Tissue tumor marker expression in normal cervical tissue and in cervical intraepithelial neoplasia, for women who are at high risk of human papilloma virus infection, are smokers, contraceptive users or in fertile age

RAGHAD SAMIR
Abstract

The aim of this research was to study the correlation between tissue tumor marker expression and HR-HPV infection, smoking, hormonal contraceptive use and sex steroids in women with cervical intraepithelial neoplasia or normal epithelium. The study investigated the expression of 11 tumor markers in cervical biopsies obtained from 228 women with different diagnoses ranging from normal cervical epithelium to various stages of CIN. 188 women were recruited at our colposcopy clinic (out-patient surgery, Department of Obstetrics and Gynecology, Falun Hospital) for laser cervical conization or a directed punch biopsy, either because of a vaginal smear (Pap smear) that showed cytological findings suggesting CIN, or because of repeated findings showing atypical squamous cells of undetermined significance (ASCUS). For 40 volunteers, punch biopsies were taken from the normal cervical epithelium. The time period for this study was 2005-2007.

Study I: 228 women, of whom 116 were tested, 64 were positive to HR-HPV. The results showed that Ki67 tumor cell proliferation index was the only marker that independently correlated to both the presence of HR-HPV and the severity of cervical lesions.

Study II: 228 women, of whom 83 were smokers (36, 9%). Smokers showed lower expression of p53, FHIT (tumor suppressor markers) and interleukin-10. Higher expression of Cox-2 and Ki-67 (tumor proliferation markers).

Study III: 195 women who were premenopausal. There was increased p53 expression (tumor suppressor) in the progestin-IUD users compared to non-users. Decreased IL-10 expression (immunological marker) was observed in both COC users and any progestin-only users.

Study IV: Serum from 80 premenopausal women was available. The main finding was that the increased levels of serum progesterone and estradiol were associated with increased Cox-2 expression (proliferation marker). Serum progesterone and estradiol levels influence cellular and extracellular proteins which have been associated with neoplastic development in normal epithelium and CIN.

Conclusion: The results of these studies support previous epidemiological findings on the role of smoking, contraceptive use and sex steroids as co-factors in development of CIN and that tumor marker expression varies in different grades of CIN.

Keywords: tumor markers, cervical intraepithelial neoplasia, smoking, contraceptive use, sex steroid hormones, HPV infection

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To my family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


II Samir R, MD; Anna Asplund, PhD; Tibor Tot, MD, PhD; Gyula Pekar, MD; Dan Hellberg, MD, PhD. Tissue tumor marker expression in smokers, including serum cotinine concentrations, in women with cervical intraepithelial neoplasia or normal squamous cervical epithelium. Obstet Gynecol 2010;202:579.e1-7.


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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ASCUS</td>
<td>Atypical squamous cell of undetermined origin</td>
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<td>CIN</td>
<td>Cervical intraepithelial neoplasia.</td>
</tr>
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<td>CPS</td>
<td>Cytology pap smear</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<td>COC</td>
<td>Combined oral contraceptives</td>
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<tr>
<td>DAB</td>
<td>3, 3-diaminobenidine</td>
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<td>HPV</td>
<td>Human papilloma virus</td>
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<tr>
<td>HR-HPV</td>
<td>High risk HPV</td>
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<td>LR-HPV</td>
<td>Low risk HPV</td>
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<tr>
<td>HSIL</td>
<td>High grade squamous intra-epithelial lesion</td>
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<tr>
<td>LSIL</td>
<td>Low grade squamous intra-epithelial lesion</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IL 10</td>
<td>Cytokine, immunosuppressive</td>
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<tr>
<td>LBC</td>
<td>Liquid based cytology</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PPV</td>
<td>Positive predictive value</td>
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<tr>
<td>MID</td>
<td>Medicated intrauterine device</td>
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<td>SCJ</td>
<td>Squamous-columnar junction.</td>
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<tr>
<td>Syst-p</td>
<td>Systemic progesterone users.</td>
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<tr>
<td>TZ</td>
<td>Transformation zone</td>
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<td>TMA</td>
<td>Tissue microarray</td>
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Introduction

Cervical cancer is the second most common female cancer worldwide and according to the most recent data, an estimated 528,000 new cases of cervical cancer occur among women each year, the vast majority of them in developing countries (1). In 2008 an estimate of 275,000 deaths from cervical cancer was registered, placing cervical cancer as the fourth most common cause of cancer death among females worldwide (2, 3). The main cause of cervical cancer is the persistent human papillomavirus infection (4), but invasive cancer is a rare complication of HPV infection, itself a rather common type of viral infection (5). HPV virus infection is the main cause of cervical cancer but it needs co-factors such as smoking, sex steroids and contraceptive use in order to invade the immune system of the host.

Since the beginning of cervical screening programs in the early sixties, mortality and morbidity from cervical cancer has been reduced. The aim of screening programs is to recognize cervical intraepithelial lesions and establish the right diagnosis in order to treat those lesions which are liable to lead to cancer. The need for more than diagnostic cytology appears reasonable, in order to decrease the unnecessary over-treatment of non-specific cervical lesion. The main purpose of many studies done in this field is to find the tumor markers which can be used as diagnostic tools or surrogate markers for HPV infection. These surrogate markers might also aid the differential diagnosis among different cervical intraepithelial lesions and help to distinguish those lesions liable to lead to cervical cancer from non-specific changes, such as atypical squamous cells of undetermined origin ASCUS.

Two things must be taken in consideration: firstly, the risk of subjecting women with low grade or non-specific lesions to repeated testing, biopsies and colposcopies, and secondly, the cost and time required in order to establish the right diagnosis.

Anatomy:
The cervix is the most inferior part of the uterus protruding into the vagina. The cervix is divided anatomically into a vaginal portion and a supra-vaginal portion. This cervical layer consists of the inner mucosa, smooth muscles. Normally in non-pregnant females the cervix length is about 3cm. The end cervix is a mucosal lining of the cervical canal (covered with a single layered columnar epithelium) while the ectocervix is the vaginal part of the cervix (covered with stratified squamous epithelium).
The term SCJ refers to the squamocolumnar junction where the metaplastic changes occur. The majority of carcinomas begin at the transformation zone of the cervix uteri where the metaplastic changes convert the columnar mature cells to a different mature cell type. The process usually involves conversion from a columnar cell to a stratified squamous cell.

The area between the congenital junction (the original squamocolumnar junction) and functional junction (post puberty) is called the transitional zone TZ.

Screening programs:
Organized screening programs using cervical cytology (pap smears or liquid based cytology) have been shown to be effective in decreasing mortality and incidence of invasive cervix cancer. Mass screening has also reduced the present incidence by more than half since the 1960s and 1970s in many high-income countries (6-8).

Cervical cancer screening was implemented in Sweden in the mid-60s and since then a significant decrease in cervical cancer has been observed. Annually around 700,000 pap smears are taken in Sweden, with a total population of 9,000,000. Approximately 600,000 are taken within the mass-screening program, the remainder being taken as part of other gynecological examinations. The overall incidence of cervical cancer declined by 67% over a 40-year period, from 20 cases per 100,000 women (world standard rate) in 1965 to 6.6 cases per 100,000 women in 2005. During the last decade, however, the incidence has stabilized (Cancer incidence in Sweden. National Board of Health and Welfare 2008).

One explanation for the stability in the cervical cancer incidence is the nonparticipation in the regular screening programs; another explanation is the poor management of abnormal smears found during the primary screening(9). According to the Swedish nationwide audit of the cervical screening program, 32% of women with invasive cervical cancer had a pre-diagnosis of abnormal smear finding under their participation in the normal screening interval (10).

Dysplasia
Dysplasia is used to describe the premalignant squamous cervical cellular changes which range from mild to moderate and severe, but dysplasia has been replaced by the term CIN which follows the Richart’s description system (7). The European guidelines for quality assurance in cervical cytology terminology recommend CIN classification for histopathological diagnosis (11).

CIN is used to describe the histological changes which are detected with biopsy and is divided into three degrees of severity. CIN I is a low grade
lesion (formerly called mild dysplasia) manifesting mildly atypical cellular changes in the lower third of the epithelium. HPV viral cytopathic effect (koilocytotic atypia) is often present. CIN II is a high grade lesion (moderate dysplasia) referring to moderately atypical cellular changes confined to the basal two-thirds of the epithelium with preservation of epithelial maturation. CIN III is a high grade lesion (formerly called severe dysplasia or carcinoma in situ). It refers to severely atypical cellular changes encompassing more than two-thirds of the epithelial thickness, and includes full-thickness lesions.

Another terminology used to describe these changes at the cytological and histopathological level is LSIL (Low-grade squamous intraepithelial lesion) and HSIL (High-grade squamous intraepithelial lesion). LSIL corresponds to CIN I and HSIL comprises CIN II and CIN III (12, 13).

**Background to cervical cytology screening**

The Pap smear (vaginal cytology) was developed by George Papanicolaou in the 1920s. Later, in 1941, G. Papanicolaou and the gynecologist Herbert Traut published the first major article on the use of vaginal smears for the diagnosis of cancer of the uterus (14). Soon thereafter, the pap smear (named after Papanicolaou), was born and is still considered a sensitive, specific, simple and effective cancer screening test. Simultaneously, Hans Hinselmann and Leitz technicians devised the first working binocular colposcopy (hinselmann H Einfuhrung in die kolposopi. Hamburg 1933). In 1925, Hinselmann published the first paper on colposcopy, and in 1933 the book ‘Einfuehrung in die Colposcopy’ was published. Colposcopy was developed further in 1925 by Hinselman and Eduardwirth, but routine colposcopy examinations were confined to Germany until the 1960s. In the United States, as early as 1929, Levy described the importance of studying the genital tract with some degree of magnification and subsequently Emmert published an article introducing colposcopy to North American physicians (Emmert F. The recognition of cancer of the uterus in its earlier stages. J AMA 1031; 97:1684). By 1932 the colposcopy technique was used in a few centers. The present form of colposcopy started in 1953 when Bolten introduced modern colposcopy in United States. Initially, it served as a tool to identify women with asymptomatic early invasive disease. Subsequently, it has also helped physicians identify pre-invasive squamous neoplasia of the cervix. At a meeting of the American College of Obstetricians and Gynecologists in Miami in 1964, a group of enthusiastic colposcopists identified the need for a Colposcopy Society. Thereafter, through the dedicated efforts of many members of the Society, various colposcopy courses were initiated. In 1981, Hamou introduced the microlpohystrescope for the examination of stained cells in vivo at high magnification.
Cytology tests/screenings:

The conventional cytology Pap smear (CPS) has been successfully used since the sixties. Cells are taken from the cervical canal and the uterine cervix, these cells smeared on a glass slide and fixed in 95% ethyl alcohol to prevent air drying. The slide is then sent to the lab for analysis. The sensitivity of this test depend on the number of the tests done within a period of time, a single test has a sensitivity for CIN II+ (70-80%) (13, 15-17).

The specificity is high, 90-96%, and the positive value PPV is about 42%. Repeating the test within an interval of 3-5 years increases its sensitivity.

In order to evaluate the sensitivity and specificity of the test we need to understand the differences between test threshold (the point when the test is positive) and the reference standard threshold (the point when the reference standard is positive). In CPS the most accurate testing is to consider LSIL as the test threshold and CIN2-3 as a reference standard (this will give as a sensitivity of 70-80% and specificity 95%) (18).

According to 2 different studies done by Soost. H, et al 1991 and Benoit AG, et al 1984 (19, 20) comparing single and repeating Pap testing, the sensitivity of any abnormality on a single test for detecting high-grade lesions was 55% to 80%. Because of the slow-growing nature of cervical cancer, the sensitivity of the repeating Pap testing is likely to be higher.

Liquid –based cytology (LBC) is another type of test that has been used recently based on the idea that cells will be sampled with a cervix–brush or with a plastic spatula and endo-cervical brush. The smear will directly immersed into a special liquid solution (20 ml preservCytSolution) and send to the lab for analysis. This method provides a thin monolayer of the cells on the slides which are easier and quicker to read than the CPS and the material can be used to do more testing such as human papillomavirus (HPV) DNA testing.

LBC has been compared with CPS in a wide range of studies, most of these studies showing an increase in the sensitivity for detection of pathological changes (LBC eliminates blood and extra derbies from the specimen) (21-24).

A meta-analysis done by E.Davey et al (24) showed that in high–quality studies CPS classified more slides than HSIL and fewer slides than ASCUS as compared to LBC. The benefits and disadvantages of these tests can be discussed as a matter of economy and accuracy. Many RCT have been done to find which test is the more reliable; Fröberg et al 2013 found no significant differences in screening performance between LBC +HPV triage and CPS(25, 26).

Sigurdsson K et al published a comparative study done in Reykjavik, Iceland which included 98477 women aged between 20-69 years old. The result of the study showed that LBC tests are no more sensitive than Pap smear in detection of high grade cytology or CIN2+histology, irrespective of age.
LBC test increased the frequency of low grade lesion below the age of 40, but decreased the total number of abnormal cytology tests over the age of 40(27).

The usefulness of cytology smear only use (CPS,LBC) in establishing the right diagnosis in cases of HILS (CIN 11-CIN111) is well known, while this usefulness is questioned in cases of atypical squamous cells of undetermined origin (ASCUS) or in LSIL. In order to establish the correct diagnosis or to reach subsequent diagnostic procedures, many different health guidelines have been introduced such as repeating the cytology, testing for HR-HPV infection, colposcopy and cervical punch biopsies or a combination of 2 or more of these.

It is well known that the progression risk of these intraepithelial lesions from CIN1 to CIN111 within 5 years is 2.6% (28), but also to be considered are the hazards of over-treatment and subjecting these women to invasive procedure (over-treatment) such as bleeding, pain, sexual dysfunction (29) and pregnancy–related morbidity. The rate of invasive cervical cancer in women with high–grade squamous abnormal cytology within 24 months is 1.44% (30). The rate in histologically verified CIN III without treatment was 31% after 30 years follow up (31). In recent years surrogate markers have been used to estimate the high risk precursor or invasive cervical cancer lesion such as P16/Ki67.

**HPV tests/screening**

Since the progression of cervical intraepithelial lesion to cervical cancer is considered to be quite slow, and the period between the establishment of HPV infection and invasive cervical cancer development is many years, it will be logical to find an effective screening test. It is important to understand that CIN lesions have a 30% risk to progress to invasive cancer if untreated within many years (31) while CIN 2 is considered as the threshold between treatment or not. This fact shows the need of finding an effective test to be used primarily or as a part of triage system. In this way the screening for HR-HPV will make it easier to decide which lesions we should treat and will help to identify women at risk (32) (33, 34). (35).

The low risk HPV testing does not identify women at risk for low risk CIN II, III (36, 37) . HPV testing has been considered as co-test for screening cytology in many countries. Primary cervical screening for HR-HPV has been found to be more sensitive in detecting CIN II+ compared to cytology but unfortunately with less specificity (38). HPV testing has been used as a part of triage screening including both cytology and HPV testing and has showed a higher sensitivity in detecting CIN III+ than cytology by itself (39). It is important to shed light on the benefit of triage screening since each test alone (as a single test) has its own problems regarding specificity. HPV testing shows less specificity among younger women (over-diagnosis regard-
ing CIN II (40), whilst cervical cytology alone is more dependable among women over the age of 50. (41).

A Swedish study done in 2011 by Gyllensten. U and et al (42) found that repeating the self-sampling HPV test can increase the specificity of cervical cancer screening of women aged 30-65. The prevalence of CIN II+ lesions in women with two positive HPV tests varied from 49% in women aged 30-39 years to 24% in women aged 50-65 years. Short-time repeat HPV testing increased the specificity for detection of CIN II+ lesions from about 94.2% to 97.8%.

**Different HR-HPV testing types**

It must be taken into consideration that HPVs cannot be cultured and the use of serology assays has its own limitations concerning the accuracy of the test. There are three types of HPV test: those tests that can detect HPV nucleic acid DNA, RNA, or the cellular markers of HPV-associated malignant transformation in clinical samples. (43, 44).

There are many types of assays available nowadays:

HPV tests are divided into two categories:

**DNA detectors:**
1. HC2 test (Hybrid capture 2 assay).
2. PCR. (Polymerase chain reaction).
3. LA. (Linear array HPV genotyping test).

**RNA detectors:**
1. HPV mRNA
   Home screening test (self-collection of cervical (and vaginal) fluid for HPV testing).

This is a new approach regarding HR-HPV testing by using an FTA card which provides a solid support that stabilizes the DNA. The sampling kits can be used accurately and the risk of damaging the sample will be very low. HR-HPV testing there with the PCR method has been found to be more sensitive for CIN II , CIN II+ compared to conventional cytology (45).

**Histopathology:**

The most common types of cervical cancer are squamous cell carcinomas and these represent about 75% of all cervical cancer - about 15% are adenocarcinomas. Neuroendocrine and other histological types constitute about 10% (46, 47).

Dysplasia is the premalignant squamous cell abnormalities that range from mild, moderate to severe dysplasia, and eventually to carcinoma in situ, but this classification has been replaced by cervical intraepithelial neoplasia (CIN). CIN is also used for histological abnormalities that are histopathologically diagnosed in cervical biopsies.
Cell biology

The cell cycle (eukaryotic cells) is programmed so that the cell will replicate its own DNA, correctly leading to the formation of two identical cells. In order to ensure that this will happen, there are many check points at which the control and repair of the DNA can be permitted. Alteration of this mechanism is the key factor in the initiation of cancer changes in all organs in the human body (Charlotte E, Gullberg U. Cell biology. Chapter 15. Denmark 2003. ISBN91-44-20047-3).

Three main phases in the cell cycle are:

**G0 (the resting phase):** in this stage the cell is considered to be inactive but is still metabolically and functionally active.

**G1: The first check point (the cell growing phase):** in this stage the cell starts to grow as a response to external factors (growth factors, cytokines etc.). The cell cycle will progress to S phase according to two mechanisms:

1- E2F transcription factor activation.

E2F (a family of transcription factors (TF)). Three of them are activators: E2F1, 2 and E2F3a. Six others act as suppressors: E2F3b, E2F4-8. All of them are involved in cell cycle regulation and DNA synthesis.

The E2F1 is normally controlled or inhibited by the Rb protein. The Rb tumor suppressor protein (Rb) binds to the E2F1 transcription factor, preventing it from interacting with the transcription machinery of the cell. In the absence of Rb, E2F1 facilitates the trans-activation of E2F1 target genes that enable the G1/S transition and S-phase.

2- Activation of the CDK4/6-cyclin D complex:

In human cells the presence of cyclins is important since their major role as eukaryotic proteins is the activation of the CDK4/6 –cyclin D complex.

This complex phosphorylates the Rb (tumor suppressor) leading to inactivation of Rb protein and enhancing the transcription factor E2F which allows the cell to enter G1-S phase.

The alteration of these two mechanisms is the key factor in the development of cancerous changes at cell level. Rb is one of the targets of the onco-genic human papilloma virus protein E7. By binding to Rb, they stop the regulation of E2F transcription factors and drive the cell cycle to enable virus genome replication. The other mechanism that plays a major role in cell cycle is the activation of P53.

P53 is considered to be the guardian of genome in the human body. Normally p53 is controlled by the negative regulator, mdm2, its main function being to inactivate P53.
P53 activates as a response to cell injury (viral infection or hypoxia) allowing the cell to repair itself and enter what is called cell arrest. Apoptosis is another form of response to injury where p53 prompts cell death instead. The mechanism behind the decision between apoptosis or repair is still considered to be complicated and difficult to explain. P53 acts also in another way by stimulating the p21 and p16 which act as inhibitors to the CDK4/6 complex, leading to the inactivation of cyclin D and preventing it from affecting Rb protein.

S (DNA synthesis).
In this phase DNA synthesis begins, where the Cyclin/cdk2 complex controls the phosphorylation of DNA polymerase, at the same time stopping another replication of DNA.

G2: the second check point is G2, in which the cell will be controlled in order to enter the mitosis phase. This process is controlled by the CDKs which are activated by phosphorylation by Mitosis Promoting Factor (MPF).

M (mitosis).

Etiology, co-factors and Risk factors

HPV infection.
Co-factors:
Smoking.
Contraceptive Use.
Sex steroids.
Genetic predilection

Risk factors:
Age.
HIV/AIDS, Immunodeficiency.
Low socioeconomic status
Poor access to health care
Multiple sex partners/ first intercourse at an early age.
High parity
Human papillomavirus infection.

Human papillomavirus belongs to the family Papillomaviridae, which includes viruses infecting many different vertebrates. They are strictly species- and organ specific. The HPV life cycle in the cervix is confined to the epithelium (48). At the border between squamous cell epithelium covering the vagina and glandular epithelium covering the uterus, the transformation zone is the target for HPV invasion (26). HPV is transmitted in desquamated genital epithelial cells which after their own degeneration leave the HPV capsids free to bind themselves to the target tissue such as TZ of the cervix, anal verge or oropharynx (the areas most exposed to micro trauma) (49, 50). HPV infection is one of the most common sexually transmitted genital infections. It is mostly clinically silent and self-limiting. Some women may remain persistent carriers of the viral infection and develop a high risk of progression to CIN and invasive cervical cancer (26). Most HPV-related lesions resolve spontaneously, and progression to cervical neoplasia is relatively rare (51-53). A key factor in permitting the disease to progress is the ability of HPV to evade the immune system and establish a persistent infection. Even when immune response is achieved there is no full protection from future HPV infections. The lifetime risk of genital HPV infection is approximately 80%. For many HPV-infected women (90%), their immune defense will generally eliminate the infection within 12 to 36 months (54). In remaining cases, progression to cytological abnormalities and CIN is observed in 5% to 10% of persistent HPV infections (26). Within a period of 7-15 years less than 1% of these infections lead to carcinoma (55, 56). According to a study by Kleter et
al, other factors such as genetic predisposition, frequency of reinfection, genetic variation within HPV type, co-infection with more than one HPV type and hormone levels may influence the clarity of the primary HPV infection.(57).

The HPV virus
The HPV has a relatively small genome (double-stranded DNA) of 8000 base pairs which are organized in 3 regions: The long control region (upper regulatory region URR) and 2 coding regions (the late L and the early region E)(58).

The virion is encased in a non-enveloped 72-sided icosahedral protein capsid. The URR is a non-coding region that is responsible for regulation of viral replication and transcription in the early region. The functional HPV genes are found only in the early region and the late region, they referred as ORF (open reading frames). Each ORF is read by the RNA polymerase. The early region is responsible for viral replication in the early HPV life cycle while the late region is responsible for capsid production late in the life cycle.

The early coding
The early coding region is divided into E1-E7.

The life cycle of HPV in the cervix mucosa and its main target in the transformation zone started with the integration of the HPV DNA with the host cell genome. Because it has no DNA polymerase, it needs to bind itself with host polymerase via E1 and E2. The cell transformation starts at this point with the help of E6 and E7 (8). E7 binds to and degrades Rb and initiates the cell division via E2F. E6 binds to and degrades p53. E6 and E7 will disable the anti-oncogene effect of p53 and Rb and will result in cell proliferations and immortalization. Thus, impairment of the tumor suppressor genes p53 and Rb is the key step in cell immortalization (8).

The late region
The two late regions L1 and L2 are responsible for the construction of the capsid of the infected HPV genome. The capsid is crucial for HPV because without the surrounding capsid the HPV DNA is considered as non-infective. The production of capsids appears to happen in the superficial layer of cervix to allow delivery of HPV infection from host till host (59).

High- and low-risk HPV infections
More than 174 HPV types are known and new types are continually being discovered (59). Of these, 13-18 HPV types are considered high-risk (HR-HPV) and 5 HPV types as moderate-risk for cervical neoplasia. Globally, HPV 16 and HPV 18 dominate in CIN and cervical cancer. According to a retrospective cross-section worldwide study, published in The Lancet by de
Sanjose S et al, approximately 70% of cervical cancer was caused by Hr-HPV-16 and-18 while HPV -31,-33,-35,-45,51,-52 and -58 contribute to the remaining 20% of cases(60).

**Co-infection with both HR-HPV and LR-HPV**

Infection with low risk HPV (HPV6,HPV11) causes condyloma (genital warts). It is well-known that LR-HPVs have low carcinogenic ability but a Swedish study by Sundström et al. 2015 found that co-infection with both high-risk HPV and low-risk HPV can decrease the risk for future invasive squamous cancer development (61). This finding can be explained by the ability of low-risk HPV 6 and HPV11 to act as antagonists to the infection with high-risk HPV 16 resulting in the hampering of the risk for invasive cancer to be lower than expected (62).

**The life cycle of human papillomavirus in the cervix:**

As mentioned above, HPVs are intracellular parasites which attack the basal layer of the cervix through minor abrasions in order to start their own life cycle. The initial attachment of HPV to the cells starts when L1 protein interacts with HSPG (heparin sulfate proteoglycans) receptors, up-regulators on the basement membrane during wound repair. This interaction will promote the internalization of the virion into the cell by causing viral structure changes that allow HPV L2 to be exposed to the local enzymes, furin or PC 5/6. These changes allow previously hidden epitopes of L1 molecules to become exposed to receptors on the newly-formed basement membrane (63). This gives the HPV its unique privilege in only infecting the basement layers of the cervical epithelium. It has been shown that by using antibodies targeting the capsid proteins L1, L2, the virion will lose its ability to attach to the basal membrane and enter the cells. The virus will be transported into the cell nucleus where an episomal reaction will start (HPV DNA is self-replicating plasmid which is not integrated with human chromosomes) and this will result in the genome replicating itself once per cell cycle during S-phase (64). The infected basal cells, which show signs of cell disruption as a result of the viral infection, continue their differentiation and migration to the epithelial surface(65). During the early stages of infection, the copy number of viral genomes is between 50 to100, and the viral genome exists as extrachromosomal plasmid or an episomal from the replicates, as the host cell chromosomes replicate (66). This viral infection prompts the host cell to proliferate abnormally inducing various kinds of lesion (Flat warts, papillary warts, CIN, cervical cancer). As it is well-known that not all individuals who have been exposed to HPV infection exhibit these changes, a question arises about other cofactors that play a major role in the outcome of the HPV infection.

These cofactors can be internal, such as genetic effects, or external, such as environmental. HPV integration into the host genome will initiate a chain
of events involving the impairment of the tumor suppressor genes P53 and Rb. E7 and E6 start to express by affecting their target proteins. E7 binds to Rb, a tumor suppressor that, as mentioned above, inactivates E2F (Transcription factor). The activation of E2F causes the transcription of genes responsible for DNA replications and cell division. E6 virus protein binds to P53. Normally P53 is responsible for triggering cell repair and even apoptosis. The main principle in the HPV infection is the need to induce DNA synthesis in the host cells in order to replicate its own viral DNA. This process is usually started with the interference of E7 with Rb, which is normally responsible for inducing apoptosis of the infected cell. The difference in the mode of action between the HR-HPV and LR-HPV is the mode of E6 and E7 capacity (67). LR-HPV is incapable of degradation of p53 and of inactivating Rb and thus cannot induce cell immortalization and oncogenesis. Single amino acid differences of the E7 protein in LR-HPV and HR-HPV lead to higher affinity of HR-HPV for RB protein and p53. This induces an alteration in the normal cell cycle, in that the cell will be totally controlled by the virus and the process of immortalization of the epithelial cells will be initiated. These changes will lead to the start of cancerous and precancerous lesions in the cervix (68, 69). Genome amplification starts in the mid-layers of the cervical epithelium. The cells will then express viral E4 and enter the cell cycle phase S to G2. When the cell cycle is finished in the upper epithelial layers, the viral L1 and L2 proteins start forming the capsid of the virus and packaging of the viral genome begins finalizing.

Figure 2. The pathway for HPV from the cervical basal cells to the shedding of new virus particles.
Figure 3. This illustration shows the effect of HPV virus on the basal cervical cells and the integration into the host genome.

- mdm2.
- P53-mdm2 complex (inactive form av P53).
- Activation of P53 by infection/Hypoxia.
- Inactivation of P53 by cancer (mutation).
- Degredation of P53 by HPV oncogen E6 (inactive form).

Figure 4. Cell biology and the pathway of tissue markers in the cell cycle during HPV infection.

- Activation of P16/P18 pathway by P53.
- Cell repair/Apoptosis.
- P16/p18 inactivt the Cdk pathway.

- Cyclin D.
- Cdk4/Cdk-CyclinD.
- Rb inactiviation by phosphorylation.
- The cell will be activated and enter G1/S phase.
- Rb inactivitation by protolysis (HPV oncogen E7).
Host immunity
The important factor in determining the risk factor of malignancy is the clearness of the viral infection by the host immune system. When there is a defect in the host immune system, HPV does not clear within a short period. HPV is mostly a transient infection with the median time to clearance of incident cervical HPV being just over 9 months, and nearly 91% of infections becoming undetectable within 2 years (70). The likelihood of persistent infection depends on the time required for elimination, so the longer the infection lasts the higher the risk of persistence (71, 72). HPV can escape the immunity of the host by different mechanisms (73):

- As the virus is not causing viremia (it is not cytolytic), there is no inflammation and activation of the host immune system.
- The area of replication is avascular area, so the accessibility of immune cells to the affected area will be limited.
- HPV E6, E7 onco-proteins interfere with type 1 interferon antiviral response (Interferon 1 stimulate both macrophages and natural killers’ cells to start an antiviral response).
- The lack of inflammation cytokine released from infected keratinocytes limits the activation of adaptive immunity.
- HPV E5 proteins promote entrapment of peptide –loaded HLA class 1 receptors in the Golgi body, and the lack of HLA surface expression allows the non-detection of the virus from CD8+cytotoxic T-cells.

Prevention:
The identification of the HPV oncogenes E6 and E7 led to the development of effective vaccines with immunological activation of HPV antibodies, but these vaccinations are at present still only directed against HPV 16 and HPV 18. However, some cross-reactions have been observed against other high risk HPV types (74). As 13-18 HPV types are considered as high-risk, conclusive results will not be available for the next 10-20 years. The HPV vaccine is made of Virus-like particles (VLPs) which are empty non-infectious viral shells consisting of recombinant L1 capsid proteins without containing any viral DNA. Immune response will be demonstrated by the formation of antibodies against L1.

The two main vaccines that are used nowadays are quadrivalent HPV vaccine Gardasil (Merck Sharp & Dohme Corp) against new infections with HPV 6, 11, 16, and 18, and bivalent HPV vaccine Cervarix (Glaxo Smith Kline Biologicals) which protects against new infections with HPV 16 and 18. Both HPV vaccines may offer some cross-protection against other HPV strains. The vaccines do not protect against strains which already have been infected.
In Sweden, qHPV vaccine is considered part of the organized school vaccination programs since May 2007 (75) and is given to girls aged 9-13 years. Catch up vaccination to girls aged 18-26 has been offered in some counties in Sweden (Stockholm). According to a Swedish study in 2012 by Leval A et al, where the effectiveness of qHPV vaccine was tested against Genital warts (short incubation period), the qHPV vaccine offered high protection against genital warts (caused by HPV 6, 11) among girls and women younger than 20 years. The effectiveness of the vaccine was 93% in the group of girls who received vaccination before the age of 14. The study could prove that the effectiveness declined with higher age at the first vaccination (76).

Smoking

Since the early 80s, smoking has been considered a co-factor in cervical neoplasms, involved in cervical carcinogenesis and the initiation of precancerous changes in the cervix (77, 78), although the biological mechanism is still unclear. Earlier it was seen as a surrogate for lifestyle factors, in particular promiscuity. In the early 80s some studies appeared that were able to adjust for sexual risk behavior, and the correlation between smoking and CIN remained. As in all epidemiological studies, residual confounding could not be ruled out. In the mid-80s cervical mucus in smokers with CIN was found to have very high concentrations of nicotine and its metabolite, cotinine. Thus, the nicotine levels were 40 times increased in cervical mucus compared to serum levels (79). It was a turning point and further studies have been published, essentially confirming the first report. There are now few objections to smoking being a biological co-factor in cervical carcinogenesis. Possible biological mechanisms however, by evaluating expression of tumor markers in CIN such as in the present study, have been poorly studied and in general included only single tumor markers.

Recent data showed that the carcinogenic effect of smoking combined with the alteration of the immunological response at the cell level can explain the poor prognosis of cervical cancer among smokers. A study in 2008 by Simen-Kapeu et al (80) found that smoking has the ability to alter the immunological response to HPV16/18, impairing the antibody response in HPV16/18-infected young women. Although the exact mechanism behind the effect of cotinine on immunological activity is not well understood at cell level, some studies had shown that the reduction in NK cells activity and Langerhans cell was noted in smokers compared to non-smokers (81-83). One study by Vaccarella et al (84) showed that the prevalence of HR-HPV infection is higher among smokers and is dose-dependent. Another study done in Finland followed 150 women with ASCUS or LSIL baseline cytology and normal colposcopy for up to 2 years with an examination interval of 6 months (these women were examined with cytology test, HPV testing). The result showed the increasing risk for medium-term development of high
grade CIN in women who smoked with baseline ASCUS/LSIL cytology compared to non-smokers. (85) The smoking effect persists over several years after smoking cessation, and smokers or former smokers have a 3-fold higher risk of developing CINII+ than non-smokers (HR=3.6; 95%CI 1.5-8.6) (86).

Oral contraceptives
A well-known epidemiological risk factor in the development of cervical cancer is the long-term use of combined hormonal contraceptives and there is some evidence that they serve as a co-factor to human papillomavirus (HPV) in cervical carcinogenesis (87). There is little evidence that the use of oral contraceptives (OC) facilitates HPV infection as such, but rather modulates the HPV-induced progression of cervical neoplasia (6, 7). In general, large epidemiological studies suffer from inability to control the effect of confounding factors. Even when the most important confounder, measures of sexual risk behavior, is included, there is the possibility of residual confounding. Biological evidence on the effects on cervical epithelium in OC users, compared to non-users, of any hormonal contraceptive in similar age groups, is required (79, 87-89). Few studies, if any, have investigated the effects of OC use on tumor marker expression in human cervical epithelium, normal or neoplastic, and then only for single biological markers.

The effects of gestagenic contraceptives, if any, have been poorly studied. The WHO Collaborative Study of Neoplasia and Steroid Contraceptives found a relative risk of 2.4 with at least five years’ use of injectable depot-medroxyprogesterone acetate, but results have been contradictory (90). Medicated intrauterine devices (MID), such as the levonorgestrel-releasing intrauterine system, are increasingly used. These differ from systemic use of progesterin as the steroid is released close to the cervix. No studies on the expression of tumor markers in cervical tissue by systemic or intrauterine progestogen-only contraceptives have, to our knowledge, been reported. In addition few, if any, studies have evaluated possible effects of serum levels and sex steroid levels on normal cervical epithelium and CIN.

Genetic predilection
Variation in the rate of cervical cancer development between many racial groups can be explained by genetic differences. According to a Swedish study by Patrick K, et al. in 1999, there is a genetic predilection to cervical cancer and its precursor forms, and the family risk for developing a cervical tumor has the same degree as those for other cancers such as prostate cancer(91).
A recent study by Wang SS et al. in 2010 showed that there are different genetic profiles that might be associated with persistent CINIII or the rapid progression to cervical cancer (92).

Ageing

Ageing is considered as one of the risk factors in the development of cervical cancer. The organised Swedish cervical screening program is divided into two categories according to European and national guidelines. The first group is aged between 23-50 years old and offered screening every 3 years; the second group is aged between 51-60 years and offered screening every 5 years. The benefit is high but the limitation of the screening test must be considered, especially for women in post-menopausal status.

The cause of this limitation is the low sensitivity of the test in detecting CIN III in these women. The older the women the higher the risk for insufficient test due to the high location of CIN changes in the endo-cervical canal. In order to improve the sensitivity of the screening, it has been suggested to include HPV testing as a useful test with high sensitivity in post-menopausal women - HPV infection is more common in young females and decreases with age (95).

Other risk factors have an indirect effect and can be explained by their link to HPV infection - such as multiple sexual partners, frequency of intercourse, anal sex and early sexual debut (53,93, 94). These risk factors are beyond the aim of this thesis.

Cervical and vaginal cancer risk in previously treated women

The changing in the treatment modalities since the 1990s may play a major role in the increasing risk for the development of cervical or vaginal cancer in women who were previously treated for cervical intraepithelial grade 3. It was proven in a study by Strander B, et al that women previously treated for cervical intraepithelial neoplasia grade 3 are at risk of developing invasive cervical or vaginal cancer. According to this study both cancers are caused by HR-HPV infection, and by the incomplete treatment of the intraepithelial changes grade 3 in the cervix, regardless of whether hysterectomy was done or not (96).

Tumor markers

Most tumor markers are proteins that are produced either by tumor cells or by the host cells as a response to the presence of cancer cells. As such, they indicate the presence of cancer and may also reflect the severity of these cancerous changes. Tumor markers can be used to identify the risk of developing cancer in asymptomatic subjects. Tumor markers assist the
pathologists in making the correct diagnosis, typing and grading the tumors, and in tracing the origin of metastasis of unknown primary tumor. In addition, some of them are used as prognostic markers while the others as predictive markers indicating the usefulness of target therapy.

A number of biomarkers (tumor markers) have been studied in CIN. The objective has generally been to find tumor marker expression that could discriminate between normal cervical epithelium and CIN, aiding the histological diagnosis of CIN grade, or as a surrogate for HPV infection. Other studies have focused on determining the aggressiveness of CIN, but with inconclusive results. During recent years much attention and extensive research has been directed towards p16, retinoblastoma protein (Rb), p53 and Ki-67/MIB1 (97). The present study evaluated expression of eleven tumor markers and their correlation, if any, to HPV infection, smoking, oral contraceptive use, and levels of serum progesterone and serum estradiol. As this was practically virgin soil we analyzed some tumor markers based on our previous results on invasive cervical cancer (98, 99) and tumor markers of possible clinical importance that were previously studied in cervical neoplasia. Tumor markers were chosen to represent different mechanisms in carcinogenesis. In our study we investigated the correlation between tumor marker expression in cervical intraepithelial neoplasia with HR-HPV infection and suspected co-factors such as smoking, serum levels of sex steroid hormones, and hormonal contraceptives. Possible response of tumor marker expression after exposure to co-factors would provide biological support for epidemiological associations. Tumor markers were divided into different groups according to their mode of action, such as proliferation markers, tumor suppressors, apoptosis, cell-to-cell interactions, angiogenesis and oncoproteins.

**Proliferation markers**

**EGFR (epidermal growth factor receptor)**

A cell surface receptor for a member of the epidermal growth factor family - Stanley Cohn received the Nobel Prize in medicine 1986 for his discovery of EGFR.

The mechanism of activation of EGFR is by binding to EGF or TGF (TGFα), resulting in auto-phosphorylation of Tyrosines inside the cell (the ability to interact with specific cell-surface receptors and transduce intracellular signals). This activation normally results in DNA synthesis and cell division.

Mutation of the EGFR gene leads to overexpression of EGFR which results in constant activation.

This constant activation produces uncontrolled cell proliferation. This is why EGFR has been associated with poor prognosis in different kinds of
cancers and is considered to be a prognostic marker especially in cervical cancers (100-103).

**Ki-67/MIB-1 (non-histone protein) is a cellular marker for proliferation.**

It is present during all active cell cycle phases (G1, S, and G2) but not G0 (the resting phase). It has also been shown that Ki-67 expression increased with advanced stages of cervical neoplasia. (104).

Ki-67 has been found to be more intensely stained in HPV-positive than in HPV-negative epithelium (105, 106). Although Ki-67 is mostly expressed in proliferative cells, a recent study by Bullwinkle J, et al showed that Ki-67 can be detected at sites linked to ribosomal RNA (rRNA) synthesis in the non-proliferative stage of the cell cycle. This finding may call into question the use of Ki-67 as a proliferative marker (107).

**Apoptosis inhibitors**

**COX-2**

Its main action is to inhibit apoptosis by enhancing proliferation, inflammation response to tumor, angiogenesis and tumor invasion, the mode of action depending on a variety of events. The mechanism of action is through the cyclooxygenase pathway which regulates the production of prostaglandins from arachidonic acid in inflammatory conditions. Cox2 acts as a mediator in this process. A high expression might indicate poor prognosis in cervical cancer. (108-110)

**Tumor suppressors**

**P53:** P53 is considered to be the guardian of genome in the human body. P53 activates as a response to cell injury causing G0/G1 cell cycle arrest allowing cell repair or apoptosis (programmed cell death)(111). P53 can be inactivated or deactivated by different kinds of cancer in which these changes led to the disability of the p53 to bind to target DNA sequences and the beginning of the repairing process. Inactive p53 mutants with a subsequent loss of tumor suppression are often found in cancer cells allowing for increased proliferation (112).

HPV E6 oncogene binds itself and inactivates P53 as the first step of viral DNA replication. Mutations of p53 that rarely occur in the cervical cancer have been found in other types of cancer, leading to the loss of the tumor suppressor function of p53 and the beginning of oncogenic activity (113).

**Rb (Retinoblastoma susceptibility gene)**

Rb is encoded as a nucleoprotein that plays a major role in the cell cycle at the G1-S phase (114, 115). The Rb tumor suppressor protein (Rb) binds to the E2F1 transcription factor, preventing it from interacting with the transcription machinery of the cell. In the absence of Rb, E2F1 facilitates the
trans-activation of E2F1 target genes that enable the G1/S transition and S-phase (116). Rb is one of the targets of the oncogenic human papilloma virus protein E7. By binding to Rb, they stop the regulation of E2F transcription factors and drive the cell cycle to enable virus genome replication.

**FHIT**

Fragile histidine triad (FHIT) acts mainly as tumor suppression and control of apoptosis. The gene involves the common fragile site on chromosome p3, where carcinogen-induced damage can lead to translocations and uncharacteristic transcripts of the gene.

These abnormal transcripts from the gene have been found in about half of esophageal, stomach, cervical and colon carcinoma.(117).

**P16**

HPV 16 has been found to integrate at FRA3B fragile site and is frequently demonstrated in cervical carcinoma.(118, 119)

P16 (cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1): p16 (p16INK4a) is a tumor suppressor protein, that in humans is encoded by the CDKN2A gene. The CDKN2A gene is frequently mutated or deleted in a wide variety of tumors. p16 plays an important role in cell cycle regulation by decelerating cell progression from G1 phase to S phase, and therefore acts as a tumor suppressor implicated in the prevention of cancers such as melanoma, oropharyngeal squamous cell carcinoma, and esophageal cancer. P16 is an inhibitor of cyclin dependent kinases such as CDK4 and CDK6. These latter kinases phosphorylate retinoblastoma protein (Rb) which eventually results in progression from G1 phase to S phase.

Several studies have investigated the usefulness of p16 INK4a expression in HR-HPV infections (112, 119, 120). In an early study, p16INK4a expression showed a high sensitivity for HR-HPV infection (83%), but a low specificity (57%).

**Cell-cell Adhesion**

**E-cadherin**

The calcium-dependent interactions among E-cadherin molecules are critical for the formation and maintenance of adherent junctions in areas of epithelial cell-cell contact. Loss of E-cadherin-mediated adhesion characterizes the transition from benign lesions to invasive, metastatic cancer (121).

**Immunological marker**

**Interleukin 10**

L-10 or human cytokine synthesis inhibitory factor (CSIF) is a cytokine considered to be a potent immuno-suppressor that depresses local virus-specific immunological responses.
Normally the human body needs both innate and adaptive immunity to identify viral infections (122). The key mechanism in HPV infection is that the HPV goes through different steps to avoid recognition by the innate and adaptive immunity. The life cycle of human papillomavirus inside the cells is in itself a good way to avoid recognition by the innate immunity APC (Antigen presenting cell).

The adaptive immunity system depends on the T lymphocytes (CD4+, CD8+) action in identifying the viral infection as a threat.

CD4+ T cells have two subsets, the th1 and th2 cytokines.

The main action for th1 (e.g. IFN-γ) is to start the cell-mediated immunity reaction (CMI), while th2 cytokines (IL –interleukins) IL-4, IL-5, IL-6, IL-10 and IL-13 activate the humoral immune responses. IL-10 is a potent immuno-suppressive cytokine and the overexpression of IL10 has been found in a varied range of cancers.

IL10 inhibits the activity of APCs and decreases the production of th1 (thus to say that IL 10 has an inhibitory effect on T-cell proliferation and inflammation) (123). In the present study IL-10 expression is neither correlated to HR-HPV status, nor to increasing histopathological grade.

**Oncoprotein**

C-myc oncoprotein induces the transcription. The HPV E6 gene increases telomerase expression through induction of c-myc and subsequent TERT gene activation (124).

![Figure 5. Main function of biomarkers and their effect on the cell cycle.](image-url)
Present investigation

Aims of this thesis

To study whether epidemiological findings of a correlation between smoking, sex steroids and contraceptive, and CIN are reflected in the expression of tissue tumor markers relevant for cervical neoplasia, thus supporting a biological role of these risk factors. The presence of HPV in tissue from the study population in women with normal cervical epithelium will also be studied for tissue tumor marker expression.

Correlations to tumor marker expression will be studied:
- Between a panel of biomarkers/tumor markers as above and HR-HPV-positive versus HR-HPV-negative cervical lesions.
- Between smoking and serum cotinine levels respectively, and tumor marker expression in cervical intraepithelial neoplasia (CIN) and normal epithelium.
- Between hormonal contraceptives - combined oral contraceptives (COC), any progestin-only contraceptives, medicated IUD (MID) or systemic progestin-only contraceptive use (Syst-P), compared to non-users, and tumor marker expression in cervical intraepithelial neoplasia (CIN).
- Between serum progesterone and serum estradiol levels and expression of tissue tumor markers in CIN and normal epithelium.
Material and methods

The study population comprised 228 women of whom 188 were recruited at our colposcopy clinic for laser cervical conization or a directed punch biopsy, because of a vaginal smear (Pap smear) that showed CIN, cytological findings suggesting CIN, or repeated findings showing atypical squamous cells of undetermined significance (ASCUS). They were consecutively recruited from the out-patient surgery, Department of Obstetrics and Gynecology, Falun Hospital, Falun.

In addition 40 healthy volunteers in fertile ages and with normal Pap smears were recruited to ensure that the study included a sufficient number of women with normal epithelium. The mean and median ages of the entire study population were 36.6 years and 34.0 years, respectively. These samples were collected between 2005-2007.

A structured questionnaire included birthday, age, last menstruation, cycle day, menopausal status, history of abnormal Pap smear, contraceptive use, if any, present or past smoking, cigarettes per day and duration, and climacteric status.

![Figure 6. The study population.](image-url)
The paraffin embedded punch biopsies or cones were stored at the Department of Pathology at Falun hospital.

The original histology slides were microscopically reviewed by a pathologist of our team and the most representative area(s) was marked for tissue microarray (TMA). Two-millimeter punch biopsies were taken from the blocks corresponding to the marked area and joined into TMA paraffin blocks, containing 24 to 30 punch biopsies. Each TMA block also included one control and one empty square to avoid diagnostic mistakes.

Immunohistochemistry was performed. In brief, glass slides were deparaffinized in xylene (2x15 min), dehydrated through graded alcohols and endogenous peroxidase was blocked (using H2O2 in 70% ethanol). Antigen retrieval was performed using Target Retrieval Solution (TRS pH 6.0 or pH9.0, Lab vision) in a de-cloaking chamber (Bio care Medical, Walnut Creek, CA) for 4 minutes at 125 °C. Thereafter the slides were immunostained in the automated staining instrument, where primary antibodies and secondary reagent were each incubated for 30 minutes at room temperature (RT). Finally, the slides were incubated with diaminobenzidine (DAB) as chromogen for 10 minutes and counter-stained with Mayer’s hematoxylin (Sigma-Aldrich, St Louis, MO) for 15 min. Slides were washed in distilled water for 10 min, dehydrated through graded alcohols to xylene, and mounted in Pertex organic mounting medium (Histolab, Gothenburg, Sweden).

Figure 7. The making of punch biopsies for TMA examination

Two-millimeter punch biopsies were taken from the blocks corresponding to the marked area and joined into TMA paraffin blocks, containing 24 to 30 punch biopsies. Each TMA block also includes one control and one empty square to avoid diagnostic mistakes.
Figure 8. The making of the slide for TMA examination.

The making of the slides using water fall microtome. The typical section is 4μm. Sections were de-paraffinized in xylene (2x15 min), dehydrated through graded alcohols and endogenous peroxidase was blocked (using H2O2 in 70% ethanol).

Antigen retrieval was performed using Target Retrieval Solution (TRS pH 6.0 or pH9.0, Labvision) in a de-cloaking chamber (Biocare Medical, Walnut Creek, CA) for 4 minutes.

The slides were immuno-stained in the automated staining instrument, where primary antibodies and secondary reagent were each incubated for 30 minutes at room temperature (RT).

The slides were incubated with diaminobenzidine (DAB) as chromogen for 10 minutes and counter-stained with Mayers hematoxylin (Sigma-Aldrich, St Louis, MO) for 15 min. Slides were washed in distilled water for 10 min, dehydrated through graded alcohols to xylene, and mounted in Per-tex organic mounting medium (Histolab, Gothenburg, Sweden).

25 °C.

Figure 9. The final stage the staining with DAB.
Occasionally, it was not possible to evaluate expression of a specific tumor marker in a specific subject. As it was not systematic, the whole study population was included and the number of evaluations thus differed with a few diagnoses between the tumor markers.

One external senior pathologist, who was blinded for clinical details, evaluated all biopsies. Frequency of stained cells and intensity of staining were diagnosed. A four-grade semi-quantitative score was used for 0 in absence of biomarker expression, 1 if 1-19% of cancer cells, 2 if 20-49% and 3 if 50% or more cells expressed of the tumor marker. Intensity of staining was graded in four steps: absent, mild, moderate and strong. In the analyses, there was in general a good correlation between proportion and intensity and the best discriminatory evaluation was used for presentation in all tables.

### Biological marker

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*Figure 10. Tumor markers included in the study and their major functions.*

**Paper 1**

High-risk HPV infection and CIN grade correlates to expression of c-myc, CD4+, FHIT, E-cadherin, Ki-67 and p16INK4a

PCR (polymerase chain reaction) for HPV detection from eight 5-µm sections from the original cervical cone was performed. In brief, standard
curves ranging from 102 to 105 copies were established for each HPV type using plasmids containing the full genome of different HPV types. A highly significant linear relationship was seen between HPV copy number and threshold cycle for all HPV types. The threshold for a positive HPV type was set to 10 copies per PCR. Sufficient and representative material from cone biopsies, in contrast to punch biopsies, was available from 116 women for analyses of 12 HR-HPV types, i.e. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59.

Ninety-four (82.5%) of the women were premenopausal while 20 women (17.5%) women were postmenopausal. The menopausal status of two women was uncertain. HPV-positivity was more common in the former group (59.6% vs. 35.0%). When postmenopausal women were excluded there were no significant differences in oral contraceptive (total 34.8%) and gestagenic contraceptive (total 24.2%) use between HPV-positive and HPV-negative women. In the entire study population 36.0% were smokers with no difference by HPV status.

The distribution of HR-HPV positivity in different stages of CIN and in normal epithelium shows a sharp increase from LCIN (mean 39.2% HPV pos) to HCIN (mean 89.2% HPV pos)

**Paper 2**

Tissue tumor marker expression in smokers, including serum
Cotinine concentrations, in women with cervical intraepithelial
Neoplasia or normal squamous cervical epithelium
Cotinine was analyzed at the Advanced Bioanalytical Services Laboratories, London.

Smokers averaged 34.9 years and non-smokers 37.7 years (p=0.06). Eighty-three (36.9%) of the women were smokers, defined as daily smoking and serum cotinine levels above 12 ng/mL, and 142 (63.1%) women were non-smokers. There were no findings of measurable serum cotinine in the latter group.

The histopathological diagnoses were distributed on 87 (38.2%) normal squamous epithelium, 31 (13.6%) ASCUS, 49 (21.5%) CIN I, and 61 (26.8%) CIN II-III. In women of fertile ages (n=195), 74 had normal epithelium, 23 ASCUS and 94 were diagnosed as CIN
Paper 3

Oral contraceptive and progestin-only use correlates to tissue tumor marker expression in women with cervical intraepithelial neoplasia

The study population (n=195) distribution was 57 OC users, 15 users of MID, 24 users of systemic progestin-only contraceptives, and 99 non-users (Fig 15). Mean age of OC users, and any progestin-only contraceptive users (Syst-P or MID) was 28.4 years and 35.5 years, respectively, compared to 35.2 years among non-users (p=0.01 and p=0.89). Distribution of histopathological diagnoses among oral contraceptive users, any progestogen-only use and the comparison groups.

CIN including borderline cases was diagnosed in 121 (62.1%) women, while in 74 (37.9%) women the cervical epithelium was considered normal. There were small and non-significant differences regarding histopathological diagnoses between OC users and any progestogen-only use, compared to non-users

Paper 4:

Increased levels of serum progesterone and estradiol correlate to increased Cox-2 tissue expression in CIN – and some pitfalls in evaluation of expression of other tumor markers.

Serum hormone levels were analyzed by standard methods at the biochemical laboratories of Falun Hospital (progesterone) and Uppsala (estradiol).

80 women - 60 patients and 20 volunteers.

Mean serum progesterone was 20.1 nmol/L, and the corresponding serum estradiol 449.2 pmol/L.

Serum hormone levels were analyzed by standard methods at the biochemical laboratories of Falun Hospital (progesterone) and Uppsala (estradiol).

Statistical analysis:

For the analyses the most explanatory cut-off level was used when the results were dichotomized.

When there was no evidence of any correlation to serum progesterone and/or estradiol, HPV status, smoking habits, contraceptive use data were dichotomized so that an equal number of patients, if possible, were included in the two groups.
For adjustments according to CIN grade, the material was divided into high-grade (CIN II and CIN III) and low-grade (borderline and CIN I) squamous intraepithelial lesions (HSIL and LSIL, respectively) and logistic regression was used. For statistical analyses, the JMP statistical package (SAS Institute) was used. Student’s t-test was used for significance Testing. The study was approved by the Research Ethical Committee, Uppsala University.
Results

Paper 1

**High-risk HPV infection and CIN grade correlates to expression of c-myc, CD4+, FHIT, E-cadherin, Ki-67 and p16INK4a.**

The aim of study I was to look for a correlation between a panel of biomarkers and high risk human papilloma virus (HPV) - positive versus HR-HPV negative in cervical lesions.

From the original cohort of 116 tissue samples which were tested for HPV, 64/116(55.2%) were positive for one more of the 12 HR-HPV subtypes. Most common were HPV-16 (N=21), HPV-18(N=14) AND hpv-33/52/58(n=15).None was positive for HPV-39, whereas occasional biopsies were positive for HPV-31,-35,-56 AND -59.

The prevalence of premenopausal status in the cohort was 94/114 (82.5%) while the prevalence of postmenopausal status was 20 /114 (17.5%). The menopausal status of two women was uncertain. HPV-positivity was more common in the former group (59.6% vs. 35.0%). When postmenopausal women were excluded there were no significant differences in oral contraceptive (total 34.8%) and gestagenic contraceptive (total 24.2%) use between HPV-positive and HPV-negative women. In the entire study population 36.0% were smokers with no difference by HPV status.

When the distribution of HR-HPV in different stages of CIN and in normal epithelium was analyzed, there was a sharp increase of HR-HPV positivity from LCIN (ASCUS+ CIN I) to HCIN (CIN11-CIN111) by the difference in the mean from 39.2% to 89.2% HPV-positive.
Table I. Expression of biological markers in HR-HPV- positive and –negative cervical epithelium of women with low grade or high grade CIN (frequency of stained cells: 0=no cells, 1=1%-19% cells, 2=20%-49% cells, 3=50% to 100% cells. Staining of c-myc could only be evaluated in 56 cases. P*adjusted for squamous intraepithelial lesion status.

<table>
<thead>
<tr>
<th>Frequency of stained cells</th>
<th>HPV-positive</th>
<th>HPV-negative</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc 1-3 vs 0</td>
<td>11(33.3)</td>
<td>1(4.6)</td>
<td>10.50</td>
<td>1.80-200.4</td>
<td>.006</td>
<td>.13</td>
</tr>
<tr>
<td>Cd4+ 3 vs 0-2</td>
<td>8(14.8)</td>
<td>1(2.7)</td>
<td>62.60</td>
<td>1.08-118.8</td>
<td>.04</td>
<td>.19</td>
</tr>
<tr>
<td>Ki-67 2-3 vs 0-1</td>
<td>36(67.9)</td>
<td>5(14.3)</td>
<td>12.71</td>
<td>4.49-42.64</td>
<td>.0001</td>
<td>.004</td>
</tr>
<tr>
<td>P16 3 vs 0-2</td>
<td>33(61.1)</td>
<td>7(18.9)</td>
<td>6.73</td>
<td>2.62-19.26</td>
<td>.0001</td>
<td>.34</td>
</tr>
</tbody>
</table>

The analysis for biomarkers showed, there were a high expression of C-myc, CD4+, Ki-67 and p16 which correlated with HR-HPV positivity but after adjustment was made for CIN grade only Ki-67 remained significant.

When the subcategorizing was done according to the histopathological assessed diagnosis and because of the small proportion of HCIN in HPV-negative tissue (n=4), only normal epithelium and LCIN were analyzed in this group. In HR-HPV negative tissue, a high expression of E-cadherin (P=0.03) and Epidermal growth factor receptor (P=0.03) correlated significantly to LCIN compared to normal epithelium. When the HPV-positive group was adjusted for normal/LCIN/HCIN, in the univariate analyses of HR-HPV positive tissue, showed that Ki-67(p=0.001), p16 (0.001), FHIT (p=0.002), E-cadherin (p=0.0004) and Rb (p=0.04) expression increased significantly with severity of the lesion.

P16 expression did not correlate to HPV status (p=0.34) but correlated significantly to histological grade (p=0.001). Ki-67 expression remained significantly correlated to HPV status also after adjustment (p=0.01) but also to histopathological diagnosis (p=0.02).
Ki-67 was the only marker that independently predicted both the presence of HR-HPV and the severity of cervical lesions.

**Paper 2**

**Tissue tumor marker expression in smokers, including serum Cotinine concentrations, in women with cervical intraepithelial Neoplasia or normal squamous cervical epithelium.**

The aim of study II was to investigate the correlation between biomarkers expression and smoking in cervical intraepithelial lesions. In this cohort the Smokers averaged 34.9 years and non-smokers 37.7 years (p=0.06).

Smoking was defined as daily smoking and serum cotinine level above 12 ng/ml. Eighty-three (36.9%) of the women were smokers, and 142 (63.1%) women were non-smokers. There were no findings of measurable serum cotinine in the latter group.

The histopathological diagnoses were distributed on 87 (38.2%) normal squamous epithelium, 31 (13.6%) ASCUS, 49 (21.5%) CIN I, and 61 (26.8%) CIN II-III. None of the 40 volunteers’ hade CIN.

**Smoking in the whole cohort:**

The result in general showed that smoking associated with lower P53 in CIN, but not in those women with normal epithelium, compared to non-smokers. Lower Interleukin -10 expression was also noted in smokers with CIN than in non-smokers
Table II. Smoking habits and correlation to tumor marker expression in cervical intraepithelial neoplasia and normal epithelium. (Frequency of stained cells was semi quantitatively 0=0%, 1=1-19% cells, 2=20-49 cells, 3=50-100% cells).

<table>
<thead>
<tr>
<th>variable</th>
<th>Smokers</th>
<th>Non-smokers</th>
<th>0R</th>
<th>P value</th>
<th>Smoker</th>
<th>Non-smokers</th>
<th>OR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Normal</td>
<td>95% CI</td>
<td></td>
<td>CIN</td>
<td>CIN</td>
<td>95% CI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>epithelium</td>
<td>epithelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P53</td>
<td>3(10)</td>
<td>5(11.6)</td>
<td>0.84 (0.16-3.74)</td>
<td>0.83</td>
<td>7(17)</td>
<td>28(35)</td>
<td>0.38</td>
<td>0.03</td>
</tr>
<tr>
<td>2-3 vs 0-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>8(25)</td>
<td>11(25.6)</td>
<td>1.01 (0.34-2.90)</td>
<td>0.98</td>
<td>15(34.9)</td>
<td>47(58)</td>
<td>0.39</td>
<td>0.01</td>
</tr>
<tr>
<td>1-3 vs 0-2</td>
<td></td>
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</tbody>
</table>

There was no significant correlation for p53 between smokers and non-smokers in normal epithelium in contrast to those with CIN where a significantly lower p53 expression was found (p=0.03). There was also a lower interleukin 10 expression in smokers with CIN (p=0.01), than in non-smokers.

There was a negative but insignificant dose-response relationship between EGFR expression and serum cotinine levels in normal epithelium (p=0.09), while there was a positive correlation between EGFR expression and serum cotinine levels in CIN (p=0.049) in women who were non-smokers.

**Smoking in fertile ages**

All cervical tissue of women in fertile ages was analyzed separately. No significant correlations between tumor marker expression and serum cotinine levels was found.

The tumor suppressors P53 (p=0.06) showed a significant under expression in smokers compared to nonsmokers in normal epithelium, the same is true regarding Fragile histidine triad (p=0.05) and immunologic marker interleukin-10 (p=0.11) they were under-expressed in smokers compared to non-smokers. The tumor markers COX 2 (p=0.04) and Ki 67 (p=0.005) were over-expressed in smokers compared till non-smokers in women in fertile ages. Women with CIN had similar patterns, but the statistical significance decreased.
Paper 3

Oral contraceptive and progestin-only use correlates to tissue tumor marker expression in women with cervical intraepithelial neoplasia.

The purpose of this study was to look for the correlation between contraceptive use as combined oral contraceptive (COC), any progestin-only contraceptive, medicated intrauterine device (MID) or systemic progestin-only (Syst-P) and tumor marker expression in cervical intraepithelial compared to non-user.

The study population (n=195) distribution was 57 OC users, 15 users of MID, 24 users of systemic progestin-only contraceptives, and 99 non-users. Mean age of OC users, and any progestin-only contraceptive users (Syst-P or MID) was 28.4 years and 35.5 years respectively, compared to 35.2 years for non-users (p=0.01 and p=0.89).

CIN including borderline cases was diagnosed in 121 (62.1%) women, while in 74 (37.9%) women the cervical epithelium was considered normal. There were small and non-significant differences regarding histopathological diagnoses between OC users and any progestogen-only use, compared to non-users.

COC users:
The results showed that a significantly increased expression of cyclooxygenase-2 (Cox-2) in cervical tissue in COC users with normal epithelium (OR 7.8, 95% CI 2.4-28.0) and in those with CIN (OR 5.7, 95% CI 1.8-20.1), compared to non-users of hormonal contraception.

A lower expression of IL 10 was found in OC users with CIN than in non-users among (OR 0.32, 95% CI, 0.10-0.90).

Progestin-only users
A lower expression of IL10 was found in any progestogen-only contraceptive users with CIN than non-users (OR 0, 37, 95% 0, 12-1, 03). The same is true regarding CK 10(0, 17, 95% CI0, 03-0, and 71).

Comparison between MID users and COC users
When a comparison was made between MID users and COC users, COX2 and Rb (Retinoblastoma protein) expression were increased in COC users, while P53 expression was increased in MID users.
Table III. OC vs MID use and correlation to tumor marker expression in the total study population (frequency of stained cells was semi quantitatively 0=0%, 1=1-19% cells, 2=20-49 cells, 3=50-100% cells).

<table>
<thead>
<tr>
<th></th>
<th>MID users N (%)</th>
<th>OC users N (%)</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3 vs 0-2</td>
<td>5(35,7)</td>
<td>40(74,1)</td>
<td>0,19</td>
<td>0,05-0,66</td>
</tr>
<tr>
<td>P53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 vs 0-2</td>
<td>4(28,6)</td>
<td>3(6,5)</td>
<td>5,73</td>
<td>1,10-33,2</td>
</tr>
<tr>
<td>Rb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3 vs 0-1</td>
<td>2(14,3)</td>
<td>20(40,8)</td>
<td>0,24</td>
<td>0,04-1,01</td>
</tr>
</tbody>
</table>

There was no tendency for correlation between P53 or Rb expressions when COC and SYST-P users where compared, but only a similar correlation to COX2-expression.

Paper 4

**Increased levels of serum progesterone and estradiol correlate to increased Cox-2 tissue expression in CIN.**

The aim of this study was to investigate the correlation between serum progesterone and serum estradiol levels and expression of tissue tumor markers in cervical intraepithelial neoplasia (CIN) and normal epithelium.

The mean and medium age was 35.7 years and 34.5 years, respectively, with a range of 22-52 years. Twenty-four (30%) women were smokers in the whole cohort. The histopathological diagnoses were distributed as 25(31,3%) cases of normal epithelium, 10 (12,5%) borderline, 18(22,5%) CIN I and 27(33,8%) CIN II-III.

The mean and medium menstrual cycle day at serum sampling was day 18, 3 and 19, 0, respectively.

The mean serum progesterone was 20,1 nmol/l, and the corresponding serum estradiol was 449,2 pmol/l.

**Progesterone level and tumor markers expression:**

- **In normal epithelium**

  EGFR (15,4 vs 36,5nmol/l; p=0,002) and CD4+ expression (22,2 vs 39,8; p=0,048) correlated to low serum progesterone levels.

- **In CIN**

  Positive or negative correlations between serum progesterone levels and intensity of expression were evident in CIN for Rb, p16, and Cox-2 in crude
analysis. After adjustment for CIN grade, only increased Cox-2 expression remained significantly correlated to serum progesterone levels.

**Estrogen level and tumor markers expression:**
When estradiol levels were analyzed there were no differences in tumor marker expression by serum levels in the normal epithelium, while in the CIN group high serum estradiol levels correlated weakly, but significantly, to increased Cox-2 expression.
Discussion

This thesis shows novel results on the correlations between tumor marker expression and HR-HPV. It is particularly designed to evaluate tumor marker expression in smokers, hormonal contraceptive users and with serum sex steroid hormone levels in cervical intraepithelial neoplasia and in normal epithelium. HPV infection is regarded as a necessary but not sufficient factor in transforming CIN to invasive cancer, and the presence of co-factors might also be one reason when there is a rapid progress of early lesions to HSIL and cervical cancer (4). Several previous studies focused on the correlation between expression of Ki-67, p16 and HPV infection in cervical intraepithelial neoplasia, while others focused on direct exposure to smoking and its unfavorable effect on tissues like lung or oral cavity. It is important to note that smoking, contraceptive use and sex steroids have been subjects of interest in many studies regarding their effect on tissue tumor markers in different types of cancers. In order to simplify our findings on a possible association between HPV infection, smoking, contraceptive and sex steroids, respectively, and tumor marker expression we will discuss findings in some tumor markers separately and thus include all studies. The possible correlations have rarely, if ever, been studied.

Tumor markers

Cox 2

Cox 2 has been used as a tumor marker in several cancer types and is involved in different inflammatory and mitogenic processes. The finding of high expression of Cox 2 might indicate poor prognosis in cervical cancer. The link between the initiation of inflammation process and cancer development has been recently explained by the discovery of the infiltration of tumor micro-environment with different types of immune cells such as macrophages, lymphocytes and dendritic cells. These cells are responsible for the release of cytokines (125) and the role of cytokines in the development of cancer in different organs is well-known. The chronic use of NSIDs (non-steroidal anti-inflammatory drugs) which are COX-2 inhibitors has been found to decrease the incidence of cancer such as colorectal cancer (126) and lung cancer (127). In this study, high COX 2 expression was close to
significant (p=0.08) in HPV-positive tissue compared to HPV-negative tissue in high-grade or low-grade CIN. This finding may indicate the early oncogenic effect of HR-HPV infection on cervical tissue. Smoking correlated significantly with high expression of Cox 2 (p=0.04) in the subgroup of fertile women in the entire study. Similar results have been found for the effect of exposure to smoke extract in other tissues such as the lung, where the exposure led to increased Cox-2 expression in fibroblast (128).

The finding of a high COX 2 expression (OR 7.8 and 5.7) in OC users is interesting and can be explained as a response to an inflammatory process or as susceptibility of cervical cells to inflammation. In many studies it has been found that the OC users are at more risk of developing cervical atopies than other contraceptive users (129).

Cox 2 expression also showed a strong staining intensity in HSIL with elevated serum progesterone levels. Progesterone has immuno-suppressive effects and there might be an increased susceptibility for HPV infection (130). Our findings indicate an adverse effect of progesterone on CIN that could confirm findings in invasive cancer. A study by Lindström et al. (131) showed increased cell proliferation measured as the number of cells in the S phase, with high serum progesterone levels in invasive cervical cancer. Many researchers have reported similar findings with high estradiol levels in HSIL. In all, a possible clinical role for Cox-2 expression could be to indicate an association to poor prognosis in cervical cancer.

**Ki 67**

Ki-67 is a non-histone protein which is specific for cells with active DNA replication. Increased proliferation has been found during pregnancy, in inflammatory processes and cancer. Ki-67 is expressed during all phases in the cell cycle except in resting stage (G0). It is unclear whether Ki-67 expression carries prognostic information in invasive cervical cancer (132). In the first paper we were able to demonstrate that Ki-67 correlated to HPV status and to the histopathological diagnosis. These results can be explained by the effect of HPV oncogene on the cell cycle, as it promotes dysregulation of the infected cells leading to proliferation and growth acceleration. This will lead to over-expression of Ki-67 as a proliferative antigen which is specific for active DNA replication. There is some uncertainty whether Ki-67 has the ability to distinguish between normal proliferating cells and infected cells (dysplastic cells). (32) (133). The finding of overexpression of Ki-67 in women smokers in fertile age indicates that there is a tendency to increased cell proliferation in smokers in general. This relation between smoking and increased cell proliferations has been noticed even in other studies done on the effect of smoking in other organs such as lungs or oral cavity. (134). Ki67 was close to significant in elevated serum progesterone in normal epithelium (P=0.06). This, however, might be explained by the increased proliferation of cells in the luteal phase of menstruation.
EGFR (epidermal growth factor receptor)

EGFR has the ability to interact with specific cell-surface receptors and transduce intracellular signals to stimulate DNA synthesis and cell division. EGFR is considered as one of the prognostic markers in different types of cancer (135, 136). The finding of high EGFR expression in CIN and low expression in normal epithelium in smokers compared to non-smokers might be explained by the increased cell proliferation in smokers in general. The high expression of EGFR in LCIN compared to normal epithelium in HPV-negative tissue indicates the early dysregulation of EGFR in the cervical squamous metaplasia and epithelial hyperplasia. Intense EGFR staining correlated with low progesterone level in normal epithelium but not in CIN. In a previous study by Lindström A.K et al (137) the finding of a strong correlation between low EGFR staining and high serum estradiol was interesting, as was the question whether the effect of these sex steroids on the cervix epithelium reverse their effect on the endometrium. It is well-known that estradiol is associated with cell proliferation and that progesterone has a suppressor effect on the endometrial growth, but its effect on the cervical epithelium has been poorly studied.

p53 (cellular tumor antigen p53, phospho-protein p53)

P53 is a tumor suppressor which is activated as a response to DNA damage leading to cell arrest at the G0/G1 phase. This arrest leads to DNA repair and the prevention of mutations. HPV E6 oncogenes bind to p53, leading to the degradation of P53 and resulting in the loss of the ability of apoptosis and tumor suppression function. In cervical cancer the wild type 53 (active p53) dominates but is degraded by HPV DNA, explaining why the expression of mutant p53 is low in cervical cancer. The finding of a correlation between p53 and HPV positivity was not conclusive in our study and it disappeared when adjusted to CIN grade. Our other results regarding smoking showed lower expression of P53 in smokers compared to non-smokers in CIN analysis. This finding of lower expression of p53 in smokers is consistent with the overall unfavorable molecular expression pattern of smoking in this study. The suppressor effect of smoking on p53 also started early in the precancerous process according to our results.

P53 and progesterone

The malignant proliferation of squamous cervical epithelium induced by HPV infection may be associated with increased progesterone receptor expression (138). Previous studies suggest that p53 function is inactivated in cervical carcinoma either by Complex Formation with HPV-E6 viral oncprotein product in HPV-positive cervical carcinoma or, rarely, by gene mutation in HPV-negative cervical carcinoma.(139, 140)
A most interesting finding in this study was the increased expression of p53 in MIUD users compared to non-users. This correlation has never been studied previously. The results were surprising as it would indicate a protecting effect of MIUD use. HPV has a tendency to transfect cells with progesterone receptors. Progesterone has previously been suggested as the major candidate hormone in cervical neoplasia due to its immunosuppressive effect and to a possible connection with HPV infection. Both HPV 16 and HPV 18 contain progesterone and glucocorticoid response elements that increase the expression of HPV E6 and E7 oncogenes, considered crucial in cell transformation, with gestagenic stimuli (141).

A study by Orbo et al. 2009 (142) showed that there is up-regulation of P53 pathway in patients treated with Mifepristone. This may be of interest as potential new therapeutic targets in endometrial hyperplasia and endometrial cancer, as candidate markers for tumor progression. Local progestin-only contraceptives are known to have a potent effect on the endometrium, and a reduced thickness of the endometrium from 6.1 to 4 mm after 4 months of use has been reported (143). The endometrial concentration of LNG with an LNG-MID, one common type of progestin used in MIDs, is 200 to 800 times higher than that of SystP (144, 145). Little is known about the effects on cervical epithelium, but it is evident by the scanty and viscous cervical mucus that there are such effects (145). There are only two small studies on cervical cytology. In one study, 162 users of LNG-MID were followed up for 5 years and were compared with users of subdermal progestin-releasing implants. Although CIN was more than twice as common in the former group, it was not a significant difference (146). Another small study did not find significantly increased rates of abnormal smears compared to expected rates, but there was no control group (147). Substantially larger studies are needed.

p 16 cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1

P16 is a gene product which acts as a tumor suppressor protein, its main action being to inhibit the inactivation of Rb by cyclin dependent kinase. Many studies showed that P16 immuno-reactivity is lost or decreased as a part of the immunological reaction towards the external threat (carcinogenic effect) (148-150). In cervical dysplasia another mechanism is established where P16 expression is increased as a response to the effect of HPV infection. HPV oncogenes E6, E7 alter the cell cycle mechanism by binding to the 2 main proteins which regulate the cell cycle. E7 binds itself to Rb protein, leading to degradation of E2F transcription factor (activation of E2F leads to cell progression). E7 causes the Rb dysfunction (151-153). The cells will react by increasing the expression of P16 in the cell and, since E2F is released by E7 and not by the effect of CDK4/6, p16 loses its effect on cell cycle progression (p16 has no more effect on the cell progression). P16 would accumulate in the nucleus and cytoplasm of the affected cell and can
be detected by immuno-staining. P16 has been used as tumor marker in cervical carcinoma. Our results of a concomitant increase of p16INK4a and Rb expression in HR-HPV positive specimens may indicate that p16INK4 in these early neoplasms still influences Rb expression. This is instead of the feedback loop with increasing p16INK4 expression as a result of decreased Rb expression caused by HPV induced inactivation, while increased Rb expression leads to decreased p16 activity. HPV-E7 onco-protein inactivates Rb protein leading to over-expression of p16. The results of p16 expression were surprising. The poor correlation between p16INK4a over-expression and positive HPV status in LCIN, and the lack of over-expression in nearly 20% of HCIN, might reflect non-integration of HPV DNA in the host genome. As we also found almost 10% over-expression in HR-HPV-negative LCIN, p16INK4a our results do not support the idea that p16INK4a is a useful surrogate for HR-HPV infection.

In this study there was an excellent correlation to severity of CIN in those who were HR-HPV positive, but not in HR-HPV negative lesions. No expression was found in HR-HPV positive normal epithelium, while a high level of expression was evident in HCIN. A substantial part (9%) of LCIN also had p16INK4a expression. Thus, the role in differential diagnosis was limited. Our findings of the use of p16INK4 expression as a histopathological adjunct are similar as those in a meta-analysis (119).

In the present study the significant correlation to HR-HPV infection disappeared in the multivariate analyses. In analyses of tumor marker expression HR-HPV positive and negative tissue have rarely been separated (154), but it would be easier to interpret the results if specific tumor marker expression is significantly correlated to HR-HPV infection.

C-myc

c –myc is an onco-protein that is predominantly located in the cell nucleus and involved in carcinogenesis through the activation of transcription of m RNA (effect proliferation, prevent differentiation, sensitize the cells for apoptosis). It has been used as prognostic marker in HPV positive cancer(155). C-myc overexpression has been detected in many cases of colorectal, hepatocellular, endometrium, mammary and prostate cancer .ref

C-myc activation occurs in advanced cases of cervical carcinoma (indicating the beginning of cancer progression, relapse and metastasis) (156). In a study published by C.D. Golijow et al (157) in 2001, there is cmyc amplification found in cellular atopies, low grade intraepithelial and high grad intraepithelial lesions (157). In this study, C-myc was highly expressed in HPV positive in the univariate analyses, which can be explained by the previous finding in a study by Bernard et al. (158) where a correlation between c-myc and HPV status was observed.
IL10

Interleukin-10 (IL-10), or human cytokine synthesis inhibitory factor (CSIF).

IL-10 is a cytokine and is considered to be a potent immuno-suppressor that depresses local virus-specific immunological responses. In the present study IL-10 expression correlated neither to HR-HPV status, nor to increasing histopathological grade.

IL-10 pattern in women with HR-HPV-associated CIN has been investigated in blood (159), cervical secretions (160) and in abnormal vaginal smears (161). These previous studies indicate a correlation to the presence of HR-HPV infections but independency of CIN grade has not been investigated. It has been reported that IL-10 expression was independently positively correlated to HCIN compared to less severe lesions, but was not useful as a surrogate marker for HPV (161).

Decreased IL-10 expression was observed both in OC-users and any progesterone-only users, possibly reflecting an effect on the immunological defense mechanism. Two studies evaluated women with normal cervical epithelium. Increased levels of IL-10 in cervical cytology of OC users were found in both studies OC (124, 162). It is therefore of particular interest that we observed an increase of IL-10 expression in users of progestogen-only contraceptives in women with normal epithelium, in contrast to those with CIN. This could indicate a switch in immuno-suppression when CIN is established. From the present results it can be concluded that exogenous steroid sex hormones influence IL10 expression, but whether the immunological balance is influenced positively or negatively remains unknown.

Co-factors

Smoking

The present study included evaluation of smoking habits and serum cotinine level in women with cervical intraepithelial lesion or normal cervical epithelium. Smoking seems to be associated with a negative molecular pattern. Lower expression of p53 and FHIT and higher expression of COX2 and Ki 67 have been found in smokers compared to non-smokers and this is true for women at a fertile age. Significant difference between smokers and non-smokers was found also in IL 10 expression in CIN only.

Low expression correlated to cotinine levels, which suggests that cotinine (and thus nicotine) are generally bystanders and that other substances affect cervical cells. It must be stressed, however, that accumulation of cotinine has been found in cervical mucus and that we had no possibility to perform such analyses. The findings of lower EGFR expression in normal epithelium and higher expression in CIN in smokers, compared to non-smokers, are interesting and deserve further studies. Our results showed positive correlations
between smoking and tumor marker expression, and we could determine objectively that smoking status was true through serum cotinine.

In a previous study on invasive cervical cancer, we found significantly lower p53 expression in smokers compared with non-smokers, as in the present study (137). A novel tumor marker, LRIG1, which is probably a tumor suppressor in cervical cancer, was found to exhibit lower expression in smokers than in non-smokers (163). The present study showed that the expression of Ki-67 is increased in smokers with CIN, which was confirmed in the previous study.

The p53 activation after several signals of cellular stress or DNA damage needs to be compromised during tumorigenesis. In cervical cancer, the human papillomavirus oncogene E6 is able to promote wild type p53 degradation, and in contrast to other cancer types, the expression of mutant p53 is low in cervical cancer (69). Our results indicate that suppression of p53 occurs very early in the presence of smoking.

Ki-67 (or MIB1) is, like EGFR, a well-known and thoroughly studied proliferation factor in cancer. It is unclear whether Ki-67 expression carries prognostic information in invasive cervical cancer (164). Our results showed a significant correlation between Ki-67 expression and smoking in fertile women which indicates an increased cell proliferation in smokers. Smoking correlated to increased Cox-2 expression in the subgroup of fertile women in this study. In tissues directly exposed to smoke, like the lung and the oral cavity, smoke extract on human lung fibroblasts has induced Cox-2 (187). Smokers have increased Cox-2 messenger RNA levels in oral mucosa, compared with non-smokers (165).

Interleukin-10 showed a significant difference in expression between smokers and non-smokers in women with CIN. Interleukin-10 is an immunosuppressor that is involved in immune response escape in cancer (166). Interleukin-10 concentrations in cervical secretions of smokers, compared with non-smokers, have been measured, but the differences were non-significant (99).

**Hormonal Contraceptives**

This study provides evidence that hormonal contraceptives influence expression of molecular markers that are used in investigations of cervical cancer. We cannot speculate whether these changes indicate an overall favorable or unfavorable pattern. Carcinogenesis is complex, and the present study only evaluates expression of 11 (although carefully selected) tumor markers. One of the main indications that there is a switch in the immuno-suppression mechanism when CIN is established is the finding of increased IL10 expression in any progesterone-only users in women with normal epithelium in contrast to those with CIN. The finding of increased IL10 expression in cervical HPV infections is well known (160, 167). From the present results, it
can be concluded that exogenous steroid sex hormones influence IL10 expression, but whether the immunological balance is influenced positively or negatively remains unknown. Cytokeratin 10 is a major marker of keratinocytes and is involved in cell differentiation. Promotion of keratinocytes and enhancement of differentiation in cervical cell lines have been associated with growth suppression (168). Cytokeratin 10 expression was also reported to be increased in thick cervical epithelium associated with cervical cytology showing mild dysplasia or ASCUS. Other studies have found decreased expression with progressive CIN and invasive cancer (169, 170), but the clinical importance of CK10 expression in cervical carcinogenesis is poorly studied (171). As CK10 expression is associated with differentiation, our findings of decreased expression in progestin-only users in CIN compared to non-users might be unfavorable. When users of MID and Syst-P were analyzed separately, no significant correlations to expression of tumor markers were observed for systemic users. In the present study, there was increased p53 expression in the progestin-IUD users compared to non-users. The role of p53 in response to DNA damage and the correlation to MID use are intriguing, but could indicate favorable effects.

**Progesterone and Estrogen**

Progesterone hormone is considered to be an important risk factor in the development of cervical neoplasia, although the present study is the first to investigate molecular biological mechanisms of these hormones.

It has been shown that HPV has a tendency to target cells with progesterone receptors. Both HPV 16 and HPV 18 contain progesterone and glucocorticoid response elements that increase expression of the HPV E6 and E7 oncogenes, which are considered crucial in cell transformation (89). Such a transformation has been reported to take place when progesterone or OC gestagens were added to cell cultures (130).

Progesterone and glucocorticoid hormones increase HPV mRNA and significantly stimulate viral replication (172, 32). An increased cell proliferation with high serum progesterone levels in invasive squamous cell cervical cancer has also been reported in a study which included more than 100 women (173). Estrogen has been reported to reduce susceptibility to primary HPV infection, but might be of no importance once an HPV infection has been established (174). Estrogens might also have a role in increasing the levels of HPV-induced apoptosis (175). Sex steroid hormones might also be involved in single steps in the neoplastic progression, similar to that of many tumor markers. Once invasive cancer has been established, high serum estrogen levels might have a positive effect on outcome, while high serum progesterone levels have been found to have a deleterious effect (173).

Some previous studies have studied sex steroid hormone interactions with HPV, in vitro experiments on cervical cancer cell lines, and animal experi-
ments. This clinical study on women with normal or neoplastic epithelium indicates that serum progesterone and estradiol levels influence cellular and extracellular proteins which have been associated with neoplastic development, in normal epithelium and CIN.
Summary

The main findings of this study were the observations that exposure to co-factors, as well as HR-HPV infection, was associated with molecular changes in cervical tissues. There was a tendency towards a “negative” pattern of tumor marker expression with exposure to co-factors. This supported the main goal of the study, i.e. lending biological support to previous epidemiological studies.

Thus, our findings included the following:

I Smoking correlated to decreased suppressor expression, i.e. p53, FHIT and IL-10, while the proliferation markers Ki-67 and Cox2 were over-expressed. Serum cotinine levels, the main metabolite of nicotine, correlated with increased EGFR expression.

II Hormonal contraceptives. In combined oral contraceptive users both Cox-2 and Ki-67 expression was increased, and IL-10 expression decreased. In MIB-users, p53 expression was increased.

III Serum sex steroid levels. Increased levels of serum progesterone and estradiol were associated with increased Cox-2 expression.

IV HR-HPV infection. Several correlations to tumor marker expression were found. Independent correlations could not be conclusively established when CIN grade was adjusted for. Ki-67 was over-expressed independently of CIN grade, but other candidates need to be further studied.
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Infektion med hög risk-human papillomavirus (HR-HPV) anses vara en viktig orsak i utvecklingen av cellförändringar i livmoderhalsen (cervical intraepithelial neoplasia, CIN) och invasiv livmoderhalscancer. Trots att HPV infektionen är den viktigaste orsaken, kan förändringar i immunsvar i livmoderhalsens celler förklara de olika graderna av cell förändringar och deras utveckling till invasiv cancer. Dessa förändringar i immunsvaren kan förklaras av närvaron av ytterligare faktorer som rökning, preventivmedel och könshormoner.


Studie I: 228 kvinnor, varav 116 var testad, bara 64 var positiva till HR-HPV. Resultaten visade att Ki67 tumör cellproliferationsindex var den enda markör som på ett oberoende sätt var korrelerad med både HR-HPV och svårighetsgraden av cellförändringar i cervix. Detta innebär att HR-HPV-infektion var associerad med ett negativt mönster av uttryck för tumörmarkörer i cervix och att Ki-67 kan användas som en surrogatmarkör för HPV-infektion.

Studie II: 228 kvinnor, varav 83 var rökare (36,9 %). Rökare uppvisade lägre uttryck av p53, FHIT (tumör suppressor markörer) och interleukin-10 och högre expression av Cox-2 och Ki-67 (tumörproliferationsmarkörer). Det sammanlagda resultatet tyder på en negativ inverkan av rökning på cervikal epitel.

Studie III: 195 premenopausala kvinnor. 57 var COC (kombinerat ppiller) användare, 15 MID (medicinsk/hormonell spiral) användare, 24 var progestin enbart användare och 99 icke-användare. Det fanns en ökad p53-
expression (tumörsuppressorn) i progestin-spiralanvändare, jämfört med icke-
användare. Minskat IL-10-uttryck (immunologisk markör) observerades hos
både kombinerade p-piller och eventuella progestinbaserade endast använ-
dare. Detta resultat visar att hormonella preventivmedel påverkar uttrycket
av de mest undersökta molekylära markörer för livmoderhalscancer.

Studie IV: Serum från 80 premenopausala kvinnor var tillgängliga, varav
25 (31,3 %) med normalt epitel, 10 (12,5 %) med epitel på gränsen till nor-
malt, 18 (22,5 %) med CIN I och 27 (33,8 %) med CIN II-III. Den viktigaste
slutsatsen var att de ökade nivåerna av serum progesteron och estradiol var
associerade med ökad Cox-2-uttryck (spredningsmarkör). Upptäckten av en
omvänt korrelation mellan höga Rb-uttryck och låg P16-uttryck med höga
progesteron nivåer, när justering för CIN grad gjordes, kunde bekräfta sam-
bandet mellan dessa tumörmarkörer i HPV-infektioner.

Serum progesteron och östrogennivåer påverkar cellulära och extracellu-
lära proteiner som har satts i samband med tumörutveckling i normalt epitel
och CIN.

Slutsats: Resultaten av dessa studier stöder tidigare epidemiologiska rön
angående rollen som rökning, preventivmedel och könshormoner har som
bidragande faktorer i utvecklingen av CIN och att tumörmarkörers uttryck
varierar oavsett graden av CIN.

Nyttelord: tumörmarkörer, cervikal intraepitelial neoplas, rökning, preven-
tivmedel, könssteroidhormoner, HPV-infektion.
References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)