia were reported as non-pathologic. Age and cytological diagnosis were recorded and HPV genotyping performed.

4.1.2 Paper II

All 95 women who attended the Department of Gynecology and Obstetrics at the Karolinska University Hospital Huddinge, Stockholm, for follow-up after treatment by C-LETZ conization between September 2005 and September 2006, were asked to participate. LBC samples were collected at two post-conization follow-up visits 18-24 months apart. HPV genotyping was performed in the archival cone material and at the first follow-up visit. In the majority of cases, the histopathology and/or Pap smear follow-up were available.

4.1.3 Paper III

We consecutively enrolled 118 women with cytological abnormality of any grade detected through the population-based primary cervical screening program in 2004. The women were referred for extended testing at the Department of Gynecology and Obstetrics at the Karolinska University Hospital Huddinge, Stockholm. The extended testing took place during 2005, two to six months after the initial screening. All women underwent pelvic examination, cytological resampling, and colposcopy. We carried out immunocytological staining of cytological samples and correlated these with cytologic and histologic diagnosis.

4.1.4 Paper IV

The study enrolled 24 subjectively healthy female volunteers between March 2007 and September 2008. All subjects underwent a pelvic exam with collection of samples for cytological diagnosis, HPV DNA analysis, diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhoea*. Each woman had an ectocervical biopsy and blood samples for HIV serology were drawn. Each woman also completed a questionnaire regarding past and current medical and reproductive history, menstrual cycle, contraception, and sexual partner status.

4.2 CYTOLOGICAL SAMPLES

In paper I and III, LBC samples were collected from the ecto- and endocervix with a spatula and a cervical brush. The cells were suspended in PreservCyt fixative and the ThinPrep 2000 Processor (both Cytyc Corporation, Marlborough, MA, USA) was used to prepare slides for cytological analysis by a cytotechnologist and/or pathologist (Linder, 1998). According to Swedish recommendations, cases of koilocytosis without signs of dysplasia were reported as non-pathologic. In paper I, cytological diagnoses were defined according to the Bethesda nomenclature system (Solomon *et al*, 2002), and for statistical reasons grouped into the following categories: within normal limits (WNL), ASCUS, LSIL and HSIL or worse (HSIL+), which included cases of atypical squamous lesions – HSIL cannot be ruled out (ASC-H) and cancer.

In paper II and IV, similarly collected samples were smeared on a glass slide, fixed in 10% ethanol, and air-dried. The slides were then stained according to Papanicolaou (Arbyn *et al*, 2007a). Pap smears were evaluated according to the CIN classification (Richart, 1973), as requested by the Swedish Society for Clinical Cytology. For the purposes of these studies, cytological results in paper II, III, and IV were translated into the Bethesda system (Solomon *et al*, 2002), excluding koilocytosis without nuclear atypia from the LSIL group. After slide preparation, the cytobrush was placed in 1 ml of sterile saline suspension (IV) or PreservCyt fixative (II) for subsequent HPV genotyping.

4.3 COLPOSCOPY AND BIOPSIES

In paper III and IV, all women underwent colposcopy using a Zeiss OMPI colposcope for magnification. In paper III, the ectocervix and distal part of the endocervix were stained with 5% acetic acid. Punch biopsies were obtained from acetowhite areas. When no acetowhite area was observed, a biopsy was obtained at the 12 o'clock position near the squamocolumnar junction and fixed in buffered 4% formalin. The biopsy material was embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin/eosin for histological diagnosis. In paper IV, biopsies were taken from the superior ectocervix avoiding the transformation zone, immediately transferred to RNAlater buffer (QIAgen, Hilden, Germany), and stored at -70ºC for future mRNA extraction and RT-PCR.

4.4 CERVICAL CONES AND BIOPSIES

In paper II, between 2002 and 2006 all women, except one, underwent loop excision electrosurgical procedure using a C-LETZ electrode (Utah Medical Products Inc, Midvale, UT, USA) after a biopsy-verified diagnosis of CIN2 or 3 (Mints *et al*, 2006). One woman with CIN2 was treated with cryotherapy. The cone biopsy from two women could not be retrieved for the study. Following excision, the cone material was paraffin-embedded, sectioned, and diagnosed according to WHO (ICD-10, Geneva 1990) and then grouped for statistical reasons into benign, CIN1, and CIN2+.

4.5 HPV GENOTYPING

In Papers I and II, a 1 ml suspension of PreservCyt fixative was centrifuged and the cell pellet lysed according to instructions in the Total Nucleic Acid Isolation kit (Roche,

Basel, Switzerland). In paper IV, 1 ml of the cytobrush saline suspension was similarly centrifuged and lysed. In paper II, archival cone material was dewaxed with xylene-ethanol. DNA was extracted from these various suspensions with the MagNA Pure LC robot and analyzed with the Linear Array HPV Genotyping Test (LA) according to the manufacturer's instructions (both from Roche, Basel, Switzerland). The use of LA in archival material has been described by Woo et al (Woo *et al*, 2007).

The LA test is a PCR and probe hybridization based genotyping assay covering 37 HPV types including 12 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), 6 pHR-HPV types (26, 53, 66, 68, 73, 82), and 19 LR-HPV types (6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39, CP6108) (Munoz *et al*, 2003). The LA kit provides biotinylated primers within the consensus L1 region of the HPV genome. The typing results are read by comparing the location of the blue lines on a probe strip with a reference guide.

4.6 IMMUNOCYTOCHEMISTRY

In paper III, after removing some of the sample for routine cytology, the remaining cell suspension was prepared using the ThinPrep 2000 Processor (Cytyc Corporation, Marlborough, MA, USA). The slides were post-fixed in acetone for 10 minutes at room temperature and air-dried for a minimum of 30 minutes. All slides were subject to "Heat Induced Epitope Retrieval" using the Epitope Retrieval Solution (Dako, Copenhagen, Denmark) at 95^{o}C for 40 minutes. Staining for $p16^{INK4a}$ was performed in groups of five samples using the CINtec kit (kit code K5339) in the DakoCytomation Autostainer. In each group analysis, a negative and a positive control were used. Counterstaining was subsequently performed according to Papanicolaou in order to facilitate identification of transformed keratinocytes and to avoid counting false positive cells, i.e. metaplastic cells or inflammatory reactive cells. The $p16^{INK4a}$ immunoreactivity was regarded as negative (-) if less than three cells per slide were stained and categorized as increasingly positive (+, ++ and +++) depending on how intense the nuclear staining was. The cytological and histological material was evaluated by different persons and independently of the $p16^{INK4a}$ analysis.

4.7 RNA EXTRACTION

In Paper IV, the cervical biopsies were thawed and disrupted in lysis buffer using a mechanical rotor, and RNA was extracted with the RNeasy kit according to the manufacturer's protocol (QIAgen, Hilden, Germany) yielding 25 µl of RNA in suspension.

4.8 REAL-TIME SEMI-QUANTITATIVE RT-PCR

In Paper IV, RNA was converted in equal dilutions to copy DNA (cDNA) in a single reverse transcriptase reaction using superscript reverse transcriptase (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) and random hexanucleotide primers (Roche, Basel, Switzerland). The cDNA was stored at -20ºC. Amplification of the endogenous control ubiquitin C (UBC), and the markers CD3, CD4, CD8, CD19, CD27, IL-2, IL-12a, IL-4, IL-10, IL-17a, HLA-DRα, TGFβ, IFNγ, PD-1, PD-L1, CTLA-4, LAG3, $IgA_{constant\ heavy}$, $IgG_{constant\ heavy}$, CCR5, CCL5/RANTES, and IL-7R cDNA was performed using the ABI PRISM 7700 sequence detection system and

commercial FAM™ dye-labelled TaqMan MGB probes and primers (Applied Biosystems Inc, Foster City, CA, USA).

UBC was chosen as endogenous control after a dilution series testing UBC, 18sRNA, and GAPDH, which showed that UBC was the most consistently highly expressed gene in our samples. Each sample and control was run in triplicate. Relative quantity of target cDNA was computed using the comparative threshold (Ct) method (Livak & Schmittgen, 2001). Ct values for target cDNA were normalized to ubiquitin C (Ct_{UBC}-Ct_{target}) and amounts of cDNA were computed using ln-transformation (2^{-dCt}). This can be read as number of target gene copies for each copy of UBC.

4.9 STATISTICAL ANALYSES

In all papers, data were analyzed with STATISTICA 6.1 software (Statsoft Inc, Tulsa, OK, USA). Pearson's Chi^2 and Yates corrected Chi^2 or Fischer's exact test (for $n<5$) were used to compare proportions, and Student's t-test to compare normally distributed continuous variables between two groups. All tests were two-sided and the null hypothesis of no difference was rejected at a significance level of $p<0.05$.

4.9.1 Paper I

After plotting proportions of HR-HPV and HPV infected women and multiple infections respectively in each age group, linear or polynomial fit was visually estimated as 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 (Chi^2). 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. This was repeated for centered age and centered age^2 to control for covariation between age and age^2, with the same results. The larger model with a second degree provided better explanation as tested with Nagelkerke R^2 test. Therefore we concluded that the quadratic function $\log(p/1-p)=b_0-b_1x+b_2x^2$ could best describe the relationship between prevalence of HR-HPV in LSIL cases and age, multiple infections and age, and HR/LR-HPV dominance and age. Logistic regressions were also performed using HPV prevalence, HR-HPV prevalence, and multiple infections as dependent variables, and age group and cytological diagnosis as categorical predictors.

4.9.2 Paper II

A composite cytological-histological outcome was defined for the study: LSIL-CIN1 and HSIL-CIN2+, including CIS and AIS. Where no histology was available, the most severe cytological result at inclusion or subsequent follow-up visit was considered as outcome of treatment. Odds ratios, reflecting the association of risk factors with outcomes, were calculated using forward stepwise logistic regression. Treatment failure was defined at two thresholds for disease: presence of a cytological LSIL or a histologically confirmed CIN1+, or presence of cytological HSIL/histological CIN2+, at one of the two follow-up visits. Accuracy parameters for prediction of treatment failure were computed, including sensitivity, specificity, positive and negative predictive value, and diagnostic odds ratio (OR). Women without CIN in the cone were excluded from the computation of accuracy.

4.9.3 Paper III

The Chi^2 exact test for trend and Spearman rank-order correlation test were used to assess correlations between immunohistological staining intensity and CIN grade. Accuracy parameters for prediction of cytological and histological outcomes were computed, including sensitivity, specificity, positive and negative predictive value.

4.9.4 Paper IV

Data were also analyzed using the GraphPad Prism 4.00 software (GraphPad Software Inc, La Jolla, CA, USA). Data were not normally distributed even after logarithmic transformation; therefore the non-parametric Mann-Whitney U test was used to compare two groups, the Kruskal-Wallis ANOVA to compare multiple groups, and Spearman rank-order correlation test to assess correlations.

5 RESULTS

5.1 PAPER I

5.1.1 Cytology results and age distribution

There were 223 cases of LSIL and 120 cases of ASCUS, including three cases of atypical glandular cells of undetermined significance (AGUS). Mean age was 33.6 years (median 32, range 22-60). Women were grouped in 5-year age groups. **Table I:1** shows the age distribution. Mean age of women with ASCUS was 35.7 years and with LSIL 32.4 years (p=0.002). In all, 73% of the women were younger than 40 years. Women with ASCUS were older than women with LSIL; 38% of women with ASCUS were 40 years or older, compared with 21% of women with LSIL (p <0.001, Chi^2).

5.1.2 HPV prevalence

A total of 282 (82%) women tested positive for one or more of the 37 included HPV types. HPV prevalence ranged from 92% in women 20-29 years to 62% in women 40-45 years. Prevalence was 67% in women over 50 years. Age-specific prevalence for all HPV types and only HR-HPV was higher in younger age groups and there was a linear decline with age (p<0.001, logistic regression, **Table I:1**).

HPV was more common in women with LSIL (207/223, 93%) than in women with ASCUS (75/120 women, 63%, p<0.001, Chi^2). Age-specific prevalence of all HPV types in women with ASCUS compared with women with LSIL is shown in upper **Figure I:1**. HPV prevalence was significantly dependent on age group (p=0.03), but did not differ significantly between ASCUS and LSILwithin each age group (p=0.098, logistic regression). However, there is an apparent tendency for increasing discrepancy with lower and especially higher age. Simple Chi^2 tests to compare proportions gave significant differences in all age groups except 30-39 years.

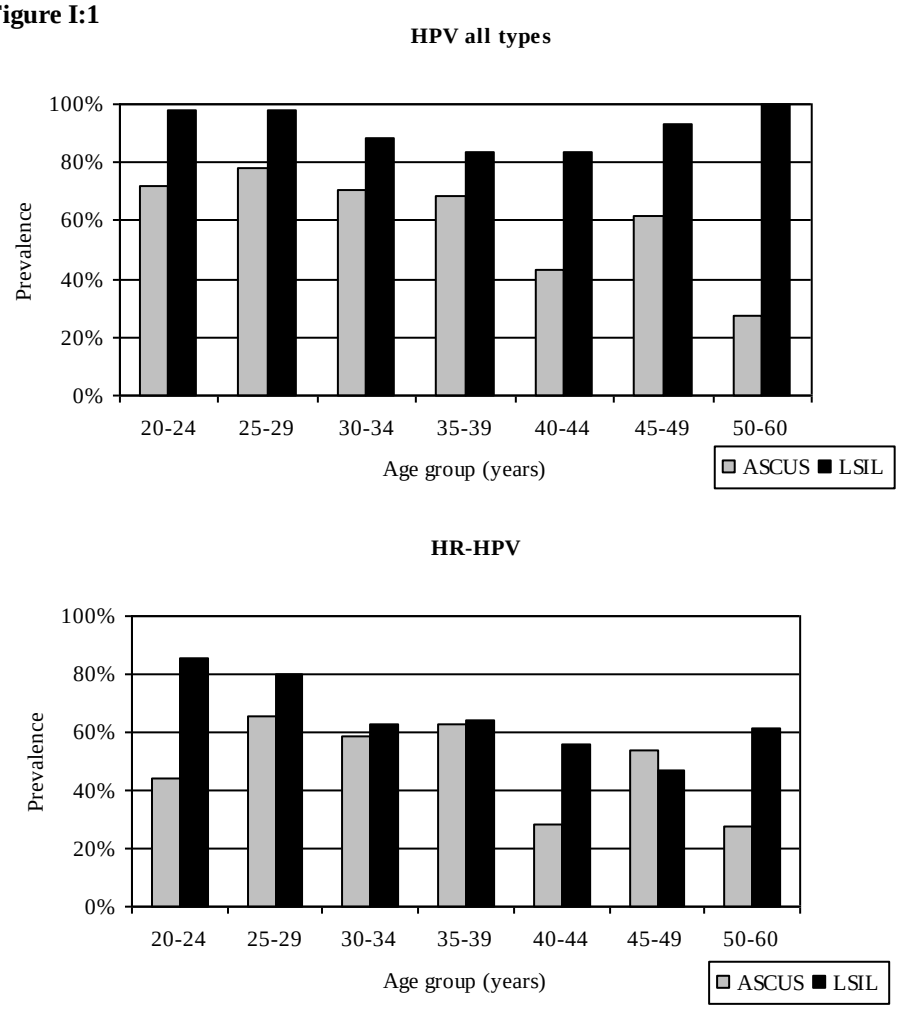
HR- or pHR-HPV was found in 181 (81%) LSIL cases, compared with 68 (57%) ASCUS cases (p<0.001, Chi^2). HR-HPV was found in 59 (49%) women with ASCUS, compared with 158 (71%) women with LSIL (p<0.001, Chi^2). Considering 13 types (12 HR-HPV and pHR-HPV 68), these were found in 60 (50%) ASCUS cases and 159 (71%) LSIL cases (p<0.001, Chi^2). Lower **Figure I:1** shows that HR-HPV prevalence was age-dependent among women with LSIL (p=0.001, logistic regression), with decreasing prevalence until the age of 50. A small increase was found in older women, but this was not statistically significant (p=0.19 for age^2). A linear correlation between HR-HPV prevalence and age was also found for ASCUS cases (**Figure I:1**). Age-specific HR-HPV prevalence did not differ between ASCUS and LSIL cases (p=0.15, logistic regression). A visual trend was apparent showing increasing discrepancy in low and high ages, though simple Chi^2 tests failed to reveal significance in any age group.

Table I:1. Age-specific HR-HPV and multiple HR-HPV prevalence in the study population .

Age group (years)	Total number of women		HR-HPV			HR-HPV and HPV68			Multiple HR-HPV		
	Cytology		Cytology			Cytology			Cytology		
	ASCUS	LSIL	ASCUS N (%)[a]	LSIL N (%)[a]	*P* (Chi^2)	ASCUS N (%)[a]	LSIL N (%)[a]	*P* (Chi^2)	ASCUS N (%)[b]	LSIL N (%)[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

[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

Figure I:1



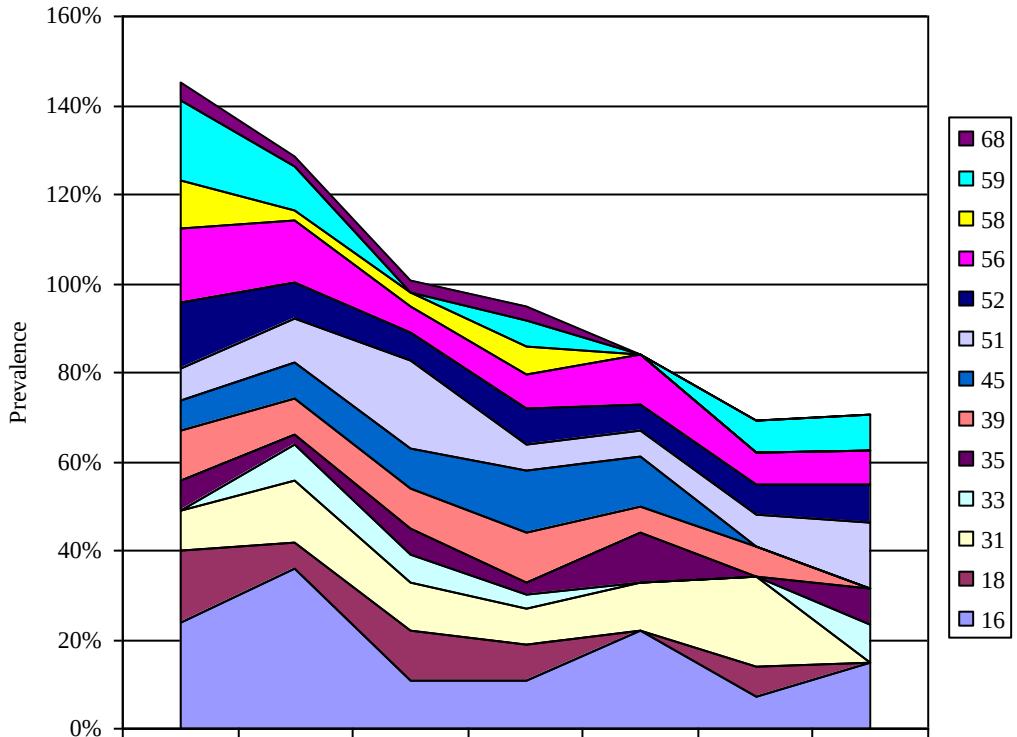
5.1.3 HPV genotype distribution

Figure I:2 shows prevalence of the HR-HPV genotypes (including pHR-HPV68) in each age group. HPV16 was the most common HR-HPV and found in 65 (23%) of the HPV-positive women, ranging from 30% (20/67) in women 25-29 years to 14% (3/22) in women 45-49 years (p=0.22, Chi^2). Remarkably, HPV18 was only found in 28 (9.9%) of all HPV-positive women (p<0.001 compared with HPV16, Chi^2). HPV51, 52, 56, and 31 were also more common than HPV18. HPV51 was equally or more common than HPV16 among women over 45 years.

Among women with ASCUS, the most prevalent HR-HPV genotype was HPV16 (16%), followed by HPV51 and 52 (10%). In women with LSIL, HPV16 was detected in 21%, followed by HPV56 in 11% (**Figure I:3**).

Table I:2 outlines women infected with HPV16, 18, clade 9 and clade 7 HPV types to elucidate the preventive potential of current HPV vaccines against low-grade cytological abnormalities. We found a shift in predominant genotypes with age. The proportion of pHR+HR genotypes decreased with age, following an inverse quadratic function with increasing age (p<0.001, logistic regression).

Figure I:2



	20-24	25-29	30-34	35-39	40-44	45-49	50-60
68	4%	2%	3%	3%	0%	0%	0%
59	18%	10%	0%	6%	0%	7%	8%
58	11%	2%	3%	6%	0%	0%	0%
56	16%	14%	6%	8%	11%	7%	8%
52	15%	8%	6%	8%	6%	7%	8%
51	7%	10%	20%	6%	6%	7%	15%
45	7%	8%	9%	14%	11%	0%	0%
39	11%	8%	9%	11%	6%	7%	0%
35	7%	2%	6%	3%	11%	0%	8%
33	0%	8%	6%	3%	0%	0%	8%
31	9%	14%	11%	8%	11%	20%	0%
18	16%	6%	11%	8%	0%	7%	0%
16	24%	36%	11%	11%	22%	7%	15%

Age group

Figure I:3

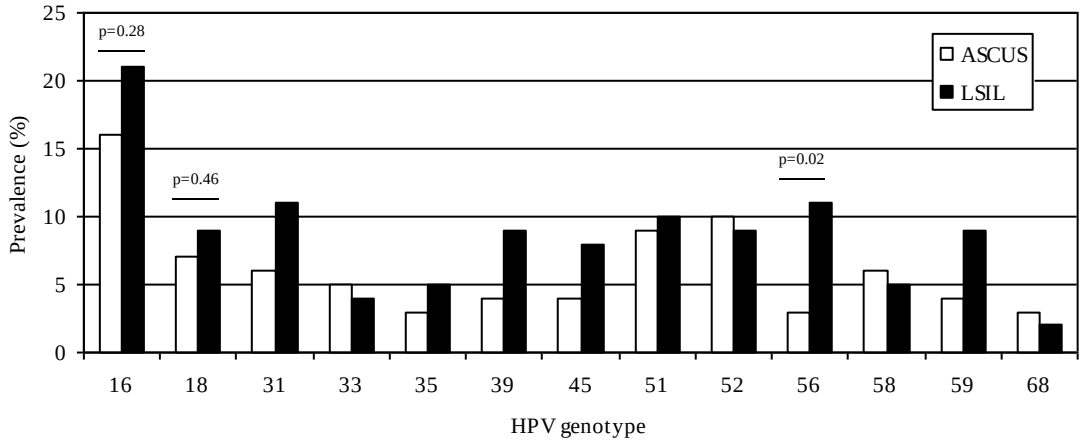


Table I:2. Preventive potential of current HPV vaccines in Stockholm County, LSIL and ASCUS.

HPV types	Diagnostic group			*p*
	All	ASCUS	LSIL	
	N (%) [n=343]	N (%) [n=120]	N (%) [n=223]	
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

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; *Clade 7 does not include HPV18 and Clade 9 not HPV16 in these cross-tabulations.

5.1.4 Multiple infections

Multiple infections were common in our material. On average, we found 2.5 genotypes per HPV-positive sample. In one LSIL sample, 9 separate genotypes were found. In all, 54% (185/343) of the women carried more than one HPV type. Women with LSIL had multiple types (61%, 136/223) more frequently than women with ASCUS (41%, 49/120, $p<0.001$, Chi^2). Taking only HPV-positive samples into account, multiple infections were equally common in women with ASCUS and LSIL (65% or 49/75, and 66% or 136/207 respectively). Age-specific prevalence of multiple infections followed a quadratic function in LSIL cases ($p=0.001$, logistic regression). This was also found for multiple HR-HPV infections among the HR-HPV-positive LSIL cases ($p=0.04$, logistic regression). Among HR-HPV-positive samples, 39% (85/217) were infected by more than one HR-HPV type and this was similar in LSIL and ASCUS samples (39.9% and 37.3%, $p=0.73$, Chi^2).

In all, there were 710 individual HPV infections. Co-infection was equally common among LR-HPV genotypes (243/268, 92%) and pHR-HPV genotypes (87/101, 91%, $p=0.21$, Chi^2). HR-HPV genotypes were somewhat less likely to be co-infected with a second HPV type (283/341, 83%, $p=0.006$, Chi^2). The HR types which were most commonly co-infected with HPV16 (clade 9) were HPV39 (28%), 45 (38%), and 59 (46%, all clade 7). Interestingly, HPV18 (clade 7) did not occur together with either HPV39 or HPV45 ($p\leq0.01$, Chi^2), but was found in 13% of HPV59 infections. The HR types most commonly co-infected with HPV18 were HPV33 (14%), 51 (14%), and 56 (17%), which did not differ from frequencies of HPV16 co-infection ($p>0.7$, Chi^2, for all). HPV16-infected women were more often co-infected with clade 7 types (38%) than similar clade 9 types (23%), though this did not reach statistical significance ($p=0.06$, Chi^2). HPV18-infected women carried genotypes from clade 9 in 43% of cases compared with similar clade 7 genotypes in 11% ($p=0.02$, Chi^2).

5.2 PAPER II

5.2.1 Inclusion follow-up and subsequent visits

Complete work-up with cytology and HPV typing was carried out at the inclusion follow-up visit. Women were grouped into an early group (<12 months) and a late group (≥12 months after conization). Thirty-three women (37%) presented <12 months and 57 women (63%) ≥12 months after conization. Categorization was based on when post-surgical HPV analysis was performed, since many women had had cytological follow-up at more than one visit. In addition, all women underwent pelvic exam, and in some cases colposcopy-directed biopsies. Characteristics of the two follow-up groups are summarized in **Table II:1**.

Table II:1. Characteristics of follow-up groups.

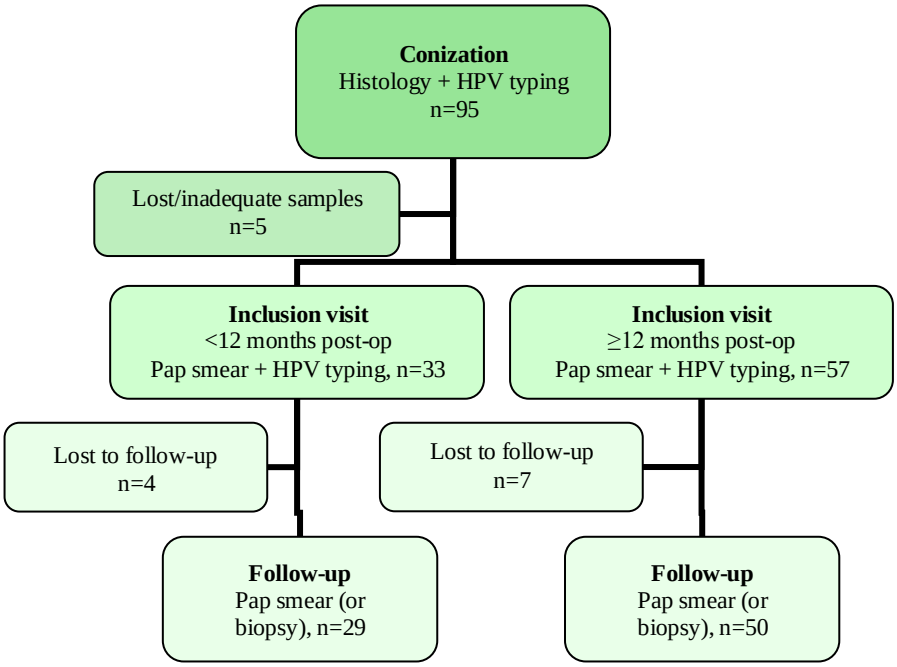
	<12 months n=33 (%)	≥12 months n=57 (%)	*p*	Total n=90 (%)
Conization data				
Mean age (years) [range]	34.5 [21-53]	36.1 [22-74]	0.44	35.4 [21-74]
Free margins	22 (67)	44 (77)	0.28[a]	66 (73)
CIN3+/AIS	17 (52)	32 (56)		49 (54)
CIN2	6 (18)	15 (26)	0.188[a]	21 (23)
CIN1	9 (27)	6 (11)		15 (17)
No CIN	1 (3)	4 (7)		5 (6)
HR-HPV positive	24 (73)	41 (72)	0.53[a]	65 (72)
Mean number of HR-HPV types[b]	1.4	1.1	0.02[c]	1.2
HPV16	10 5 (30)	19 (33)	0.77	29 (32)
HPV18	2 (6)	3 (5)	0.87	5 (5)
HPV31	5 (15)	6 (11)	0.52	11 (12)
HPV33	4 (12)	7 (12)	0.98	11 (12)
HPV51	3 (9)	2 (4)	0.27	5 (6)
HPV52	1 (3)	4 (7)	0.43	5 (6)
Inclusion visit data				
HSIL+/CIN2+	2 (6)	3 (5)		5 (6)
LSIL/CIN1	3 (12)	12 (21)	0.57	15 (18)
LSIL+/CIN1+	5 (18)	15 (26)		20 (23)
HR-HPV positive	14 (42)	15 (26)	0.23[a]	29
Mean number of HR-HPV types[b]	1.6	1.5	0.82[c]	1.5
HPV16	4 (12)	1 (2)	0.03	5 (6)
HPV18	2 (6)	2 (4)	0.57	4 (4)
HPV31	0 (0)	2 (4)	0.28	2 (2)
HPV33	3 (9)	3 (5)	0.48	6 (7)
HPV51	2 (6)	1 (2)	0.27	3 (3)
HPV52	0 (0)	6 (10)	0.05	6 (7)

HPV= human papillomavirus, HR= high-risk, CIN= cervical intraepithelial neoplasia
[a]Pearson's Chi^2, [b]restricted to HR-HPV-positive subjects, [c]Kruskal-Wallis

A total of 79 of 90 (88%) patients had a second follow-up visit with Pap smear and in some cases including colposcopy and biopsy. Mean total follow-up time was 39 months (median 34, range 4-115). In the <12 months group, mean time to the inclusion

follow-up visit was 6.5 months (median 7, range 2-11) and to the second follow-up another 18 months (median 18, range 9-29). In the ≥12 months group, the corresponding mean time to inclusion follow-up visit was 35 months (median 24, range 12-105) and to the second follow-up an additional 16 months (median 15, range 4-28). See **Figure II:1** for flow-chart.

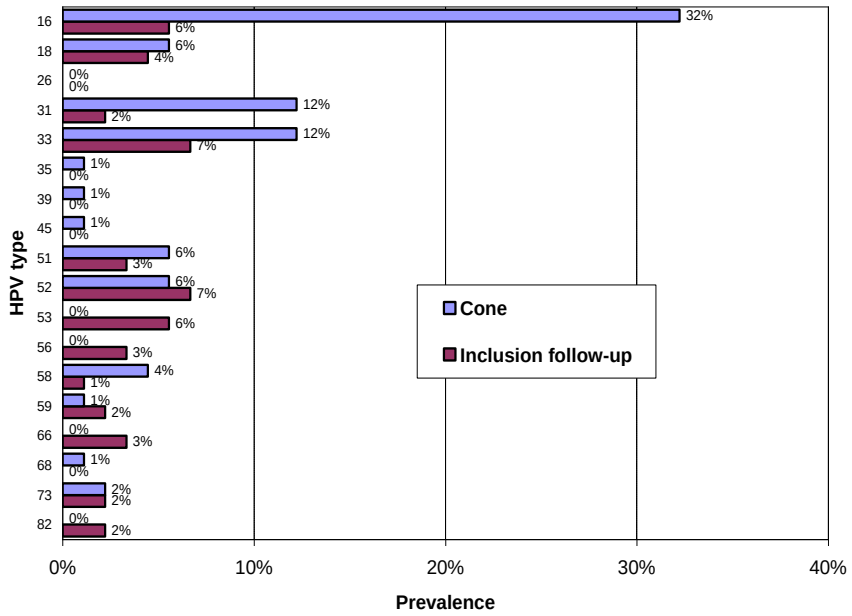
Figure II:1. Flow-chart of study.



5.2.2 HPV prevalence and distribution in cone specimens

HPV was detected in 65 of 84 (77%) cone specimens. In 6 cases material was insufficient. The most common HR-HPVs were HPV16, 18, 31, 33, 51, and 52, each of which occurred in more than 5% of cases. Prevalence of each HPV type is illustrated in **Figure II:2**. A single HR-HPV was found in 55 (85%) of the HR-HPV-positive cones, 9 (14%) had a double infection, and one a quadruple infection.

Figure II:2. Prevalence of HPV types at time of conization and at inclusion follow-up visit.



5.2.3 HPV prevalence and distribution at inclusion follow-up

Fourteen (42%) women were positive for HR-HPV at <12 months compared with 15 women (26%) at ≥12 months after conization (p=0.12). The number of virus types in each HR-HPV positive woman was similar in both groups (**Table II:1**). The most common HR-HPV were 16 (17%), 33 (21%), 52 (17%), and 53 (17%). Multiple infections were common. Nineteen women (21%) carried a single genotype and 10 (11%) had a multiple HR-HPV infection (7 with double, 1 with triple and 2 with quadruple infection).

The HR-HPV prevalence at the inclusion follow-up visit was unaffected by positive margins (p=0.22) or age at conization (p=0.19). Remarkably, when a cone contained a CIN3+ lesion, HR-HPV was *less* frequently found <12 or ≥12 months later, compared with those who had less advanced CIN (p=0.02). HR-HPV positivity at the inclusion follow-up visit was higher in women who were HR-HPV-positive (34% in HR-HPV-positive cone specimens vs. 21% in HR-HPV-negative cone specimens, p=0.29) or had multiple HR-HPV infections at conization (29% in single vs. 60% in multiple infection, p=0.12), but differences were not statistically significant. Overall, nine (10%) women were positive for the same HPV type as in the cone biopsy, including 6 at <12 months after conization and 3 at ≥12 months after conization (p=0.09). The persistent HPV types were 6, 16 (two women), 33 (three women), 51, 58, and 59. We noted that three of 11 (27%) HPV33 infections persisted and only two of 29 (7%) HPV16 infections.

5.2.4 Histopathological findings in the cone specimens

A total of 90 women were included. Five (5.6%) cone specimens contained no neoplasia, 15 (17%) contained CIN1, 21 (23%) CIN2, 46 (51%) CIN3, and three (3.3%) AIS. Sixty-six (73%) of the cones had free margins. The remaining 24 (27%) cones had positive margins. Prevalence of severe lesions in the original cones was similar in the early and the late groups (**Table II:1**), as was the proportion with free margins.

5.2.5 Cytological and histopathological diagnoses at follow-up

Of 33 women whose inclusion follow-up visit was at <12 months after conization, two (6%) had HSIL, four (12%) LSIL, and 27 (82%) were within normal limits (WNL). Of the 57 women whose inclusion follow-up visit was at ≥12 months, three (5%) showed signs of HSIL, 12 (21%) LSIL, and 42 (74%) were WNL. No statistically significant differences were found between these groups (p=0.57). At the second follow-up, two (7%) HSIL, two (7%) LSIL and 25 (86%) WNL were found in the early group (n=29), compared with three (6%) HSIL, 6 (12%) LSIL, and 41 (82%) WNL in the late group (n=50). No difference was found in disease recurrence in these samples either (p=0.86). The cumulative rate of HSIL in the 79 women who attended both follow-up visits was 7 (9%), LSIL 17 (21%), and WNL 55 (70%).

5.2.6 Determinants of treatment outcomes

Twenty two treatment failures were identified at the first or second follow-up visit (5 with HSIL+/CIN2+, 17 with LSIL/CIN1) among the 85 cases in which CIN had been identified in the cone. Margin status and presence of CIN3+ in the cone were poor

predictors of treatment outcome (sensitivity <50%, DOR ≤2.5) (**Table II:2**). All residual HSIL/CIN2+ cases had cones containing CIN2+ disease (sensitivity 100%), but this criterion was associated with low specificity (19%). Presence of pHR- or HR-HPV types predicted 100% of residual HSIL/CIN2+ at 73% specificity. Considering only 13 types showed equal sensitivity, but higher specificity (86%, *p*(McNemar)<0.01). Persistent HR-HPV infection (13 types) detected high-grade residual disease with 60% sensitivity and 95% specificity, resulting in a PPV of 43%, which exceeded the PPVs of all other criteria. One persistent HPV51 and two persistent HPV33 infections were responsible for 3 of the 5 cases involving residual high-grade disease. Considering LSIL or CIN1 to be thresholds for treatment failure resulted in lower sensitivity and higher specificity values. Persistent infection with HPV6, HPV16, and HPV59 each caused one case of residual LSIL/CIN1.

Table II:2. Predictors of treatment outcome at second follow-up visit.

Criterion	Outcome	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Margin status	CIN2/HSIL+	40.0	72.5	8.3	95.1
Cone containing CIN3+	CIN2/HSIL+	20.0	40.0	2.0	88.9
Cone containing CIN2+	CIN2/HSIL+	100	18.8	7.1	100
HR-HPV+ (13 types)	CIN2/HSIL+	100	86.1	31.3	100
HR-HPV+ (18 types)	CIN2/HSIL+	100	73.4	19.2	100
Persistent HPV	CIN2/HSIL+	60.0	93.7	37.5	97.4
Persistent HR-HPV	CIN2/HSIL+	60.0	94.9	42.9	97.4
Margin status	CIN1/LSIL+	40.9	76.2	37.5	78.7
Cone containing CIN3+	CIN1/LSIL+	40.9	36.5	18.4	63.9
Cone containing CIN2+	CIN1/LSIL+	72.7	14.3	22.9	60.0
HR-HPV+ (13 types)	CIN1/LSIL+	52.4	92.1	68.8	85.3
HR-HPV+ (18 types)	CIN1/LSIL+	61.9	79.4	50.0	86.2
Persistent HPV	CIN1/LSIL+	28.6	96.8	75.0	80.3
Persistent HR-HPV	CIN1/LSIL+	23.8	96.8	71.4	79.2

5.2.7 Occurrence of residual or recurrent SIL – multivariate analysis of predictors

In a stepwise multivariate logistic regression, using residual/recurrent low-grade SIL/CIN or worse disease as outcome, presence of HR-HPV infection at follow-up inclusion, type-persistence, and period of inclusion were used in the model as statistically significant predictors. Odds ratios were 7.3 (95% CI: 1.3-40.2) for late inclusion, 5.0 (95% CI: 1.3-19.7) for presence of HR-HPV infection at the inclusion follow-up visit, and 11.9 (95% CI: 1.3-110.5) for persistence. Margin status, severity of CIN in the cone, number of HPV types in the cone or at follow-up, and identification of the individual types at follow-up were all excluded from the model. Logistic regression using high-grade SIL/CIN as outcome was not possible, since the outcome was perfectly predicted by presence of HR-HPV at follow-up (sensitivity 100%).

5.3 PAPER III

The mean age of included women was 34.1 years (median 32 years, range 21 to 60 years). In 111 (94%) of the 118 cases the cell suspension sufficed for immunocytochemistry. Five of these cases were excluded because the tissue was insufficient for histological examination, leaving 106 cases with all three analyses performed.

5.3.1 Cytological findings

Thirteen (12%) of the 106 cases had benign findings on cytological analysis, 40 (37%) cases had CIN I, 30 (28%) cases had CIN II, 14 cases CIN III, and two cases of squamous cell carcinoma (SCC) were found. Two cases were classified as ASCUS and three cases as ASC-H. Glandular atypia was rare with only one case displaying atypical glandular cells – uncertain significance (AGUS) and one case of adenocarcinoma in situ (AIS).

5.3.2 Histological findings

In the histological assessment, one of the suspected SCC was considered to be CIN II, and one ASC-H was considered to be AIS. A total of 19 (18%) of the 106 cases were benign, 26 (25%) women had CIN I, 28 (26%) CIN II, 30 (28%) CIN III, one SCC, and two were found to have AIS. The three cancers were included in the group of CIN2+ (**Table III:1**).

Table III:1. Intensity of $p16^{INK4a}$ immunocytochemistry staining and *histological* diagnosis.

Intensity of $p16^{INK4a}$ staining	Histological diagnosis			Total
	WNL N (%)	CIN1 N (%)	CIN2+ N (%)	N
-	14 (74)	20 (77)	15 (25)	49
+	3 (16)	4 (15)	12 (20)	19
++	2 (11)	2 (7.7)	24* (39)	28
+++	0	0	10** (16)	10
Total	19	26	61	106

*Two cases of AIS, **one case of SCC. Spearman Rank correlation coefficient Rho 0.52, $p<0.001$, Chi^2 exact test for trend, $p<0.001$

5.3.3 $p16^{INK4a}$ expression in relation to CIN grade in histology

Immunocytochemical staining for $p16^{INK4a}$ was carried out along with conventional Papanicolaou counterstain (**Figure III**). Of 106 patients, 10 (9%) showed strong $p16^{INK4a}$ reactivity, 28 (26%) moderate, and 19 (18%) weak. Reactivity was absent in 49 samples. Most benign cases were negative, but three showed weak reactivity and two moderate reactivity. Three quarters of CIN2+ cases were identified by $p16^{INK4a}$ staining and more than half showed moderate or strong reactivity (**Table III:1**). Degree of reactivity to $p16^{INK4a}$ antigen correlated with the severity of CIN as certained by histology (**Table III:1**). The sensitivity of $p16^{INK4a}$ immunostaining for detecting a histologically confirmed CIN2+ was 59% (95%CI 49-69), the specificity 94% (95%CI 68-100), and PPV 98% (95%CI 89-100).

5.3.4 p16^{INK4a} expression in relation to cytological abnormality

The correlation between p16^{INK4a} immunoreactivity and severity of the cytological abnormality was stronger when the diagnosis was based on simultaneous routine cytology (**Table III:2**). Only one benign case with weak reactivity (7.7%) was found. Some reactivity, usually weak, was seen in low-grade lesions, while two thirds of high-grade lesions were moderately or strongly stained (**Table III:2**). The sensitivity of p16^{INK4a} immunostaining to detect a high grade lesion on cytology was calculated to be 60% (50-70), the specificity 100% (77-100), and PPV 100% (92-100).

Figure III. Increasing nuclear staining intensity (brown color) with increasing grade of CIN (I-III).

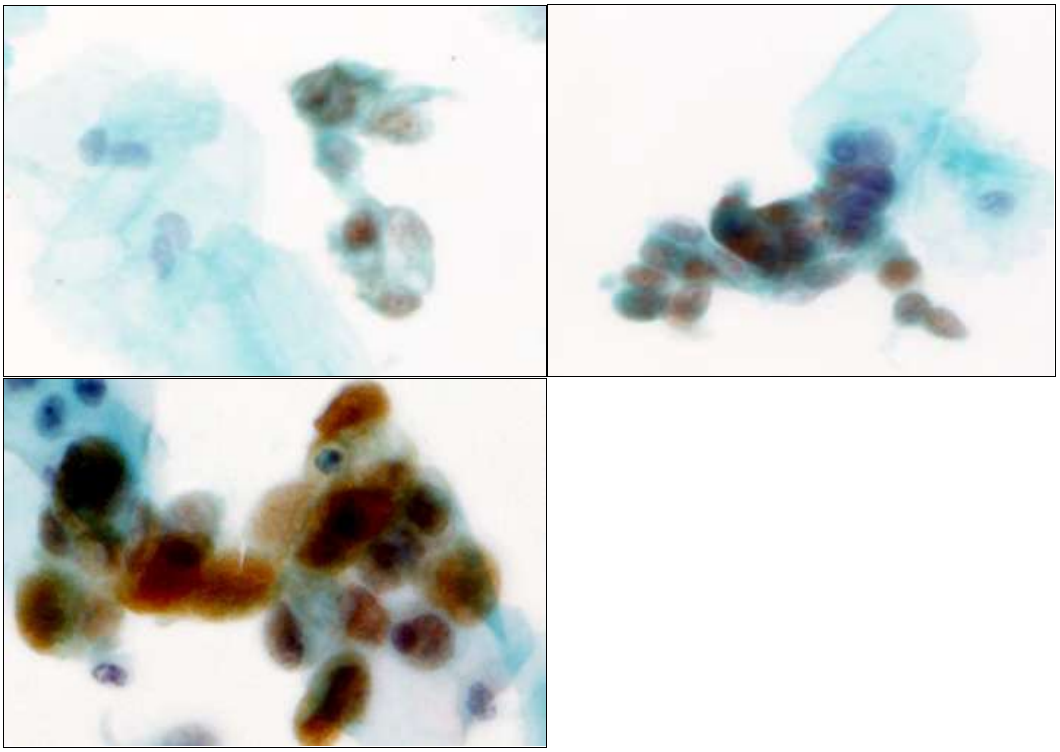


Table III:2. Intensity of p16^{INK4a} immunocytochemistry staining correlated with *cytological* diagnosis.

Intensity of p16^{INK4a} staining	Cytological diagnosis				Total
	WNL	ASCUS	LSIL	HSIL	
	N (%)	N (%)	N (%)	N (%)	N
-	12 (92)	2 (67)	30 (73)	5 (10)	49
+	1 (7.7)	1 (33)	7 (17)	10† (20)	19
++	0	0	4 (9.8)	24* (49)	28
+++	0	0	0	10** (20)	10
Total	13	3	41	49	106

†One case of suspected SCC not confirmed in histology, *one case AIS, **one case of SCC. Spearman Rank correlation coefficient Rho 0.70, $p<0.001$, Chi^2 exact test for trend, $p<0.001$.

5.4 PAPER IV

5.4.1 Distribution of HPV genotypes and cytological findings

A total of 22 of 24 (92%) women examined here had cytological results within normal limits, one had ASCUS and one woman had LSIL. Eleven (46%) were positive for any HPV type; 8 (33%) of them carried HR-HPV types. None of the women was diagnosed with HIV, Chlamydia, or gonorrheal genital infection, although one woman's results from Chlamydia PCR analysis and N. gonorrhea culture were missing. Two women had additional findings of yeast infection, and one had signs of bacterial vaginosis. These three women, including the two with minor cytological lesions, were designated as a group with an "additional cervical condition". No relevant differences in sexual or reproductive histories were noted between HPV-positive and negative subjects (**Table IV:1**).

Table IV:1. Entry characteristics of study subjects.

Entry data	All	HPV status		p[a]
		HPV-positive	HPV-negative	
	N (%)	N (%)	N (%)	
Number of women	24	11 (46)	13 (54)	0.56
Steady relationship	18 (75)	7 (63)	11 (85)	0.35
Oral contraception	7 (29)	5 (45)	2 (15)	0.18
Progesterone IUD	4 (17)	0	4 (31)	0.10
Condom use ≥50%	7 (29)	4 (36)	3 (23)	0.66
Sex partner change previous 12 months[b]	6 (25)	3 (27)	3 (23)	1.00
Ever pregnant	13 (57)	4 (40)	9 (69)	0.16
Additional cervical condition[c]	5 (21)	2 (18)	3 (23)	1.00
Preovulatory menstrual phase	2 (8.7)	1 (10)	1 (7.7)	0.95
Postovulatory menstrual phase	10 (43)	4 (40)	6 (46)	0.95
Age (median) [range]	32 [24-50]	31 [24-46]	32 [26-50]	0.66

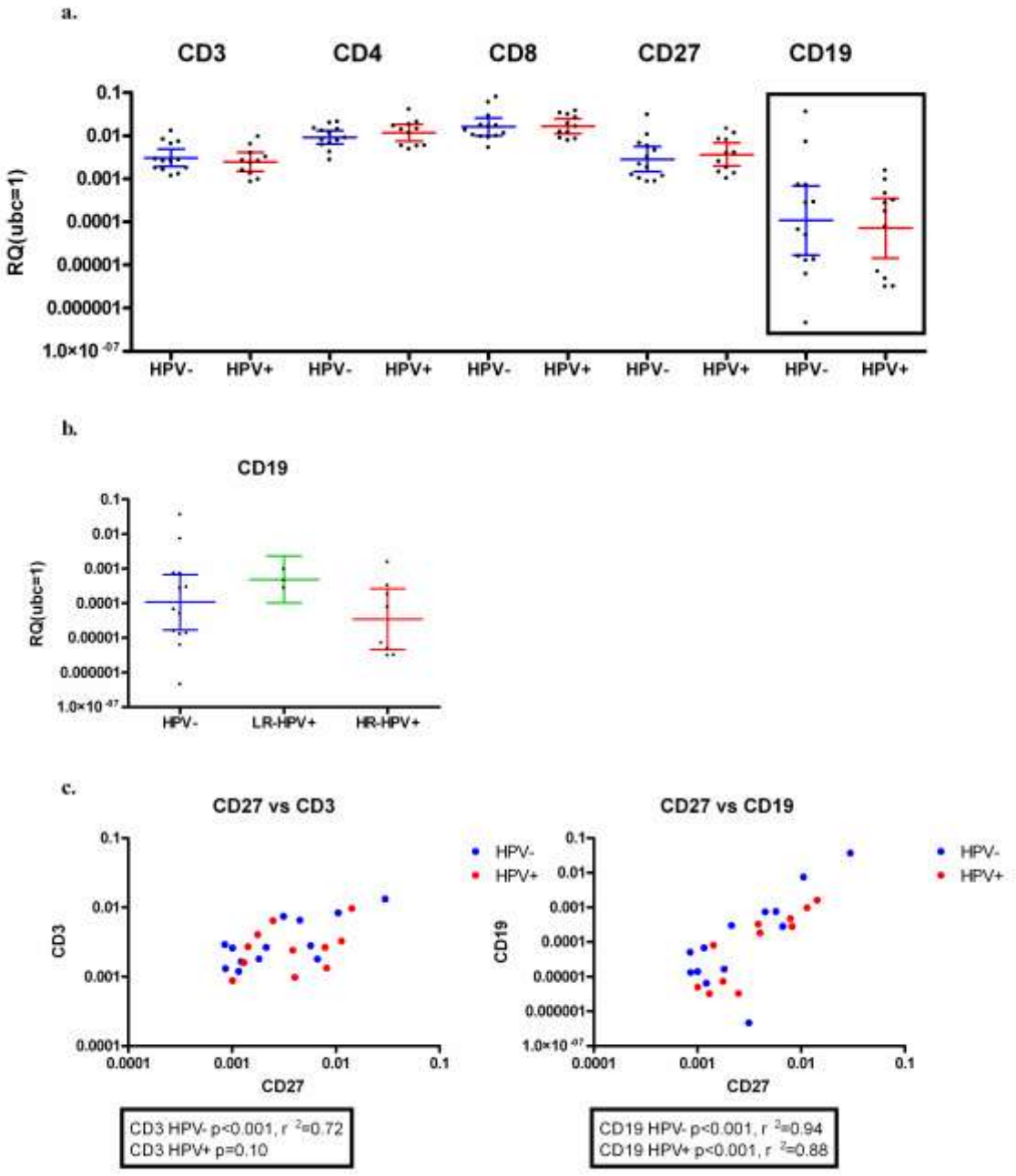
[a]Mann-Whitney U and Chi^2/Fischer's exact test, [b]reported more than one sex partner previous 12 months
[c]Yeast infection, bacterial vaginosis or ASCUS/LSIL in the pap smear

5.4.2 Expression of phenotypic markers

Expression of the T phenotypic markers CD3, CD4, and CD8, and B phenotypic markers CD19 and CD27 was calculated in relative quantity compared to the endogenous control UBC (**Figure IV:1a**). However, the presence of these cellular phenotypes did not differ significantly in HPV-positive women compared to the HPV-negative group (p>0.5).

CD19 expression was widely dispersed in HPV-negative women and women positive for LR- or HR-HPV (**Figure IV:1b**), raising the question of whether outliers represent different compositions of B cells, with very high or low relative frequencies of CD19-negative cells such as memory B cells and plasma cells. Since no markers specific for human plasma cells are available, quantities of the memory B cell marker CD27 were evaluated and showed a linear correlation with CD19 (**Figure IV:1c**), arguing against an imbalanced subtype distribution. Not forgetting that CD27 is also expressed by T cells, we tested further and found that the total CD27 expressed here correlated better with the amount of CD19 from B cells than with CD3 from T cells (**Figure IV:1c**).

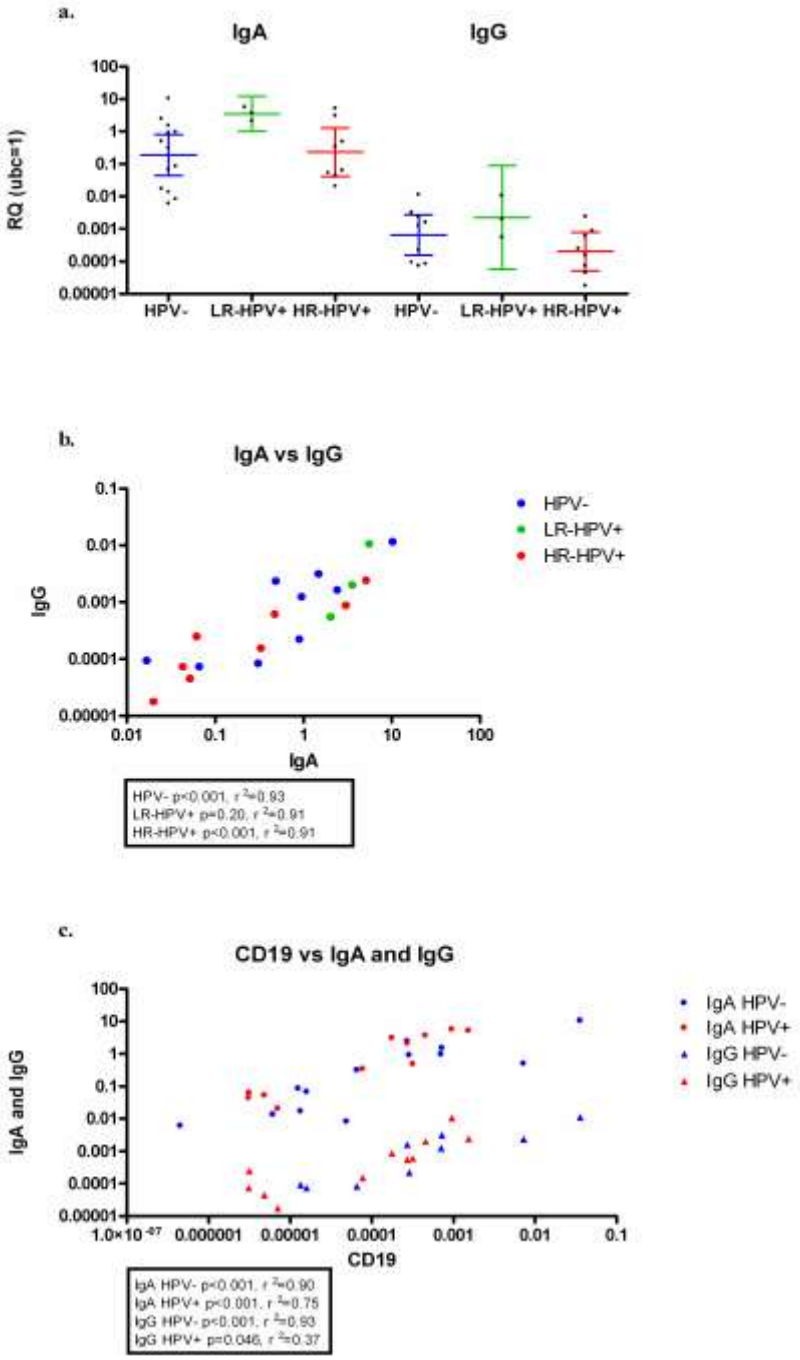
Figure IV:1. Phenotypic T and B cells markers.



5.4.3 Expression of immunoglobulins

Expression of immunoglobulin mRNA was used as a marker for plasma cell presence. IgA was detectable in all women, but IgG was absent in four women who were HPV-negative. There was no difference in the expression of neither IgA nor IgG in HPV-negative vs. HPV-positive women (**Figure IV:2a**). IgA expression correlated well with that of IgG in all women, although too few LR-HPV-positive women were included in the study to reveal a significant difference from null (**Figure IV:2b**). Immunoglobulin expression correlated linearly with both CD19 (**Figure IV:2c**) and CD27 (data not shown), although neither CD19 nor CD27 should be expressed by plasma cells.

Figure IV:2. Immunoglobulins and the B cell marker CD19.



5.4.4 Cytokine profiles, immunoregulatory receptors and ligands

As **Figure IV:3a** depicts, expression of the proinflammatory cytokines IL-2, IL-12, IL-17, and IFNγ, RANTES/CCL5, its receptor CCR5, and HLA-DR was similar in the HPV- negative and positive women. Nor was any difference evident when sub-grouping HPV-positive women into LR- or HR-HPV carriers (data not shown). The antiinflammatory cytokines TGFβ, IL-4, and IL-10 were expressed in a mutually similar fashion (**Figure IV:3b**). IL-4 mRNA expression was below the detection limit in all patients. Additionally, as outlined in **Figure IV:4**, neither immunoregulatory receptors nor ligands differed to a significant extent between HPV-positive and negative women.

Figure IV:3. Proinflammatory and antiinflammatory cytokines.

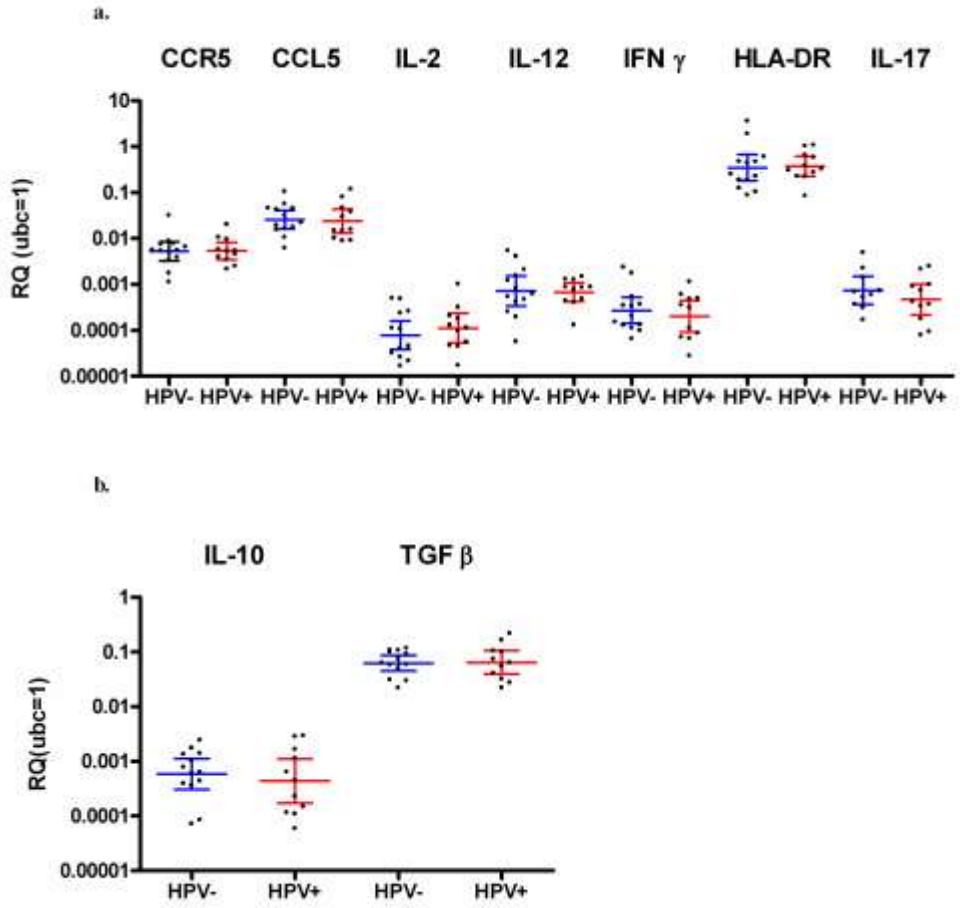
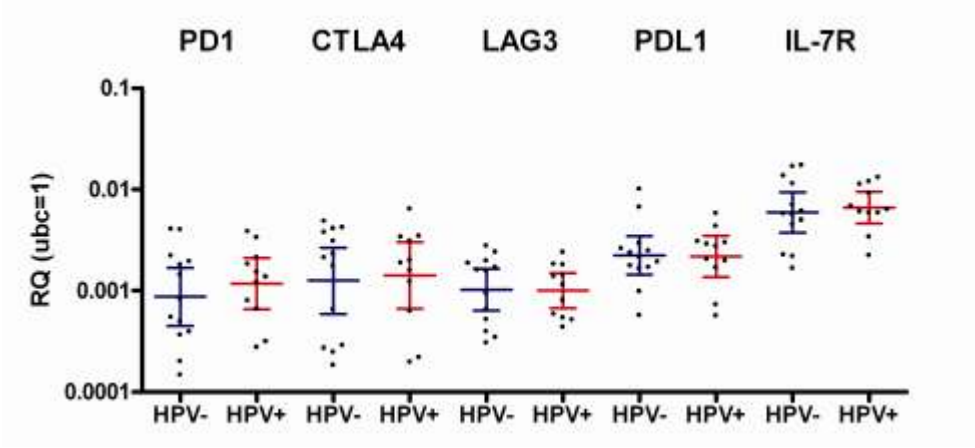


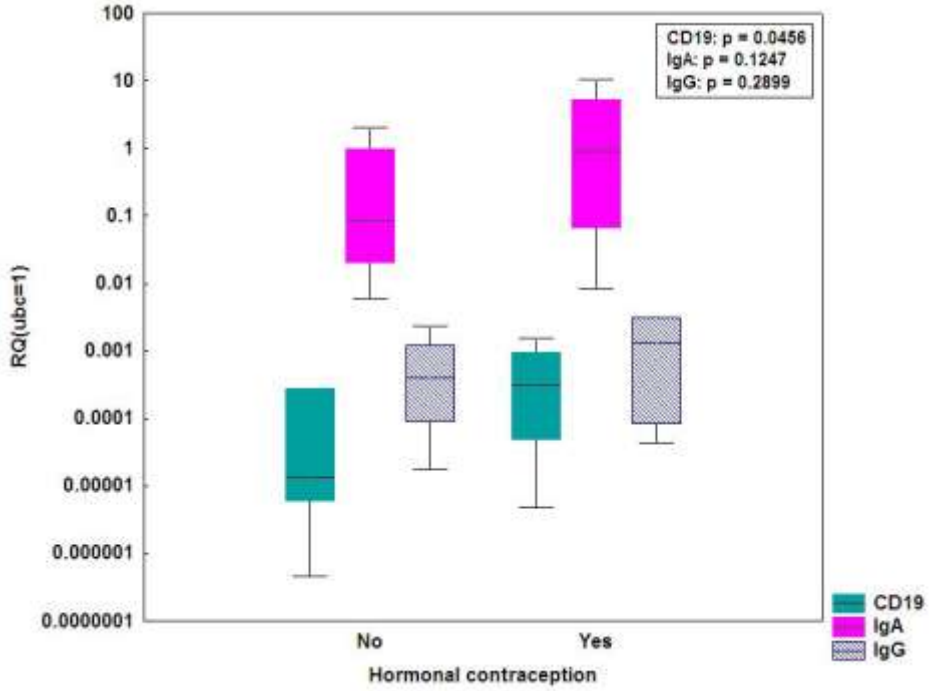
Figure IV:4. Immunoregulatory receptors and ligands.



5.4.5 Clinical correlates of cytokines and phenotypic markers

Surveys of hormonal exposure, treatment with inhaled or oral corticosteroids, sexual and reproductive history, or age (data not shown) revealed only one distinctive result, and that was expression of the phenotypic marker CD19. CD19 expression was significantly higher in women currently using oral contraceptives or progesterone intrauterine devices compared with women not using contraception (**Figure IV:5**). Ig expression was not associated with hormonal exposure. No significant correlations with clinical parameters were found among the pro- or antiinflammatory cytokines, immune regulatory receptors, and corresponding ligands.The two women with LSIL or ASCUS showed immune activity within the interquartile range for expression of all markers. One woman demonstrated extremely strong expression of CD3, CD8, CD19, CD27, IgA, IgG, PD-1, IL-7R, IFNγ, and CCR5, despite lack of clinical or laboratory signs of inflammation or infection.

Figure IV:5. B cell markers and hormonal contraception.



6 DISCUSSION

6.1 HPV GENOTYPING IN TRIAGE AND POST TREATMENT

Viral persistence is believed to be the most important risk factor for cervical cancer. Yet, this is true only for certain oncogenic types (Schiffman *et al*, 2005). Assessment of viral persistence requires HPV genotyping. Genotyping is also necessary to monitor the effects of vaccination and to explore the importance of multiple infections.

6.1.1 Influence of age and time on HPV prevalence

A slight but non-significant increase in overall HPV prevalence was observed in women with ASCUS or LSIL over 45 years. Multiple infections increased in women over 50 years, as well did the proportion of LR-HPV in older women. Other have reported similar age-related patterns of HPV epidemiology in women without CIN (Bosch *et al*, 2008; Castle *et al*, 2006; de Sanjose *et al*, 2007) and in women with CIN (Cuschieri *et al*, 2004). We also described an age-dependent decrease in HR-HPV prevalence in LSIL cases, but not in ASCUS cases. Only the youngest women (20-24 years) differed in HR-HPV prevalence between ASCUS and LSIL cases.

We showed that about 56% of women cleared all HPV infection <12 months after conization, which is consistent with several recent studies (Chao *et al*, 2004; Costa *et al*, 2003; Fen *et al*, 2004; Kreimer *et al*, 2006). A trend was shown toward decreasing number of infected women over time. Viral clearance is a time-consuming immunological process, which is enhanced by the surgical treatment and probably not due to the aging of these women. Age and incomplete surgical excision have been proposed as risk factors in treatment outcome (Alonso *et al*, 2006; Costa *et al*, 2003; Kreimer *et al*, 2006), although age, the histological finding of free resection margins, and presence of CIN3+ in the cone were poor predictors of treatment outcome in our study.

6.1.2 Are there hormonal effects?

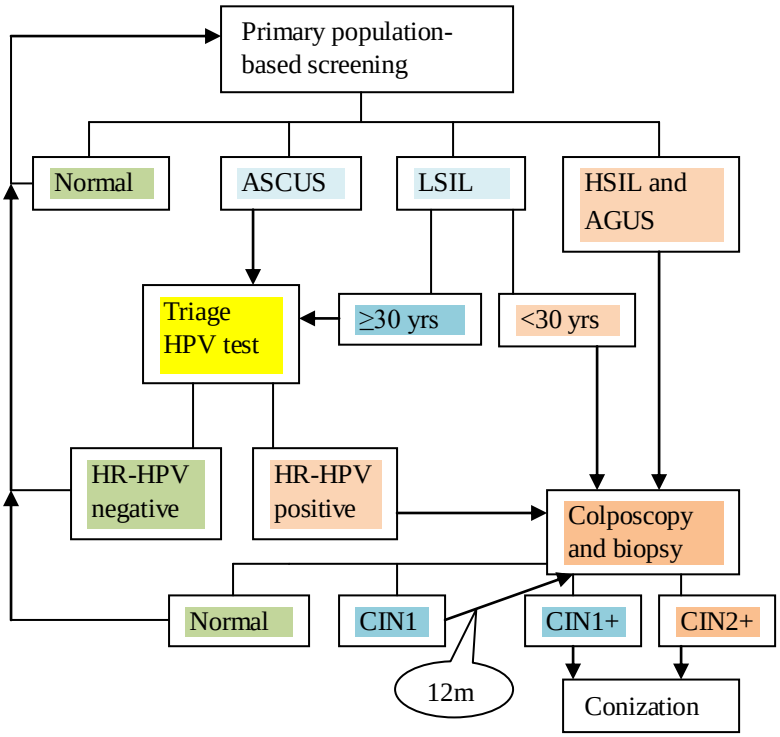
The age-related differences in HPV prevalence in Paper I may be related to sexual behavior and therefore increased exposure, since many young women encounter several partners and the older women have divorced, remarried, and generally meet sexually experienced men (Stevenson & Wolfers, 2007). The increase in older women may also be explained by menopause-induced hormonal and immunological changes that could facilitate HPV DNA detection or reactivate latent infections from exposures earlier in life. Some evidence suggests a hormonal effect on oncogene expression at least for HPV16 and 18, via the action on progesterone and 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). Oral contraception, which is frequently used by young women, 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. In Paper IV, we found an interesting correlation between hormonal contraception and expression of CD19 mRNA. To our knowledge, no studies have explored whether other

ovulatory hormones such as gonadotropins, inhibin, or prostaglandins could influence the immune response to HPV or HPV induced carcinogenesis.

6.1.3 Implications for triage

Triage of ambiguous smears with HPV testing is becoming internationally accepted and recommended for ASCUS lesions (Arbyn *et al*, 2005). HPV triage testing of LSIL has been said to have limited potential, based on the ALTS report of high prevalence of HR-HPV in LSIL cases (83%) (ALTS, 2000). In Paper I, average prevalence of HR-HPV was higher in LSIL (71%) than in ASCUS cases (49%), but lower than in the ALTS report. The difference in HR-HPV prevalence might be due to another definition of LSIL or to a lower mean age in the ALTS. ALTS used HCII and defined 13 HPV types as high-risk, including HPV68. This should not introduce any major difference, since HPV68 without co-infection with "our" HR-HPV was found only in one ASCUS case and in one LSIL case. One study suggests that women with LSIL aged 35-64 years may benefit from HPV triage (Ronco *et al*, 2007). In our study, only the youngest women (20-24 years) differed in HR-HPV prevalence between ASCUS and LSIL cases when using 5-year intervals for age grouping. The largest drop in HR-HPV prevalence in LSIL cases was seen after age 30 (83% in women <30 years vs. 60% in women ≥30 years, $p<0.001$). This suggests that age 30 or even lower is a suitable cut-off point for HR-HPV triage in LSIL, if consideration is only given to the prevalence argument. Histological follow-up has been performed in a subgroup of these women, showing that 48% of women over 30 years with LSIL in cytology were HR-HPV-negative and histologically negative; thus they could safely have remained in the three-year interval screening program (Froberg *et al*, 2008). The number of colposcopic and histological follow-ups after a LSIL cytology result could therefore be cut in half by HPV triage in women over 30 years. In **Figure 9**, we present a suggested flow-chart for triage HPV testing.

Figure 9. Suggested flow-chart for triage HPV testing.



6.1.4 Impact of multiple infections

We found that the prevalence of multiple infections in women with ASCUS and LSIL decreased with age until 50 years, whereafter a significant increase was observed. It is speculated 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. It is tempting to explain multiple infections in the younger age group with multiple partners, although cervical ectopy, lack of cross-protectoral immune responses, and the use of oral contraceptives also may increase susceptibility to multiple HPV infection. Epidemiological cancer data from Statistics Sweden (SCB) reveal two peaks in cervical cancer incidence; one at 35-40 years and one at 65-69 years, which suggests increased susceptibility also in the post-menopausal women. Multiple infection increases the probability of having at least one oncogenic HPV type. It is still unclear whether co-infections with certain types will exert a synergistic effect on malignant transformation or if cancer can arise at multiple sites in the cervix (Guo *et al*, 2001). The importance of multiple infections needs to be elucidated in longitudinal studies with histological confirmation of progressive CIN.

After conization, multiple HR-HPV types in the cone and type-specific persistent infection in particular increase the risk of recurrence. Previous reports state that HR-HPV positivity (not true persistence) after conization is associated with recurrence (Almog *et al*, 2003; Kreimer *et al*, 2006; Nagai *et al*, 2000; Song *et al*, 2006). Type-specific persistent infection occurred in 10% of the women after conization, supporting the results from another Swedish study (Elfgren *et al*, 2002). However, clearance of virus was similar after single or multiple infection (Kreimer *et al*, 2006). Interestingly, we found that a severe lesion (CIN3+) in the cone was associated with a significantly better clearance. This may be explained by the theory of lower viral production in severe lesions (Doorbar, 2006; Doorbar *et al*, 2005; Elfgren *et al*, 2002; Longworth *et al*, 2004; Munger, 2002) or the fact that CIN3+ is often treated with greater margins removing the entire infected area.

6.1.5 Implications for vaccination strategies

The observed high co-infection rates of HPV16 with certain HPV types, such as HPV39, 45, and 59, may be due to differences in susceptibility to certain genotypes (all clade 7) (Liaw *et al*, 2001). We found that HPV18-infected women were more likely to be co-infected with non-related clade 9 HPV types, than similar clade 7 HPV types, disputing specific susceptibility. Our data support cross-protection from infection with HPV from the same clade. Up to 60% of women with minor cytological abnormalities would potentially benefit from catch-up vaccination provided full cross-protection can be assumed (Castellsague *et al*, 2008).

As far as we know, Paper I is the only study to describe the prevalence of HPV genotypes in ASCUS and LSIL cases among women of all ages in Sweden before the implementation of public HPV vaccination. The relative frequencies of individual HR-HPV types were similar to those found from primary screening in a Swedish study of 5696 women aged 32-38 years (Swedescreen) (Naucler *et al*, 2007c). In middle-aged women, HPV16, 31, and 33, represent the highest population-attributable proportion in development of CIN2+. HPV18 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 cervical carcinoma, after 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. Our data support the opinion 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" (Cuschieri *et al*, 2004).

6.1.6 Importance of HPV genotyping in post-conization follow-up

Presence of HR- or pHR-HPV types predicted 100% of residual HSIL/CIN2+ after conization with 73% specificity. Considering only the 13 HPV types included in HCII showed equal sensitivity, but higher specificity (86%). The presence of type-specific persistent HR-HPV infection (13 types) detected high-grade residual disease with 60% sensitivity and 95% specificity, resulting in a PPV of 43%. These data suggest that HPV typing could be used for more focused follow-up. Although HPV genotyping assays are generally more expensive than cocktail-based HR-HPV tests, genotyping is indispensable for identifying truly persistent infection, which is believed to be a prerequisite for progression to cervical cancer. However, not all treatment failures which involved high-grade lesions were associated with type-specific HPV persistence in our study. It may be speculated that these viral types were present, but not represented in the cone. These strains may have initiated a parallel oncogenic process that evolved during the course of follow-up. Polyclonal origin has been suggested by microdissection studies of cervical cancer (Guo *et al*, 2001). Absence of persistence therefore cannot be proposed as a reason to interrupt follow-up or to exclude risk of recurrent or progressive disease.

It is noteworthy, that some women were HPV-negative, but found to have an abnormality in cytology. HPV tests are not 100% sensitive and low-grade abnormal cytology does not always represent true CIN (Baker, 2002). Absence of any HR-HPV infection after conization should however be a clear signal of treatment success and follow-up could be limited to combined cytology and HR-HPV testing at 6 and 24 months after treatment (Bais *et al*, 2009; Jordan *et al*, 2008; Zielinski *et al*, 2003). HPV-testing in the follow-up period after conization is no longer a controversy and is recommended after convincing meta-analyses of its benefits (Arbyn *et al*, 2008a; Paraskevaidis *et al*, 2004; Zielinski *et al*, 2004). It is also cost-effective (Coupe *et al*, 2007). To our content, it will be introduced in the coming edition of the Swedish Society of Obstetricians and Gynecologists' (SFOG) guidelines, which are now available at www.sfog.se. Some prudence is still warranted, since discordances of HPV test results and cytology will need follow-up by colposcopy and clinical evaluation (Aerssens *et al*, 2009). Women with a history of CIN treatment also remain at higher risk for developing cervical cancer than women in the general population (Kalliala *et al*, 2005; Soutter *et al*, 2006; Strander *et al*, 2007b).

6.2 MARKERS OF POTENTIAL PROGRESSION TO CERVICAL CANCER

6.2.1 P16^{INK4a} in triage

Accumulation of p16^{INK4a} in squamous cell nuclei should indicate presence of oncogenic HPV with some degree of deregulated transcription, thus cells on the verge of malignant transformation (von Knebel Doeberitz, 2002). Detection of p16 ^{INK4a} could therefore be more specific for oncogenic potential than the mere presence of HR-HPV. In Paper III, we visualized the accumulation of p16^{INK4a} in cells from the same suspension that had shown cellular abnormalities on routine staining. This allowed us to avoid many errors associated with repeated sampling, and ancillary analysis became a possible tool to sharpen the primary cytological evaluation. We also evaluated the nature of immunoreactive cells by counterstaining with Papanicolaou. We found that transformed keratinocytes show nuclear staining as opposed to the cytoplasmic staining seen in metaplastic and glandular cells in inflammation, which is unrelated to the precancerous process (Negri *et al*, 2006). A nuclear scoring system has also been proposed to facilitate identification of transformed cells (Trunk *et al*, 2004; Wentzensen *et al*, 2005). Papanicolaou counterstaining may be less time-consuming, since it integrates cytological evaluation with immunocytochemistry. Yet, neither of these improvements dismisses the subjectivity of the interpreter.

The presence of dysplastic lesions correlates with p16^{INK4a} immunoreactivity. Most normal cells did not express p16^{INK4a} and strong reactivity was solely confined to highly neoplastic cells, which is in full agreement with previous studies (Carozzi & al, 2006; Murphy *et al*, 2003). A statistically significant correlation was found between the intensity of p16 ^{INK4a} reactivity and the lesion severity. We noted that a quarter of the histological high-grade lesions completely lacked p16^{INK4a} reactive cells. This corresponds to the expected prevalence of false negative cytology results and could therefore be samples devoid of diagnostic cells, in which triage of any sort would be useless. Most low-grade lesions showed no or faint p16^{INK4a} reactivity, which was interpreted as signs of non-transformed cells or impending regression (Kalof & Cooper, 2006). However, the lesion may still contain HR-HPV (Andersson *et al*, 2006b). Ideally, the presence of p16^{INK4a} accumulation demonstrates oncogenic transformation by HR-HPV, but absence of immunoreactivity cannot exclude an infection with long-term oncogenic potential.

One possible use for p16^{INK4a} immunostaining in triage is to assess ASCUS and LSIL as a first step for detection of potentially precancerous lesions, since it has up to 96% specificity (Andersson *et al*, 2006a; Cuschieri & Wentzensen, 2008; Meyer *et al*, 2007; Murphy *et al*, 2003; Negri *et al*, 2006; Wentzensen *et al*, 2005; Yoshida *et al*, 2004). Stain negative samples could be submitted to HPV DNA analysis to maintain sensitivity. Another potential use for p16^{INK4a} may be to detect precancerous cells that otherwise would have remained undetected with conventional Papanicolaou staining, but the sensitivity was limited in our study and in other studies (Carozzi & al, 2006; Duncan *et al*, 2008). Yet, it is known that the majority of histologically confirmed high-grade lesions spontaneously resolve (Holowaty *et al*, 1999; Nasiell *et al*, 1983; Westergaard & Norgaard, 1981), and it is plausible that p16^{INK4a} expression may actually be more specific for lesions with invasive potential.

6.2.2 Immune response markers in HPV infection

Results from Paper IV provide a unique baseline for the constituent mRNA expression of a panel of cytokines and cellular markers in the ectocervices of subjectively healthy volunteers with asymptomatic HPV infection. No differences in inflammatory or adaptive immune response regulation were found in this material, suggesting that HPV DNA positivity with high- or low-risk types, in the absence of dysplastic lesions, do not affect the local mucosal immune response. Yet, we did observe a significant association between exposure to hormonal contraception and elevated CD19 expression.

Few cytokines have been studied in cervical samples from women without CIN and no correlation has been found between them and ongoing HPV infection (Gravitt *et al*, 2003; Scott *et al*, 2006), which supports our results. In women with HPV16-positive CIN, decreased levels of IFNγ and TGFβ mRNA and increases of IL-10 have been observed (El-Sherif *et al*, 2000; El-Sherif *et al*, 2001). Other researchers recently found that elevations in IFNγ *and* IL-10 were associated with decreased odds of having CIN 2 or 3 and thus probably represent a proper immune response in these women (Scott *et al*, 2009). Using immunohistochemistry, more IL-2 and IL-4 expressing cells as an indication of immune activation in HPV-positive women have been observed (Behbahani *et al*, 2007). Immunohistochemistry enables identification of cells expressing a certain target molecule, whereas RT-PCR detects the amount of expressed mRNA, which may or may not be related to the number of cells. With respect to HIV susceptibility, the amount of target molecules such as CD4 and CCR5 may be more important than the number of cells. In essence, asymptomatic HPV infection alone does not seem to evoke a measurable inflammatory response or attract possible HIV target cells: rather, oncogenic transformation or additional immunogenic conditions are required.

We assessed other covariates of cytokine expression, but were unable to repeat previous observations of increased IL-10, IL-12, or IFNγ mRNA expression in women with current or recent use of oral contraceptives (Gravitt *et al*, 2003; Scott *et al*, 2006). Howwever, we did observe higher levels of CD19 mRNA in women engaged in hormonal contraception. CD19 is a phenotypic B cell marker that is lost during the late stages of B cell differentiation. With immunohistochemical technology, CD19+ lymphocytes have been located in follicle-like structures in the subepithelial layers of the cervical mucosa (Johansson *et al*, 1999), but Paper IV is the first report to identify CD19 mRNA in the cervix. Endometrial aggregates of CD19+ lymphocytes increase in size during mid-cycle and the secretory phase of the menstrual cycle (Yeaman *et al*, 1997), supporting the finding of hormonal influence on CD19+ cells. We did not observe any correlation between hormonal contraception and Ig expression. It may be speculated that high levels of CD19 without change in IgG or IgA expression represent hormonally blocked B lymphocyte maturation and, ultimately, an impaired adaptive immunological response. Use of combined oral contraceptives has long been regarded as an independent risk factor for HPV infection, CIN, and cervical cancer (reviewed in (Castellsague & Munoz, 2003)), but has come into question, partly because no underlying mechanisms is evident and partly due to confounding HPV infection (Syrjanen *et al*, 2006). Here, we provide one clue to the understanding of how hormonal contraception may influence host defences.

Most cytokines, phenotypic cell markers, immunoregulatory receptors, and ligands analyzed here have never before been characterized in the cervical mucosa. We recognize the inherent limitations of the cross-sectional design of our study, since we

do not know the duration of the subjects' HPV infections and, therefore, cannot identify which, if any, of the women will fail to clear the virus. Ideally, immunological host factors associated with viral persistence should be studied in longitudinal studies of HPV infection. We considered the possibility of false HPV DNA negativity in the LBC sample of one case that showed several signs of inflammatory activation in the cervical biopsy, even though the sensitivity of Linear Array is very high (Giuliani *et al*, 2006; Monsonego *et al*, 2008). Since none of the women had a clinically overt lesion, we may not have taken the biopsy at the site of infection/inflammation. The limited sample size of 11 HPV-positive and 13 HPV-negative women may have hampered our ability to detect small differences between these groups. Nevertheless, clinically relevant major differences may be visible even in a small group of women. Most importantly, the present study will serve as a pilot study and baseline for future studies of immune response markers in women with HPV infection and cervical dysplasia.

7 CONCLUSIONS AND FUTURE DIRECTIONS

We found that HR-HPV prevalence and genotype pattern are similar among women with ASCUS and LSIL after the age of 30 years, supporting an age limit of 30 years or even lower for triage testing in LSIL cases and no age limit in ASCUS cases inorder to improve effectiveness of the screening program. Our study is the only Swedish study of HPV genotypes in women of all ages attending primary screening before the implementation of a public vaccination program. It provides a basis for future trend analyses of HPV epidemiology and for studies of cross-protection of vaccination. It is of interest to note that HPV18 was the sixth most common HR-HPV type after HPV16, 51, 52, 56, and 31. This may reflect future distribution in cervical cancers or merely inform of types frequently seen in dysplasia but not in cancer. Multiple infections were observed in 2/3 of the cases. Longitudinal studies are needed to elucidate the importance of multiple infections in cancer progression.

Our second study confirms previous publications, that over an extended time period, general HR-HPV testing without typing after conization is equally informative for prediction of treatment failure, since both persistent and new infection may be associated with recurrent disease (Arbyn *et al*, 2005; Arbyn *et al*, 2006). Follow-up could be limited to combined cytology and HR-HPV testing at 6 and 24 months after treatment, and women showing a negative result on both tests could safely be referred back to the regular screening program (Bais *et al*, 2009; Froberg *et al*, 2008; Jordan *et al*, 2008; Zielinski *et al*, 2003). HPV-testing in the follow-up period after conization is no longer a controversy and is recommended after convincing meta-analyses of its benefits (Arbyn *et al*, 2008a; Paraskevaidis *et al*, 2004; Zielinski *et al*, 2004) and is cost-effective (Coupe *et al*, 2007). To our content, it will be introduced in the coming edition of the Swedish Society of Obstetricians and Gynecologists' (SFOG) guidelines, which are now available at www.sfog.se. Some prudence is still warranted, since discordances of HPV test results and cytology will need follow-up by colposcopy and clinical evaluation (Aerssens *et al*, 2009).

Many researchers endeavor to find a uselful molecular marker in order to more specifically distinguish women at risk for progression of CIN lesions. We showed that the demonstration of $p16^{INK4a}$ accumulation in the cell nucleus is a clinically feasible way to enhance the presence of dysplastic cells in LBC samples. Immunohistochemistry does however not dismiss the need for special training of the interpreter and is associated with a considerable degree of subjectivity. Such staining may play a role as an ancillary test for selected cases at the pathologist's discretion to aid in distinguishing between premalignant cells and reactive atypias. Sensitivity is however insufficient to replace HPV testing and needs to be improved, though it is possible that $p16^{INK4a}$ expression could actually be more specific for lesions with invasive potential and only show negative results in cases that will regress. One must keep in mind that HPV infections and related lesions are usually transient, particularly in young women, and overtreatment of these women is a serious problem. A future project would therefore be to assess the clinical course of women with $p16^{INK4a}$ positive and negative LSIL and ASCUS. One could also envision a screening strategy in which

HPV-positive Pap smears are triaged with the help of this marker, which would contribute to a more evidence-based individualized clinical management.

For the first time we have also shown that several immune molecules are transcribed in the local environment of the cervix suggesting their active role in the mucosal immunity of the lower genital tract. Specifically, we have measured the mRNA expression of cytokines, phenotypic immune cell markers, immunoregulatory receptors and ligands, as well as immunoglobulins involved in mucosal immunity within the ectocervix of HPV-positive and negative women. The fact that no significant differences were found in the two groups of women suggests that the immune response is not activated in the cervices of HPV-positive women without CIN. The surprising difference among these subjects was significantly higher levels of the B cell marker CD19 in women who use hormonal contraception. Future studies will involve cytological follow-up and HPV genotyping of these women and women in follow-up after conization to identify immunological patterns associated with clearance or progression. These patterns may give a clue to new molecular markers to help distinguishing women harboring HPV with a malignant prospect.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer i livmoderhalsen orsakas av ett sexuellt överförbart virus, humant papillomvirus (HPV). HPV är en av våra vanligaste sexuellt överförbara infektioner och ger hos de flesta inga symtom, hos många könsvårtor och hos några få cellförändringar. Ett fåtal av dessa utvecklar livmoderhalscancer. I Sverige ställs diagnosen livmoderhalscancer hos ca 450 kvinnor årligen. Globalt sett är det den näst vanligaste cancerformen hos kvinnor. Införandet av vaccination mot HPV kommer att minska antalet kvinnor som får sjukdomen, men 1/3 av fallen kommer inte att förhindras eftersom de orsakas av andra HPV-typer än de som ingår i vaccinet. Ungefär 150 HPV-typer är kartlagda, varav 12-13 stycken har cancerframkallande effekt och kallas högrisk-HPV. Fortsatt deltagande i den gynekologiska cellprovskontrollen är det viktigaste sättet att förhindra utvecklingen av en cellförändring till cancer. Den gynekologiska cellprovskontrollen är avsedd att förbättra kvinnors hälsa, men skapar också problem och oro. Ett cellprov är nämligen inte helt tillförlitligt utan missar nästan 1/3 av de som borde upptäckas och påvisar felaktigt cellförändring hos 1 av 5. Flera procent av proverna visar lindriga cellförändringar som motiverar uppföljning. Hos endast ett fåtal kvinnor skulle de lindriga förändringarna utvecklas till en behandlingskrävande sjukdom. I merparten av dessa fall förbättrar alltså inte den gynekologiska utredningen kvinnans hälsa. Att testa för högrisk-HPV skulle fånga upp alla som någonsin riskerar att få cancer, men mer än 90% av de som har en högrisk-HPV-infektion läker ut utan behandling eller utveckling av cellförändring. Förbättrad känslighet i det första kontrollprovet och ökad specificitet i de prover som tas vid en uppföljning skulle minska behovet av vidare utredning. För de kvinnor som på detta sätt slipper utredning är det en stor fördel, inte minst för kvinnornas psykiska välbefinnande.

Målet med denna avhandling var att identifiera tidiga virusrelaterade och immunologiska riskmarkörer hos kvinnor med cellförändring för att kunna förutsäga en senare utveckling av cancer. Nya känsliga och specifika tester som skulle kunna förbättra den gynekologiska hälsokontrollen är av stor värde.

Det första delarbetet testade betydelsen av olika HPV-typer i lätta och oklara cellförändringar. Vi undersökte 343 cellprover med sådana förändringar från kvinnor i södra Stockholms län och fann att 53% hade flera HPV-typer i cellförändringen och att förekomsten av flera HPV-typer var högst hos de yngsta (<25 år) och de äldsta (>50 år) kvinnorna. Förekomsten av högrisk-HPV sjönk med åldern, särskilt hos de med lätta cellförändringar och var lika hög hos alla kvinnor oavsett cellförändring efter 25 års ålder.

Den andra studien undersökte värdet av HPV-test efter konisering, dvs. bortoperation av cellförändring. Totalt 90 kvinnor kom på två kontroller efter koniseringen. Antalet återfall efter operation registrerades och förekomsten av olika HPV-typer jämfördes mellan det första besöket och i den bortopererade biten av livmodertappen. Ett positivt HPV-test efter konisering förutsade alla återfall av måttlig och svår cellförändring och överdiagnosticerade mindre än 1 av 6. Om samma HPV-typ var kvar efter konisering hade kvinnan en mycket hög risk för återfall, men det var även de med nya HPV-typer

som fick återfall. Därför kan ett allmänt HPV-test förutsäga återfall med hög känslighet.

Det tredje delarbetet testade hypotesen om att påvisande av $p16^{INK4a}$ (en cancermarkör) skulle kunna användas för att skilja HPV-orsakade cellförändringar från andra inflammatoriska förändringar, något som i praktiken kan vara en svår differentialdiagnos. Vätskebaserade cellprover från 118 kvinnor som kallats för utökad provtagning pga. fynd i den allmänna cellprovskontrollen testades med immunfärgning för $p16^{INK4a}$. I stort sett alla fall som visade sig vara normala med vävnadsundersökning visade också ingen eller svag $p16^{INK4a}$ -färgning. Immunfärgning av $p16^{INK4a}$ i vätskebaserade cellprover kan således användas för att förbättra den gynekologiska cellprovskontrollen, som ett första steg för att skilja ut prov som inte behöver analyseras avseende högrisk-HPV. Analys av $p16^{INK4a}$ har dock en begränsad känslighet när det gäller att visa förekomst av högrisk-HPV, så det förefaller motiverat med fortsatta studier riktade mot alternativa markörer.

Den fjärde studien mätte 19 olika immunförsvarskomponenter i livmoderhalsen med PCR-teknik hos 24 frivilliga friska kvinnor. Det visade sig att 11 av dessa bar på HPV utan symtom. En HPV-infektion påverkade inte någon av de mätta komponenterna, men kvinnor som åt p-piller eller hade hormonspiral hade högre värden av ämnet CD19, vilket antyder att hormoner kan påverka antikroppsproducerande celler.

Sammanfattningsvis visar avhandlingen att HPV-testning har stort värde i uppföljningen efter operation av cellförändringar för att tidigt upptäcka återfall, men att typbestämma HPV inte ökar testets känslighet. Vi typbestämde lätta och oklara cellförändringar hos kvinnor i olika åldrar för att visa att spektrumet inte skiljer sig åt mellan dessa grupper särskilt efter 30 års ålder. HPV-test bör därför utföras på alla kvinnor över 30 år med lätta och oklara cellförändringar för att kunna avstå från vidare undersökning av de som inte har cancerframkallande högrisk-HPV. Testning med cancermarkören $p16^{INK4a}$ kan möjligen användas som ett första steg för att skilja ut prov som inte behöver analyseras avseende högrisk-HPV. Immunförsvarskomponenter i livmoderhalsen påverkas inte av en HPV-infektion, men fortsatta studier är nödvändiga för att utröna om någon komponent kan användas som markör för att hitta det fåtal kvinnor som får cancer så småningom.

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