Human Papillomavirus Load
and Cervical Carcinoma

BY
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Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Uppsala, Wednesday, May 19, 2004 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

**Abstract**


Human Papillomavirus (HPV) is a key factor in the development of cervical cancer. Out of the more than 100 known HPV types 13 are considered oncogenic. In addition to presence of the virus several other factors have been proposed to influence risk of cervical cancer. This thesis focuses on viral load and HLA class II alleles as risk factors for cervical cancer.

To enable quantification of the most common oncogenic HPV types, a real-time PCR-based assay was developed and evaluated in terms of technical sensitivity and specificity.

This assay was then employed on archival smears from 457 cases and 552 controls to assess associations between viral load and cervical carcinoma in situ (CIS). Whereas the data indicate a pronounced dose dependent effect of HPV 16 load on the risk of CIS, other HPV types only seem to increase CIS risk at higher viral loads. These effects were observed even when cytology indicated that cells were normal.

We then investigated viral load as a risk factor for invasive cervical carcinoma (ICC) in a retrospective study comprising 139 cases and 550 controls. Viral load contributed similarly to the risk of ICC as to the risk of CIS.

Finally, associations between HLA class II alleles, viral load and CIS were investigated. Carriers of the DRB1*1301 allele were less prone to infections and high viral loads of HPV 31 and -18/45. Moreover, DRB1*1301 had a protective effect against CIS among women infected by HPV 31 or -18/45. In contrast, carriers of DRB1*1501 and DQB1*0602 were more susceptible to infections and high viral loads of HPV 16.

These results indicate that HPV load may have HPV-type specific effects on cervical cancer risk. Furthermore, HLA class II alleles may confer either susceptibility or protection against cervical cancer by acting on the HPV infections preceding tumor development.

**Keywords:** cervical cancer, human papillomavirus, viral load, HLA class II

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ISSN 0282-7476
ISBN 91-554-5962-5

urn:nbn:se:uu:diva-4256 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-4256)

Printed in Sweden by Universitetstryckeriet, Uppsala 2004
To Lisa
List of Papers

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

I Real-time PCR-based system for quantification of Human Papillomavirus types associated with high risk of cervical cancer.
Moberg M., Gustavsson I., Gyllensten U.
*Journal of Clinical Microbiology, July 2003, p. 3221-3228*

II Type-specific associations of Human Papillomavirus load with risk of developing cervical carcinoma in situ.
Moberg M., Gustavsson I., Gyllensten U.
Submitted

III High viral loads of Human Papillomavirus predict risk of invasive cervical carcinoma.
Moberg M., Gustavsson I., Wilander E., Gyllensten U.
Manuscript

IV HLA class II allele control of HPV titer in carcinoma in situ of the cervix uteri.
Beskow A., Moberg M., Gyllensten U.
Submitted

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<td>ASCUS</td>
<td>Atypical squamous cells of undetermined significance</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>CIN</td>
<td>Cervical intraepithelial neoplasia</td>
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<td>CIS</td>
<td>Cervical carcinoma <em>in situ</em></td>
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<td>Ct</td>
<td>Threshold cycle</td>
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<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>FDA</td>
<td>The Food and Drug Administration</td>
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<td>HC</td>
<td>Hybrid Capture (commercially available HPV test)</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HPV</td>
<td>Human Papillomavirus</td>
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<td>HSIL</td>
<td>High-grade squamous intraepithelial neoplasia</td>
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<td>HSV</td>
<td>Herpes simplex virus</td>
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<td>ICC</td>
<td>Invasive cervical cancer</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>ISH</td>
<td><em>In situ</em> hybridization</td>
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<td>LCR</td>
<td>Long control region</td>
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<tr>
<td>LSIL</td>
<td>Low-grade squamous intraepithelial neoplasia</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>OC</td>
<td>Oral contraceptive</td>
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<td>OR</td>
<td>Odds ratio</td>
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<td>Pap</td>
<td>Papanicolaou</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>RB</td>
<td>Retinoblastoma gene</td>
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<td>RB</td>
<td>Retinoblastoma protein</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RR</td>
<td>Relative risk</td>
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<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
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<tr>
<td>VLP</td>
<td>Virus-like particle</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction

The role of infection in common disease has been increasingly recognized in recent time. A specific example is cervical cancer (‘livmoderhalscancer’ in Swedish), which is caused by a group of viruses. Since virtually no cervical cancers arise in absence of infection, it has been proposed to include virus testing when trying to identify women at risk for this cancer form. In this thesis we have investigated whether the amount of virus in clinical samples is associated with the risk for cervical cancer and if the association differs between individual types of the virus. In addition, we have explored the contribution of certain parts of the immune system to risk for infection of the carcinogenic viruses and the risk for cervical cancer.

Cervical cancer

Each year, an estimated 471,000 women are diagnosed with cervical cancer. This places cervical cancer as the second most common cancer form among females in the world 1. Cervical cancer arises in epithelial cells of the cervix uterus, which is the lower part of the uterus and forms a narrow canal between the vagina and the uterine body cavity (Figure 1).

Figure 1. Schematic illustration of the uterus. The cervix connects the vagina with the uterine body cavity.
There are two major types of cervical cancers. The most common form, cervical squamous-cell carcinoma, derives from squamous cells. Squamous cells are scale-like cells constituting the majority of the epithelia of the lower cervix. The other form, cervical adenocarcinomas, constitutes approximately 10-15% of all cervical cancers and arises in cervical glandular cells. Cervical carcinoma is the first cancer recognized by the World Health Organization (WHO) to be 100% attributable to infection. The virus involved in development of the disease is called Human Papillomavirus (HPV).

**Human Papillomavirus**

HPV belongs to the large Papillomaviridae family whose members can be found among birds as well as mammals. The virus consists of a double-stranded deoxyribonucleic acid (DNA) molecule of about 8,000 basepairs, covered by an isocahedral protein shell (Figure 2).

HPVs are divided into types and variants depending on the sequence homology. If two HPVs differ more than 10% in the L1 gene they are regarded as separate HPV types. If the homology is 2-10% they are variants of the same type. The slow mutation rate combined with the substantial heterogeneity of today’s over 80 characterized HPV types, dates the origin of the virus before emergence of *Homo sapiens* and the migration out of Africa.

HPVs infect epithelia and certain HPV types are responsible for cutaneous ailments such as hand and foot warts. A subgroup of more than 40 HPV types preferentially infect mucosal surfaces and are most frequently found in the anogenital tract. HPV types belonging to the mucosal subgroup are often

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* A very common 20-sided crystal-like configuration, with each capsomere forming an equilateral triangle.
referred to as either ‘high-risk’ or ‘low-risk’ in reference to their carcinogenic potential. This distinction was originally based on the prevalence of HPV types in cervical cancers 6-8 and was further confirmed as high-risk HPV types proved more potent in immortalizing epithelial cells than low-risk types in vitro 9-11.

The natural life cycle of HPV

Epithelia are characteristic for their constant loss and renewal of cells. Therefore, HPV has to gain access to the epithelial basal cells to avoid being thrown out with the shedding of cells. The basal cells are situated above the basal membrane and are the stem cells of the epithelium. HPV gains access to the basal cells through mild abrasions or micro-lesions in the tissue (Figure 3). Subsequent cell entry is mediated by surface heparan sulfate in combination with other as of yet unknown receptors 12. Once inside the basal cell, HPV transfers its genome into the nucleus in an as of yet unknown manner. Within the nucleus, the HPV genomes are established as extrachromosomal elements. The number of HPV genomes is then increased to approximately 50-100 copies, which are subsequently replicated on average once per cell division using the host cell’s DNA replication machinery 13,14. While remaining in the basal layer of the epithelium, HPV keeps protein production down to a minimum. It expresses mainly E6 and E7 plus minor amounts of other early genes. Expression of E6 and E7 in basal cells is thought to modify the cellular environment in order to facilitate subsequent viral replication as the infected cells differentiate into keratinocytes. Keratinocytes normally exit the cell cycle and in some epithelia the nuclei are shut down and degraded. Further, the E5 protein is proposed to increase the levels of mitogenic factors, which increase basal cell proliferation and delays the differentiation 15,16. This serves the purpose of expanding the number of infected cells. After the infection is firmly established, HPV has to move to the epithelial surface and assemble virions to spread. These events are initiated by expression of E1 and E2. E2 is thought to suppress the expression of E6 and E7 17,18. This allows the host cells to stop proliferating, differentiate into keratinocytes and move to the suprabasal epithelial layers. As expression of E6 and E7 is reduced, E1 binds to the long control region (LCR) and attracts the host cell’s DNA replication machinery, which starts to replicate the HPV genome as a rolling cycle 14. When infected keratinocytes reach the upper epithelial layers, HPV starts to express E4, L1 and L2, which are needed for viral assembly 19,20. Viral particles are produced in abundance and spread by contact to repeat the cycle in the next host.
The immune system

Immune responses can be divided into adaptive or innate responses. The innate immunity provides quick response by relying on mechanisms in place before infection. The principal components of the innate immunity are barriers such as epithelia, phagocytic cells, complement proteins, the coagulation machinery and cytokines. The adaptive immunity can be divided into either humoral or cell-mediated immunity. In brief terms, B cells and their antibody production mediate humoral immunity, while T cells are central in cell-mediated immunity.

The human leukocyte antigen (HLA), or the major histocompatibility complex (MHC) as it is also referred to, plays a major role in mounting immune
response to infections. The MHC contains genes encoding several central proteins of the immune system (Figure 4).

Figure 4. Arrangement of the human MHC loci.

The genes of the HLA class I and class II loci are highly polymorphic. They encode proteins responsible for presenting foreign peptides to the T cell receptors. HLA class I proteins are expressed in nucleated cells and present intracellular peptides to CD8+ T cells. HLA class II proteins are expressed in antigen presenting cells (APC), such as macrophages, dendritic cells and Langerhans cells. These cells type phagocytose proteins, process them into peptides and present them to CD4+ T cells via the HLA class II molecule.

In general, both innate and adaptive immune responses are mounted as response to viral infections. Infected cells often express and secrete the cytokines interferon (IFN) -α and -β to alert the surrounding. These cytokines prepare cells for virus infections in two ways. Firstly, they up-regulate expression of HLA class I molecules for presentation of intracellular viral peptides to T cells. This way, subsequently infected cells are more likely to be able to mount a cell-mediated immune response. Secondly, they induce neighboring cells to express a number of enzymes interfering with viral replication. To escape immune response, viruses often interfere with expression of HLA class I molecules. The innate immunity’s answer is natural killer cell (NK) mediated killing of cells lacking HLA class I on the surface. Adaptive immunity against viral infections is mediated by either antibodies or cytotoxic T lymphocytes (CTL). Antibodies block viruses from entering the cells, whereas CTLs eliminate the infection by killing infected cells presenting viral peptides by HLA class I.

**HPV and the immune response**

To spread and prosper, sexually transmitted viruses like the anogenital HPVs have to remain in the host until they can be passed to the next individual upon sexual contact. In order to persist, HPV has evolved a battery of strategies to evade the immune system.
HPVs go through their complete lifecycle in epithelia. This way, they avoid the humoral immune response of the circulatory system. The low profile is further maintained by keeping protein levels down to a minimum. The genes of the virus have been found to contain codons that are uncommon in mammals. This reduces the usable transfer ribonucleic acid (tRNA) pool, which in turn limits the translation of messenger (m) RNA into proteins \(^{21-23}\). As long as it remains in the lower epithelial layers, HPV restricts gene expression to the early proteins E1, E2, E5, E6 and E7. These proteins are mainly located to the nucleus, placing them mainly out of reach for patrolling APCs. The proteins also actively suppress the immune response. E7 has been shown to down-regulate production of IFN-\(\alpha\) and -\(\beta\) \(^{24,25}\), which results in reduced HLA class I expression. E5 is thought to interfere with HLA class II maturation by inhibiting acidification of endosomes, which is normally required for the degradation of phagocytosed proteins into presentable peptides \(^{16,26}\). Since HPV refrain from lysing cells in the lower epithelial layers, the contact between HPV proteins and APCs is mainly restricted to phagocytosis of dying keratinocytes. There are indications that such naturally occurring phagocytosis may mediate peripheral tolerance to the E7 protein \(^{27}\).

How HPV contributes to cancer development

High-risk HPVs are potent inducers of proliferation and have been shown to immortalize human cells, i.e. make them immune to the normal limitations on life span. The main mechanisms behind these crucial steps towards cancer are the interactions of HPV proteins E6 and E7 with the P53 protein and the retinoblastoma protein (RB) (Figure 5).

E6 from high-risk HPV mediates degradation of P53 proteins via the ubiquitin proteolysis pathway \(^{28-31}\). The protein P53 is a central gatekeeper for cellular growth and division and is found in mutated forms in many cancer types. Normally, expression of the \(P53\) gene is activated by conditions stressful to the cell, such as DNA damage, hypoxia and low levels of ribonucleoside triphosphate \(^{32}\). Aggregation of the protein P53 induces two major events: cell growth arrest in the gap 1 (G1) phase of the cell cycle or apoptosis. Removal of the protein makes the cells insensitive to DNA damage and enables them to evade apoptosis.

The E7 proteins of high-risk HPVs form complex and interfere with the RB protein. The \(RB\) gene is member of a gene family that also includes \(P107\) and \(P130\). Together, the proteins of this family repress genes governing cell cycle progression, apoptosis and differentiation. Proteins of the RB family interact with transcription factors such as the E2Fs. These play crucial parts in activating genes whose expression is required for DNA replication and the entry of cells into the DNA synthesis (S) phase of the cell cycle. By binding promoters sensitive for E2Fs, RB represses gene expression. When RB is
inactivated, e.g. by forming complex with E7, the cells are more prone to entering and remaining in S phase.

Figure 5. Mechanisms of HPV’s oncogenic proteins. E6 and E7 interact primarily with P53 and RB. The cell-cycle control is relieved, which ultimately transforms the cell.

By hitting central anti-cancer pathways, E6 and E7 render cells with phenotypes prone to accumulate mutation, insensitive to the anti-proliferation stimuli and emergency brakes normally acting to keep cell division in strict order. As a consequence, cells infected by high-risk HPV could be considered having acquired mutator phenotypes in accordance with the hypothesis put forward by Loeb et al. in 1974. The impaired P53 function may also render cells with phenotypes needed for the survival of subsequent advance stage tumors. As tumors grow large they risk outgrowing their vascular supply of oxygen, causing hypoxia in affected cells. Loss of P53 function has at least two beneficial effects on an oxygen-deprived tumor. P53 normally sends the cell into apoptosis as a response to hypoxia. Impaired P53 response leaves the cells less sensitive to lack of oxygen. Further, P53 has been found to inhibit angiogenesis by inducing the antiangiogenic factor thrombospondin. Loss of P53 may tip the balance over in favor for angiogenesis, which is crucial development of large tumors.
Risk factors contributing to the risk for cervical cancer

The importance of HPV in cervical cancer development is firmly established but while virtually all tumors contain the virus, far from all who contract infections end up with cancer. Therefore, the other risk factors than mere HPV presence have been investigated in attempts to clarify the etiology of cervical cancer.

Directly HPV-related risk factors

Persistent HPV infection has been shown to be highly associated with risk for incident premalignant stages of cervical cancer\(^{37,38}\). It seems as if certain individuals are unable to clear their contracted HPV infections. The underlying determinant of viral persistence is not yet clear and there may be several contributing factors.

Although somewhat controversial, certain HPV-type variants have been proposed to be more aggressive than others. A South American study found that women with non-European HPV variants tended to have more persistent infections\(^ {39}\). Authors have also reported non-European variants of HPV 16 and -18 to be associated with severe dysplasia and to have higher prevalence in invasive cancers than in premalignant stages of the disease compared to the European variants\(^ {39,40}\).

HPV is often found integrated in the human genome in cervical tumors\(^ {41-45}\). As the circular HPV genome is linearized, the break usually occurs within the L2 gene. This brings L2 expression to an end, which in turn is thought to remove E2’s silencing effect on E6 and E7 genes. Raised levels of E6 and E7 increase proliferation, which could result in a selective advantage of the affected cells\(^ {17,43}\).

Persistence has also been associated with high viral load\(^ {46,47}\). Although it is unclear whether viral load influences cancer development or is a result of cancer development, high viral loads have been proposed as risk marker for cervical cancer.

Risk factors indirectly related or not related to HPV

Compromised immunity

Both women infected by Human immunodeficiency virus (HIV) and patients receiving immunosuppressive treatment are at increased risk for cervical cancer\(^ {48-51}\). The underlying mechanism is likely an increased opportunity of HPV to establish persistent infections caused by the impaired cell mediated immunity. HPV prevalence and persistence have indeed been found to be higher in both groups compared to that in normal controls\(^ {52,53}\).
Genetic susceptibility
By mining the Swedish Cancer Register, Dr. Magnusson et al. could show that shared genes explained 27% of the liability to cervical cancer. The most thoroughly examined candidates for mediating such genetic susceptibility are polymorphisms at codon 72 in the p53 gene and alleles of the HLA class II locus.

A recent meta-analysis on p53 polymorphism found the proposed risk genotype (Arg/Arg) not to have any effect on preinvasive lesions. However, a slight increased risk was seen for both squamous cell- and adenocarcinoma (odds ratio [OR] 1.5, 95% confidence interval [CI] 1.2-1.9 and OR 1.7, 95% CI 1.0-2.7 respectively). The authors concluded that the seen differences could very well be caused by departure from Hardy-Weinberg equilibrium in the control groups due to methodological issues.

There is a strong biological plausibility for HLA to have effect on risk for cervical cancer. HLA is central in mediating protection against infections by presenting foreign antigen to T cells. Association between HLA class II and cervical cancer development has been described in several studies. HLA class I has been investigated in a lesser extent due to a past lack of robust genotyping assays. The HLA class II haplotype most consistently described to affect the risk of cervical cancer is the protective DRB1*1301-DQB1*0603.

Oral contraceptives
Epidemiological studies yield somewhat conflicting data regarding associations between use of oral contraceptives (OC) and cervical cancer. A meta-analysis, only including HPV positive women, reported longer use than 10 years to increase the risk by two-fold (relative risk [RR] 2.5, 95% CI 1.6-3.5). A mechanism behind the increased cancer risk may involve an effect of OCs on HPV’s protein production. Steroid hormones, such as progesterone, induce glucocorticoid responsive elements, which are similar to the regulatory sequences of HPV.

Smoking
In a recent pooled analysis of eight case-control studies using only HPV positive women, current tobacco smoking was found to increase the risk for squamous cervical carcinoma (OR 2.3, 95% CI 1.3-4.0). Several mechanisms have been proposed for the association between smoking and cervical cancer. Smoking is known to affect carcinogenesis in areas not directly exposed to smoke from other cancer forms such as pancreatic, kidney and bladder cancer. Nitrosamines and other carcinogens have been found in the cervix of smokers. Further, the immune response of the cervix has been shown to be impaired in smokers by counting number of Langerhans cells and other markers for immune function.
Parity
Several studies have reported associations between high numbers of full term pregnancies and cervical cancer\textsuperscript{64-66}. Several mechanisms have been proposed to be involved such as hormonal-, trauma- and immune influences.

Other risk factors
More risk factors are under investigation. The age at viral exposure is known to influence the risk of several other cancer forms, such as Hepatitis B induced liver cancer\textsuperscript{67} and Epstein-Barr virus induced Burkitt’s lymphoma\textsuperscript{68}. Whether the association seen between age at first intercourse and risk of cervical cancer is an artifact of mere risk of HPV exposure or if it increases the risk among HPV-exposed remains to be shown.
Other infectious agents such as Chlamydia trachomatis and Herpes simplex virus (HSV)\textsuperscript{2} are suggested to have co-factorial roles in some cases of cervical cancer but the associations are weak and inconsistently found\textsuperscript{69-71}. Proposed explanations include enhanced access to basal membrane and increased proliferation due to inflammation.

HPV vaccines
Today, large efforts are being invested in developing vaccines against HPV in order to reduce the burden of cervical cancer. Provided effective vaccines are developed and made available, this approach will significantly reduce the burden of cervical cancer and perhaps ultimately eradicate this cancer form.

Prophylactic vaccines
Prophylactic vaccines are those that are administered in order to immunize the receiver before infection occurs. These vaccines are often injected and elicit humoral immunity (antibodies). Most prophylactic vaccines in clinical trials today use virus-like particles (VLP) as antigen. VLPs are in essence empty viral capsids, consisting of the L1 and L2 proteins or solely of L1\textsuperscript{76}. VLPs are ideal for prophylactic vaccines since they induce high levels of neutralizing antibodies\textsuperscript{77}. The route of administration has been a concern when developing prophylactic vaccines against HPV. Since the virus is exclusively present in epithelia, it has been questioned whether a humoral immune response in response to injected antigens will suffice to clear infection. Several alternative ways of delivering the VLPs have been suggested, such as intranasal or oral immunization\textsuperscript{78,79}. In favor of using these alternative routes is the chance of generating mucosal immunity (IgA) and the prospect of developing simple vaccines that are affordable for developing countries. However, there are several disadvantages of vaccines inducing mucosal im-
munity. Relatively little is known about mucosal immunity and current mucosal vaccines do not induce a long-lasting immunity. In addition, the amount of antigen absorbed is generally low and varies widely among individuals. In spite of this, the research on mucosal vaccines is likely to continue, since it is the preferable strategy for large-scale vaccination programs in developing countries. However, a recent study indicated humoral immunity to be effective against HPV. In a randomized clinical trial comprising 2392 women, all 41 cases occurred in the placebo group employed. The prophylactic VLP vaccine employed both reduced persistent HPV 16 infections and associated premalignant cervical disease.

Therapeutic vaccines
Therapeutic vaccines are given as treatment when the infection is already established. Once HPV enters the cell, antibodies are unlikely to helping clearing the established infection. Instead, therapeutic vaccines have been aimed at activating a CTL response. For a therapeutic vaccine to be successful against HPV and cervical tumors, the chosen antigens must be expressed in all stages of infection and in tumors. Consequently, most groups have focused on the proteins E6 or E7. Since E6 and E7 are carcinogens, investigators have tried to develop peptide vaccines using only the part of the protein needed for presentation to T-cells. Peptide vaccines are well tolerated and there are indications of clinical response when combined with the right adjuvants. Another approach to avoid the oncogenic effect of HPV proteins has been to engineer recombinant proteins. This approach has the advantage over peptide vaccines of not being restricted to a certain epitope. Several studies employing fusion proteins of E6 or E7 in combination with other proteins have proved successful against genital warts in early trials. Several other strategies are being investigated, such as vaccination by using viral vectors or naked DNA. However, given of the heterogeneity and genomic disarray in advanced tumors, it is likely that parts of a tumor will either not express the antigen or be defect in the antigen presenting ability. As a consequence, therapeutic vaccines may only be suitable for premalignant stages of cervical cancer.

At the present, several prophylactic vaccine trials are being carried out and the emerging results seem promising. If vaccines prove effective and provided they are made available in developing countries, where the toll of cervical cancer is the greatest, there is a chance of great reduction in the worldwide burden of the disease.
Screening

Screening is defined as testing apparently healthy individuals in order to classify them as likely or unlikely to have a certain disease. People at risk for the disease are further investigated and those who are found to have the disease are treated. The goal of reducing morbidity and mortality among the screened is reached by early diagnosis and treatment. To be suitable for screening, a disease has to go through a phase during which it is detectable but unnoticed if not tested for. Further, there should be a treatment that benefits from detecting cases at an early stage.

Cytological screening

An early step on the road towards cervical cancer screening was taken in 1928, when Dr. Papanicolaou first reported of his Pap-staining technique. Cells sampled from the vagina and cervix were smeared onto a glass slide and stained to enable inspection by microscopy. In 1954, Dr. Papanicolaou presented a system for grading the Pap-stained smears based on the degree of certainty that malignant cells were present. In 1968, the “descriptive” system based on morphological criteria was introduced and embraced by the WHO. In 1978, the concept of cervical intraepithelial neoplasia (CIN) was introduced. Although based on histology, the system was used quite frequently among cytologists. In 1988, the Bethesda system was introduced in the United States, which reduced the number of categories in an effort to increase reproducibility within and between laboratories. Table 1 lists the respective classification systems.

Cytological screening for cervical cancer has been an overall success. Countries where screening has been widely accessible have experienced a 40-80% drop in mortality from the disease. In such countries, women who develop invasive cervical cancer (ICC) are mostly those who are screened irregularly or completely fail to attend screening. Although Pap smears have revolutionized the cervical cancer prevention and lowered incidence of the disease, there are some flaws to this standard technique. One such, affecting especially undeveloped countries, is the demand for skilled interpreters of the smears. Another is the varying sensitivity. A recent study including referral of women with completely negative test results for colposcopy reported a sensitivity as low as 40%. A thorough meta analysis performed by the US Agency for Health Care Policy and Research reported an overall sensitivity of 51%. Specificity in the same studies were reported to be 91.6% and 97%.
Table 1. Nomenclature of Pap smear diagnoses of the different steps in cervical cancer progression. Each column represents the diagnoses of a classification system.

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<tr>
<td>Class 1</td>
<td>Negative for malignant cells</td>
<td>Negative</td>
<td>Within normal limits</td>
</tr>
<tr>
<td>Class 2</td>
<td>Inflammatory atypia</td>
<td>Reactive &amp; reparative changes</td>
<td>ASCUS§</td>
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<tr>
<td>Class 3</td>
<td>Mild dysplasia</td>
<td>CIN* 1</td>
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<td>Class 4</td>
<td>Carcinoma in situ</td>
<td>Invasive carcinoma</td>
<td>Invasive carcinoma</td>
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<td>Class 5</td>
<td>Invasive carcinoma</td>
<td>Invasive carcinoma</td>
<td>Invasive carcinoma</td>
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§ Year the system was introduced. * Cervical intraepithelial neoplasia. § Atypical squamous cells of undetermined significance. ¶ Low-grade squamous intraepithelial lesion. † High-grade squamous intraepithelial lesion

Liquid based cytology represents an improvement of conventional cytology and is estimated to increase the sensitivity to approximately 80%. Although more sensitive, liquid based cytology is still expensive compared to the traditional Pap smear. This is partly an effect of the market being dominated by a few commercial interests. Even if prices were to drop due to increased competition, liquid based cytology still requires the costly infrastructure that restrains countries with more humble medical budgets from introducing conventional Pap smear screening.

HPV in screening
Following the general acceptance of HPV’s etiological role in cervical carcinogenesis, large efforts have been focused on validating the usefulness of HPV testing to predict the risk of cervical cancer development. Such tests could be a cost-effective alternative for the areas of low resources where the burden of cervical cancer is greatest. Of course, provided HPV testing proves reliable and efficient in regard to sensitivity and specificity. Compared to cytology, HPV testing is more objective in evaluation of samples. If sensitive self-testing devices were to be developed, the technique opens up for centralization and automation that would reduce the overall cost for laboratory equipment and personnel.

The HPV tests available today are primarily qualitative. The majority of published studies use either PCR (My09/11 or Gp5+/6+ primers) or an FDA approved RNA probe hybridization assay called Hybrid Capture 2.
(HC2) (Digene Corp., Gaithersburg, MD USA). In a comprising Chinese women ages 35-45, the HC2 was evaluated on both self-collected samples and samples collected by gynecologists. Sensitivity of identifying women with ≥CIN2 was remarkably high (95%) when gynecologists collected samples but somewhat lower (83%) for self-collected samples. The results should be seen in the light of the fact that the prevalence of cervical cancer and its preclinical stages is high in rural China, where the study was conducted. Another fact to keep in mind when considering HPV tests in screening is that the prevalence of the virus is far greater in young age groups than in older women. This pattern probably reflects the sexual behavior of young age groups. Consequently, qualitative HPV screening in young women would lead to low specificity due to numerous transient infections.

For HPV tests to be of value in primary screening where screening is already in place, it would require other markers to distinguish transient infections from those who pose an important cancer risk. Several such markers have been proposed, including viral integration, persistence, HPV-type variants and finally viral load, which we have focused on in the studies comprising this thesis work.
Thesis work

Aims

Paper I
To develop an assay for detection and quantification of the most prevalent HPV types associated with high risk for cervical carcinoma.

Paper II
To investigate whether the risk for cervical squamous cell carcinoma in situ is dependent on the HPV load in cervical Pap smears. Several high-risk HPV types are examined.

Paper III
To examine whether the association seen between HPV load and risk for cervical squamous cell carcinoma extends to invasive cervical carcinoma.

Paper IV
To study the associations between HLA class II alleles and the viral load of HPV 16, -31 or -18/45.
Results and discussion

Definition of the case-control material used in papers II, III and IV

Setting
In 1967, a screening program was organized by the health care system in Uppsala. Women at ages 30-49 were invited for Pap smear collection approximately each third year. From 1972 and onwards the starting age for screening was lowered to 25 years of age. In addition to the smears from the organized screening, smears from opportunistic screening were also included in the register. These account for about 75-80% of the registered smears. Cases and controls were selected from the 146,889 women with a total of 732,287 smears in the cytology register in Uppsala County between the years 1969 through 1995.

Subjects
Cases and controls were selected from the women having at least one smear registered in the Uppsala cytology register during the years 1969-1995. To be eligible for our study subjects had to fulfill the following criteria:

- Born in Sweden
- Less than 50 years of age at entry
- First smear registered as negative for abnormal squamous cells

Merging the eligible cohort’s records with the National Cancer Registry identified subjects diagnosed with cervical squamous carcinoma in situ. These were included in the study as cases. Controls were selected from the eligible cohort by matching on birth year and date of entry to the cohort (±90 days) to individual cases. In addition, controls were not permitted to have developed carcinoma in situ or carcinoma up until the date of their corresponding case’s diagnosis.

To reduce the risk of false classification of smears, a professional cytotechnician reviewed all the first smears from cases and controls. Cases with falsely negative smears were excluded. Controls with falsely negative first smears were replaced. Further, to minimize the risk of false classification of case status, a skilled pathologist reviewed pathology specimens from cases. Subjects not having carcinoma in situ were excluded. These procedures produced 504 risk sets, consisting of one case and one or two matched controls. The reason for adding several controls to a risk set was the higher number of registered smears among cases compared to controls. Five controls were found to having had hysterectomy performed prior to the diagnosis date of

* Collection of cases and controls had been performed as part of previous studies 85,86.
their corresponding case. Exclusion of these risk sets rendered 495 cases and 672 matched controls.

Paper I – Real-time PCR-based system for simultaneous quantification of Human Papillomavirus types associated with high risk for cervical cancer

The advent of PCR increased the sensitivity of ascertaining HPV presence in clinical material compared to previously used methods. Semi-quantitative measurements obtained with PCR pointed to significance for viral load in development of cervical cancer and its premalignant stages. Early studies proposed viral load as a way of discriminating the underlying CIN grade when cytology results are uncertain\(^{97-100}\). These studies focused on HPV 16 alone and the employed assays’ variability was poorly documented. In 1999, Swan et al. called for reassessment of viral load’s clinical usefulness for triage of prevalent disease. Their results indicated loads of HPV 16, -18, -31 and -45 to vary greatly within disease grades and could not be used for discriminating CIN1, CIN2 and CIN3. The accuracy of the results can be questioned as they were obtained by endpoint PCR. A subsequent study from our group showed strong association between HPV 16 load and the risk for cervical carcinoma \textit{in situ} (CIS) by using a more precise HPV assay\(^{101}\). This encouraged us to explore whether similar associations could be seen for other HPV types as approximately 40-50\% of cervical tumors contain other HPV types than HPV 16\(^{102}\).

This report describes an assay for detecting the most commonly HPV types associated with cervical cancer. The employed method is referred to as Taqman and is a quantitative PCR technique. It involves running a standard PCR reaction to which fluorescent probes are added. The probe is an oligo designed to match the target DNA between the primer sites and labeled with two distinct fluorophores: a reporter dye at the 5’ end and a quencher dye at the 3’ end. As long as the reporter is in proximity to the quencher, the probe emits light of a quencher specific wavelength upon illumination. The emission is switched to a reporter specific wavelength if reporter and quencher are separated. The Taq-polymerase used in the PCR reaction has 5’ exonuclease activity. In other word it removes and degrades encountered single-strand DNA in its way during extension (Figure 6). As a consequence, the amount of released reporter dye is proportional to the generated as long as probe is bound to each PCR fragment used as template by the Taq-polymerase. Recording the emitted reporter light during each extension phase generates a picture of how PCR accumulates over time. This is used to extrapolate the initial amount of target DNA in samples (Figure 7).
Figure 6. Schematic overview of the Taqman principle. The polymerase encounters bound probe during extension. As the probe is cleaved and displaced the emitted light shifts to a reporter specific wavelength.

Figure 7. Illustration of how reporter signal is increasing over time in a Taqman reaction. The Ct value is equivalent to the time at which the reporter intensity reaches a set threshold value (usually based on the variation seen early in the early reaction). The Ct value is inversely proportional to amount of template DNA present in the reaction prior to PCR.

When designing primers and probes, we tried to optimize the balance between covering as many HPV types as possible while running as few reactions as possible per sample, partly because clinical material is often scarce, and partly to keep costs of reagents and labor down. The number of probes was limited to three per reaction to reduce background signal. The developed assay is based on three parallel taqman reactions. The first reaction detects and quantifies HPV 16, -18, -31, and -45; the second HPV 33, -35, -39, -52,
and -67; and the third a human gene, used as a reference for the amount of cellular material in samples. The same probe is used to detect HPV18, and -45, which generates a summary measurement of these types when both are present in a sample. Likewise, the closely related HPV 33, -52, -58, and -67 share the same probe in the second multiplex reaction.

Several experiments were carried out to secure the technical performance of the assay. Sensitivity and specificity were estimated using standard curves of known quantities of HPV-carrying plasmids with added human high molecular weight DNA to simulate the conditions in clinical samples. The performance in multiple infections was evaluated by adding an extra HPV containing plasmid to a reaction similar to the ones mentioned above. Here, the emphasis was placed on multiple infections containing HPV 16 since these were considered to be most likely to be encountered in vivo. Further, we evaluated the reproducibility of the assay by estimating the variation due to difference in reagents batches, technician performing the analysis or date of analysis.

To enable straightforward and affordable quantification of the most common high-risk HPV types, several HPV types were quantified simultaneously in each reaction. However, designing a multiplexed PCR-based assay for simultaneous quantification of HPV is associated with severe obstacles. The restriction number of probes per reaction, in combination to the relatively low similarity in DNA sequence between the HPV types we tried to quantify, severely reduced the number of eligible primer sites. This may have contributed to the somewhat large variation obtained. In conclusion, acknowledging that the variation of our assay may reduce the power in subsequent investigations, the benefits of the assay were judged to overweigh the drawbacks.

Paper II – Type-specific associations of Human Papillomavirus load with risk of developing cervical carcinoma in situ

Here, the assay described in Paper I was employed in the case control material described above to explore the relationship between high-risk HPV load in cervical Pap smears and the risk of developing CIS.

To reduce the number of cases and controls excluded due to missing samples of insufficient DNA in samples we decided not to use the risk set classification. The introduced bias is assumed to be negligible since cases and controls as groups are still similar on initially matched variables such as birth year and date of entry into the cohort (Figure 8).
The mean viral load of all HPV positive smears from each woman was used as an approximation of infection history. Odds ratios for individual HPV types were estimated by logistic regression. Introducing the number of smears per woman in the analyses as a possible confounder did not change magnitude or direction of the exposure-associations seen in the crude analyses. Of the examined HPV types, only HPV 16 showed increased viral loads in cases compared to controls. For this HPV type, the risk of CIS showed increased with higher viral load reaching a tremendous level in the last percentile (OR 185, 95% CI 25.6-999). HPV 31 and -18/45 loads differed little between cases and controls. Either as a consequence of viral load not contributing to risk of CIS in infections by these types or our study not being large enough to statistically reveal differences (viral load estimates tended to cover a large range). When loads of these types were introduced in logistic regression, mainly percentiles of higher viral loads were at elevated risk for the CIS but at seemingly much lower ORs compared to the magnitudes of HPV 16. The last group of viruses (HPV 33, -52, -58 and -67) is phylogenetically related to HPV 16 and 31. Neither these HPV differed in load between cases and controls. The risk of CIS did not follow a linear trend with viral load and further efforts to identify underlying HPV types would be necessary to resolve possible importance of viral load in infections of these types.

To examine whether viral load in smears, collected at a stage before cytology reveals atypia, is associated with subsequent development of CIS we restricted the analysis to the first smear of cases and controls. Again, among those infected, cases harbored higher HPV 16 load than controls. The risk of CIS increased with higher HPV 16 loads. As for the other HPV types, no clear differences were evident in viral loads between cases and controls. When estimating the contribution of viral load to the risk of CIS, only the women with extreme viral loads were at elevated risk.
Paper III – High viral loads of Human Papillomavirus predict risk of invasive cervical carcinoma

Studies examining the role of viral load in development of cervical carcinoma have focused on the premalignant stages of the disease. Prospective studies without intervention at premalignant stages are of course immoral from an ethical aspect. Retrospective studies with invasive cervical cancer (ICC) as endpoint are difficult set up since archival clinical material is hard to come by. Most women who develop ICC in countries where smears are archived are those who fail to attend screening. However, findings based on premalignant endpoints may not automatically apply to ICC.

About 12-50% of all women with CIS develop ICC if left untreated \(^\text{103}\). A commonly proposed determinant for such progression is viral integration into the human genome. Integration is associated with rapid disease development and poor prognosis \(^\text{104,105}\). Integration represents a dead end in the life cycle of HPV. As the circular shape of the genome is lost the virus stops replicating. In the few clinical samples having been analyzed integrated HPV are found in few copies per cell. The number of integrated HPV copies range from a few copies to several hundreds \(^\text{106-108}\). Infected keratinocytes in culture have been reported to contain between 100 to more than 3500 episomal HPV copies \(^\text{109}\). If the \textit{in vitro} findings are transferable to keratinocytes \textit{in vivo}, HPV in episomal state are present in larger numbers per cell compared to integrated HPV. Accordingly, high viral loads in CIS patients may reflect infecting HPV being in episomal state. In contrast, lower viral loads in CIS patients could reflect carrying HPV in a mainly integrated form. This raises the question examined in this study: whether viral load really contributes to the risk of ICC.

Because of its large size and long coverage in time, the computerized cytology register in Uppsala offers a unique opportunity of identifying smears from individuals having contracted invasive cervical cancer. By merging the pathology and cytology registers, we were able to identify individuals with registered Pap smears prior to diagnosis of ICC up until 1999. The idea was to reuse the population controls used in study II. Consequently, the search for cases was restricted to match the birth years and smear collection dates of the controls. Two individuals among the cases were also found among the controls used in study II, and were therefore excluded from the control set.

As anticipated, the registry contained few smears from the cases. To avoid bias due to difference in number of smears per woman in cases compared to controls we chose to select up to three smears per woman by random. The randomization did not take the smears’ cytological results into account and yielded 139 cases and 550 controls. Introducing the mean viral load per woman into a logistic regression model produced ORs similar to that found for CIS in study I.
To avoid the possibility of confounding by smears from cases more often containing neoplasia, we tried to identify a single smear per woman, representing an early phase of the infection leading to the subsequent disease development. From each woman the latest collected smear was selected with the following inclusion criteria: a) smears had to be negative for dysplastic cells, b) the time-span to a preceding abnormal smear had to be at least 3 years. The selection produced 64 cases and 503 controls.

Although exact figures are not comparable due to differences in study design, the obtained results are generally similar to those obtained for CIS. The effect of viral load on risk for invasive cervical cancer is evident for HPV 16. No association between HPV load and risk for the disease was seen for the other HPV types examined. However, the negative findings should be interpreted with caution due to the limited power of our studies, brought about by the variation in viral load estimates and the sample size of the study combined with the rarity of HPV types other than HPV 16.

**Discussion papers II and III**

Our results indicate that HPV 16 load indeed does affect the risk for development both of CIS and ICC. It is plausible that high viral load asserts its effect on risk for CIS and ICC in an indirect fashion. That is, high viral loads may affect the risk of subsequent events that are prerequisites for cancer development. If viral integration is a prerequisite for transition to ICC and viral load increases the risk of ICC, it is conceivable that viral load has effect on the risk of viral integration. This remains to be shown both in vitro and in vivo, but a possible mechanism could be found if the underlying meaning of a high viral load was ascertained. If high viral loads reflect widespread infections as proposed by Sun et al.\textsuperscript{110}, the high number of infected cells would increase the risk of integration. Especially since integration is likely to be a random event, caused by the genomic instability brought about by the expression of E6 and E7 from high-risk HPV\textsuperscript{111}.

Although our results indicate that high loads of HPV 16 convey increased risk for cervical cancer, the clinical usefulness remains to be shown. During time spent on our studies, several others have published reports investigating viral load’s association with risk for cervical cancer and its precursors. The majority use HC2 to measure viral load and while some find viral load be positively associated with increased risk for prevalent or incident disease\textsuperscript{47,110,112-115}, others do not\textsuperscript{116-119}. Recent studies employing quantitative PCR to estimate HPV load show association between viral load and prevalent or incident disease more consistently\textsuperscript{101,120-124}.

A factor that may contribute to the discrepancies in findings of studies employing HC2 is the assay’s inability to distinguish between high-risk HPV types. If HPV types differ in aggressiveness, as indicated by us as well as others\textsuperscript{121,122,125}, results obtained with HC2 may be blurred and misleading as long as underlying HPV types are not resolved.
However, despite associations found between viral load and cervical cancer several issues remain to be answered before viral load can be said to be of clinical value. Viral load seems inappropriate for determining lesion severity underlying an unclear cytological observation. Considerable viral-load variation has been observed within histopathological grades of the disease making it hard to define uniform cutoff values. In addition, viral load has been reported to be associated with lesion size rather than lesion severity. A study where participants were sampled densely reported substantial variation in viral load over time within individuals. Provided the observed variation is not caused by the specific technique employed, it may reflect either actual viral-load fluctuations in the tissue or difficulties in collecting samples in a uniform manner. Consequently, the variation may complicate interpreting the meaning of a single viral-load estimate from a woman. Our results indicate a possible role for viral load of HPV 16 in estimating the risk of future cervical cancer development. If HPV tests are introduced in screening, inclusion of viral load estimates could be informative. Not as a predictor of underlying disease, but rather as an indication of need for more intense follow-up in patients harboring high viral loads.

Paper IV – HLA class II allele control of HPV load in cervical carcinoma in situ of the cervix uteri

Previous studies have shown that genetic inherited variation affects the risk of cervical cancer. The most thoroughly example studied of such variation are the highly polymorphic HLA class I and class II alleles. The products of these genes are central in mounting adaptive immunity to infection as well as tumors. Epidemiological studies have shown that some HLA class I and class II alleles convey increased susceptibility to cervical cancer while others protect against the disease. Our group has previously showed that HLA class II alleles DRB1*1501 and DQB1*0602 increases the risk of cervical carcinoma in situ and elevates the risk for HPV 16 infection. In addition, carriers of the same alleles were more prone to have higher content of HPV 16 in their Pap smears.

In this study we merged the viral-load data generated in study II with HLA class II information to examine the allelic effect on cancer associated with infection of HPV 31, -18/45. It is conceivable that the proteins of HPV 31, -18 and -45 differ from that of HPV 16 in properties determining affinity to certain HLA class II molecules. Therefore the individual HLA class II alleles may act on infection and cancer in HPV-type specific manners. Stratification on both HLA class II carrier status and uncommon HPV types requires large studies to yield sufficient power. To our knowledge, the case-control material used here is one of the larger collected for investigating HLA class II importance for HPV-associated cancer development.
Our results point to a protective effect of especially DRB1*1301 on HPV 31 and -18/45 related CIS. Compared to controls, the frequencies of DRB1*1301 were lower in cases positive for HPV 31 or HPV 18/45. In addition, the frequency of DQB1*0603 was lower than expected in cases positive for HPV 18/45 indicating a protective effect of this allele as well. We did not observe increased susceptibility for CIS in HPV31 or -18/45 infected cases carrying DRB1*1501 or DQB1*0602 as seen previously for HPV 16. This negative finding could reflect a type specific effect of these alleles, but it could also be caused by lack of power. Protective alleles have been hypothesized to be more easily discernable in epidemiological studies than risk alleles. The authors hypothesized that a single protective HLA allele would suffice to more efficiently bind and present the antigen, while risk alleles can easily be cancelled out by the presence of other more protective alleles.

Next, we investigated the risk modulating alleles’ affect on viral load. The previously described increased HPV 16 load in carriers of the haplotype DRB1*1501-DQB1*0602 was confirmed with the new HPV assay. Carriers of the haplotype DRB1*1301-DQB1*0603 had lower viral loads than non-carriers among those infected by HPV 31 or HPV 18/45.

In conclusion, our results confirm that certain HLA class II alleles influence the risk of cervical cancer, the risk of being HPV infected and the risk of high viral load. The impact of HLA class II alleles on cancer risk is likely indirect, resulting from the susceptibility or resistance to the virus. Different HLA class II alleles seem to differ in efficiency of presenting viral antigens to T helper cells. However, there are other conceivable explanations for the observed associations. HLA class II alleles could in fact exert their risk enhancing/reducing effect as APCs present tumor antigens after phagocytosis of dead tumor cells. Although this study did not examine this plausibility directly, our findings as well as earlier studies support involvement of HPV even when restricting the analysis to an early phase of infection, before cervical smears are positive for dysplastic cells.

Even though certain HLA class II alleles elevate the risk for cervical cancer, it seems that this knowledge at the present is of little value in improving screening for the disease. Beskow et al. earlier assessed the value of using carrier status of the DRB1*1501-DQB1*0602 haplotype together with HPV 16 load to predict the development of CIS. The analysis showed no additional value of the haplotype status in the predicting the disease once a viral load is estimated.

The allele-specific association of HLA and viral load may have implications for the development of effective vaccines against HPV infection. HLA class II alleles are associated with cancer development to a higher extent than HLA class I alleles. This indicates that alleles of HLA class II are of importance in mounting immune response against the virus. Consequently,
carriers of risk alleles may need separate routines when and if large-scale vaccination programs are introduced. This possibility validates further investigations in the interactions between products of susceptibility loci, HPV and development of cervical cancer.

Future perspectives

HPV tests in cervical cancer screening

The relatively high rate of false-negative cytology results has inspired investigators to evaluate HPV testing as a way of increasing the sensitivity of cervical cancer screening. Opponents to HPV testing argue that cytology alone has been successive in reducing cervical cancer mortality and morbidity in countries where organized screening is offered. In addition, the abundance of transient infections and lack of treatment for HPV infections make it impractical to follow up all infected individuals. Supporters maintain that virtually no one develops cervical cancer without being HPV infected and that HPV testing therefore poses a logical approach for secondary prevention that is worthy further validation. Especially in countries lacking the resources needed for setting up and maintaining effective cytology screening. In a recent meta-analysis HC2 testing was identified to have higher sensitivity and equal specificity compared to repeated Pap-smear collection as a triage for CIN2 or higher in patients with equivocal cytology results. Even though the sensitivity of qualitative HPV testing may be higher than that of cytology, there is room for improvement. Efforts are being made to identify sensitive and specific markers for infections at high risk for progressing to cervical cancer. Viral load and integration have been proposed, as ways of increasing the specificity of HPV tests but the value of both approaches remain uncertain. Based on our results, viral load mainly seems to have relevance for HPV 16 related tumors. Although HPV 16 is the most prevalent HPV type, about 40% of tumors contain other HPV types and require other means of discerning infections leading to cancer. The use of viral integration as a triage for severity of lesion is still yet to be validated and technical issues remain to be solved. To determine the physical state of the HPV genome, investigators often use either in situ hybridization (ISH) or PCR-based techniques. A commonly employed approach in the PCR assays is the use of low relative amounts of L1 or L2 to E6 or E7 as an indicator of integration. A risk with this strategy is that the integration has to occur within the amplified E1/E2 region for the assay to work. Others have used more precise PCR protocols for examining the physical state of the viral genome. However, these approaches are often elaborative, which is likely to limit the clinical value. A disadvantage in common for all
PCR techniques is risk of missing viral integration in the presence of high viral loads co-infecting episomal genomes. It is also likely that clusters of cells harboring mainly integrated genomes are surrounded by less severe lesions. This may further obscure the results from PCR-based assays.

ISH targeting HPV has been demonstrated to generate a punctuated pattern representing integrated DNA localized to the nucleus of cells. A more diffuse pattern represents episomal HPV. The previously poor sensitivity of ISH has recently been improved by using tyramide-based assays. However, the high sensitivity (single HPV copies) raises several questions. Setting a threshold as low as single HPV copies increases the risk for false positives, as unspecific binding is likely to resemble the punctuated pattern of integrated HPV. Moreover, single episomal HPV-copies may be confused with integration in a similar fashion. However, future studies will resolve the technical issues of HPV-directed ISH. To its advantage, ISH has been indicated to be applicable to thin-layer cervical smears. This would enable using the technique in adjunct to thin-layer cytology, which is increasingly being used in many countries today. In spite of the promising results with HPV-directed ISH, the technique shares a drawback in common with all other DNA or RNA based assays for detecting and evaluating HPV infections. This drawback is the difficulty of designing a straightforward test covering the multitude of HPV types found in cervical tumors. However, a recent development may have found a way to circumvent this problem.

Investigators have identified a biomarker that may be present in all high-risk HPV infections. As mentioned before, the E7 protein of high-risk HPV disables RB and induces proliferation. Proliferation is normally tightly regulated by the cyclin-dependent kinase inhibitor P16. P16 acts in a feedback loop to increase the amount of unphosphorylated RB (the active kind). But as RB is inactivated in high-risk HPV infections, the feedback loop is bypassed and P16 accumulates in response to the excessive proliferation. Promising studies have indicated immunostaining for P16 to be a both sensitive and specific marker of high risk HPV infection and severe cervical lesions both in biopsies and cytological samples. If used inadjunction to cytology, P16 staining may be a way of improving the current screening programs.

Although new strategies for improving screening are emerging, it should be recognized that part of the false-negative diagnoses in screening are due to reasons that are likely to remain regardless of technical improvements. For instance, suboptimal sampling will continue to affect the diagnosis outcome regardless of the following technical strategy for identify markers associated with disease in the samples.
Will vaccination be the end of screening?

As vaccines against HPV are on their way does this mean that screening will be obsolete in a near future? The short answer to the question is: ‘no’. Several issues contribute to this answer. Some of them are directly related to the efficiency of the vaccines and others concern the problem of positioning the vaccine so that it is publicly accepted.

First of all, the vaccines are not available yet. And even when they are, there will still be several generations already exposed to HPV. Prophylactic vaccines are not meant to eradicate already established infections, but rather protect against new ones or at least reduce the viral load to prevent transfer of the virus. This means all that have been sexually active are at risk for cervical cancer and should continue being screened.

Secondly, the duration of the time during which HPV infections can be contracted poses a challenge. For a HPV vaccine to be effective it should be delivered before the infection takes place, i.e. preferably before the first sexual intercourse. This age varies in different countries but to be on the safe side it would be best to vaccinate children at ages 10-12 or perhaps as infants. This poses two problems. To give protection during the most sexually active ages the immunization would have to last for perhaps 20-30 years. In the case booster shots are needed, there will be a loss of coverage. Further, vaccinating infants or children against a sexually transmitted disease is likely to be a matter of debate in many cultures.

Thirdly, it is feasible that individuals will respond differently to vaccination. As indicated from the results of study IV and other studies on associations between the HLA alleles and HPV infection, women differ in their ability of mounting immune response to the virus. Consequently, it is likely that vaccines will not provide 100% protection on a population basis.

Lastly, we return the issue of the plethora of HPV types found in tumors. Vaccines emerging today are directed mainly against HPV 16 or HPV 16 and 18. Even if vaccines were expanded to cover HPV 31 and -45 they would still only prevent 80% of cervical cancers worldwide (provided vaccines conveyed 100% protection).

In conclusion HPV vaccination is likely to significantly reduce HPV infection and cervical cancer burden, but the need for screening of some form is likely to remain in the foreseeable future.
Acknowledgements

I would like to express my sincere gratitude to all former and present colleagues of the Rudbeck Laboratory for creating a stimulating environment. In particular I would like to thank:

*Ulf Gyllensten*, for generously offering me a place in the group, sharing scientific knowledge, never-ending optimism and encouragement, and always having the door open in spite an extremely busy schedule.

*Erik Wilander*, for maintaining an unbeatable cytology register and sharing with us non-clinicians. None of this would have happened without it. Well perhaps Paper I, but anyway.

Former and present member of the cervix group in no particular order: *Patrik Magnusson*, for great stories, many laughs and sharing stimulating thoughts about science, politics and other tricky issues, such as worn out clutches. *Anna Beskow*, for relieving frankness, watering the plants and teaching me almost all there is to know about pregnancies. *Inger Gustavsson*, for reminding me that there are important things in life besides science and skillfully doing the fair share of the lab work. *Malin Engelmark*, for being a kind, ambitious and outspoken addition to the diminishing group. *Jessica Magnusson*, for reminding me to take coffee brakes and filling in the gaps that Anna missed concerning baby stuff. *Agneta Josefsson*, for paving the road before me by extracting an enormous amount of Pap smears.

Charlotte, Hanna, Jonas, Veronika, Åsa Johansson, Jenny von S, Kalle, Tomas Bergstöm, Anna and all other members.
Special thanks to Mathias, Alistair and Graciela Elgue for proofreading.

Friends outside work who’s much appreciated company have carried Lisa and I through these years.

My family in Skövde, especially: Murat, Hatune, Benjamin, Daniel, Loma, Maria, Susanne, Mona, Maha, Ibrahim, Evelina, Ballota-Emilie, Rana and Mikael. Thanks for generously making me a part of suroyoyo community and adopting me into your bigheartedly family.

My family in Mälardalen: Svante, Ami, Anders, Kaje, Palle, Britta and all the other Mobergs and Fagerholms. Thanks for endless moral support during these years and all the fun times at family gatherings. I love you all and hope to get to see more of you now that this thesis business is completed.

And finally: Lisa, my adorable other half. Thanks for all the laughs, love and support during our journey together. I consider myself blessed for having you by my side in life.
I love you, habibti!
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 Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)