Human Papilloma Virus

Association with vulvovaginitis and genital intra-epithelial neoplasia

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Abstract

In many women with gynecological complaints such as itching, burning, discharge, and fissures causing dyspareunia, examination of the vulvovaginal mucosa reveals hyperkeratotic and papillomatous changes. Polymerase Chain Reaction (PCR) technique revealed 64% of such lesions to harbour Human Papilloma Virus (HPV)-DNA, whereas Southern blot (SB) technique showed 50% to be positive for HPV-DNA.

Women with papillomatous lesions were more often HPV-DNA positive than those with flat hyperkeratotic lesions. The virus-induced vulvovaginitis described was sometimes the sole cause of atypical Pap-smears. However, papilloma virus infections in vulva and vagina were often accompanied by neoplasia.

In women with an atypical Pap-smear, signs of HPV were observed by colposcopy in 58% of cases, by cytology in 21%, by histopathology in 53% and by HPV-DNA hybridization techniques in 46%. Colposcopy, cytology and histopathology were more sensitive than SB and Filter In Situ Hybridization (FISH) in detecting HPV in benign epithelium and in mild to moderate dysplasia. The FISH technique, when applied to cell samples and the SB technique for biopsy material proved equally sensitive when benign tissue and mild to moderate dysplasia were analysed. However, in women with severe dysplastic lesions, use of the SB technique on biopsy material proved more sensitive than FISH.

In lesions with severe dysplasia, HPV-DNA was very often present (67% of CIN III lesions). HPV 16, which is capable of oncogenic transformation, was found in 54% of such tissue.

At follow-up after laser treatment of genital intra-epithelial neoplasia, HPV could be detected in 38% of cases. This indicates that HPV may affect the entire mucosa of the lower genital tract, even when not clinically detectable. Thus, to eradicate the virus, systemic therapy would appear to be required.

Vulvovaginal HPV infection is an entity with characteristic symptoms, morphological changes and oncogenic potential. Certain HPV-types are associated with the development of genital intra-epithelial neoplasia. The diagnostic methods presently available are not, however, sensitive enough for detection of HPV-infection and there is no effective treatment currently available. It would therefore be premature to suggest the introduction of a screening program for certain oncogenic HPV-types. Further study of the natural history of the virus is needed before any step can be taken toward using HPV screening in efforts to prevent cervical cancer.
Hon har räknat stjärnornas vägar och är där de stimt i den ändlösa rymden går. Hon sitter vid instrument och bestick och själv är hon bara ett ögonblick. Själv är hon bara ett bloss i vind, ett födsloskri och en fårad kind.

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

I. Bodén E, Eriksson A, Rylander E, von Schoultz B.  
   Clinical characteristics of papillomavirus-vulvovaginitis.  

II. Bodén E, Rylander E, Evander M, Wadell G, von Schoultz B.  
    Papilloma virus infection of the vulva.  

III. Bodén E, Evander M, Wadell G, Bjersing L, von Schoultz B, Rylander E.  
     Detection of human papillomavirus in women referred for 
     colposcopy. A comparison between different diagnostic 
     methods.  

IV. Bodén E, Rylander E, Evander M, Wadell G.  
    Follow-up of HPV-DNA positive women after 
    laser-removal of genital intra-epithelial neoplasia.  
    Submitted for publication 1991.

V. Bodén E, Rylander E, Evander M, Joelsson I, Wadell G.  
   Detection of minimal copy numbers of HPV-DNA by 
   polymerase chain reaction in women with vulvovaginitis.  
   Submitted for publication 1991.
ABBREVIATIONS

The following abbreviations are used in the text:

CIN   Cervical Intra-epithelial Neoplasia
DB    Dot blot
DNA   Deoxyribonucleic Acid
FISH  Filter In Situ Hybridization
HPV   Human Papilloma Virus
HSV   Herpes Simplex Virus
OC    Oral Contraceptives
PCR   Polymerase Chain Reaction
SB    Southern blot
STD   Sexually Transmitted Disease
VAIN  Vaginal Intra-epithelial Neoplasia
VIN   Vulvar Intra-epithelial Neoplasia
ABSTRACT

In many women with gynecological complaints such as itching, burning, discharge, and fissures causing dyspareunia, examination of the vulvovaginal mucosa reveals hyperkeratotic and papillomatous changes. Polymerase Chain Reaction (PCR) technique revealed 64% of such lesions to harbour Human Papilloma Virus (HPV)-DNA, whereas Southern blot (SB) technique showed 50% to be positive for HPV-DNA.

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In women with an atypical Pap-smear, signs of HPV were observed by colposcopy in 58% of cases, by cytology in 21%, by histopathology in 53% and by HPV-DNA hybridization techniques in 46%. Colposcopy, cytology and histopathology were more sensitive than SB and Filter In Situ Hybridization (FISH) in detecting HPV in benign epithelium and in mild to moderate dysplasia. The FISH technique, when applied to cell samples and the SB technique for biopsy material proved equally sensitive when benign tissue and mild to moderate dysplasia were analysed. However, in women with severe dysplastic lesions, use of the SB technique on biopsy material proved more sensitive than FISH.

In lesions with severe dysplasia, HPV-DNA was very often present (67% of CIN III lesions). HPV 16, which is capable of oncogenic transformation, was found in 54% of such tissue.

At follow-up after laser treatment of genital intra-epithelial neoplasia, HPV-DNA could be detected in 38% of cases. This indicates that HPV may affect the entire mucosa of the lower genital tract, even when not clinically detectable. Thus, to eradicate the virus, systemic therapy would appear to be required.

Vulvovaginal HPV infection is an entity with characteristic symptoms, morphological changes and oncogenic potential. Certain HPV-types are associated with the development of genital intra-epithelial neoplasia. The diagnostic methods presently available are not, however, sensitive enough for detection of HPV-infection and there is no effective treatment currently available. It would therefore be premature to suggest the introduction of a screening program for certain oncogenic HPV-types. Further study of the natural history of the virus infection is needed before any step can be taken toward using HPV screening in the effort to prevent cervical cancer.
INTRODUCTION

Genital warts

Genital warts have long been associated with promiscuity. Condyloma is a word of Greek origin which means 'a round swelling adjacent to the anus'. These warts aroused little interest in ancient times and physicians of the Roman world had no firm opinion about their background. The outbreak of syphilis in Europe toward the end of the 15th century, however, led to an increasing interest in the etiology of genital diseases, including warts. Genital warts were then considered to be merely a manifestation of the syphilitic disease and were not differentiated from condyloma lata. Subsequently, the view of genital warts as being a sign of syphilis was abandoned and a new theory was put forward. The warts were now thought to be caused by gonorrhea, since they were often present in women with this disease. In the 19th century the term 'gonorrheal warts' was coined.

Gemy (1893) was the first author to suggest that common skin warts and genital warts might be related. His conclusion was based on the histological similarities between these manifestations (1).

Early in the present century the viral etiology of genital and common skin warts was verified. Cell-free extracts from human skin warts and genital condylomas were inoculated into volunteers. The recipients developed warts at the inoculation sites a few weeks later (2). The production of warts by inoculating filtrates of genital warts into non-genital skin, and filtrates from non-genital warts into genital mucosa, convinced the observers that all types of warts in humans must be caused by the same virus. The 'unitary theory' was proposed. Many believed that genital warts were a part of 'wart dermatosis'.

The viral etiology was finally confirmed by Strauss et al. (1949). By applying electron microscopy these authors demonstrated virus particles in common skin warts (3). Dunn and Ogilvie (1969) observed similar particles in extracts from penile, anal and vulval warts (4).

Evidence of a sexual transmission of genital warts was published by Barret et al. based on the observation of the frequent occurrence of penile warts among US service-men returning from the Korean War (5). The fact that after an incubation period of 4–6 weeks their wives developed vulvar warts led to the assumption that genital warts are a venereal affliction.
Oncogenic potential

Condylomata and flat skin warts have been reported to undergo malignant transformation. In the 1930’s, when the principles of tumour virology were formulated, squamous carcinomas were experimentally induced with papilloma viruses in rabbits (6). Chemical carcinogens (such as tar) greatly accelerated the oncogenic capacity (7). Despite these findings, a lack of interest in Human papilloma virus (HPV) infections followed for a long period.

Much later, in 1977, zur Hausen proposed that HPV plays the part of a sexually transmitted oncogen (8). Since then, an increasing interest in this field has been evident. Nowadays it is realized that most HPV infections are subclinical and that condylomata acuminata represents the ‘top of the iceberg’.

In 1983, HPV-DNA was identified in cervical cancer tissue (9). This HPV-type, designated no. 16, was found to be present in almost two-thirds of all cervical cancer specimens.

Human papilloma virus

Papilloma viruses belong to the papova virus group. They are small, circular, double-stranded DNA viruses. Papilloma viruses cannot be propagated in cell cultures. Individual papilloma viruses show great differences in species specificity, predilection site, and degree of oncogenicity.

All papilloma viruses exhibit a similar pattern of genetic organization. The viruses are named according to species and subclassified into types according to nucleotide sequence. Within the same species, any new isolate which contains less than 50% sequence homology (by DNA hybridization in liquid phase under stringent conditions) is designated as a new type and numbered in order of discovery. Human papilloma viruses (HPV) comprise the largest group, with today more than 70 types, about 30 types with tropism for the genitoanal areas have been identified (Table I). Almost half of the presently known HPV types have been isolated from the skin of immunosuppressed individuals.

HPVs infect primarily the basal layer of the epithelium where they remain as episomes, that is, they are not integrated in the host chromosomes. In this situation the replication is highly restricted (10). The replication is regulated by encoded proteins and depends on the differentiation of the epithelial host cell. The ability of the virus to replicate increases with the cell-maturation, thus virus expression is more prominent in the superficial cell layers (11,12).
Table I. Human papilloma virus types affecting the lower female genital tract [Modified from Syrjänen (13)].

<table>
<thead>
<tr>
<th>HPV-type</th>
<th>Associated with</th>
<th>Predilection site</th>
<th>Oncogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV 6</td>
<td>Condyloma acuminatum</td>
<td>vulva vagina cervix</td>
<td>Low risk</td>
</tr>
<tr>
<td></td>
<td>CIN</td>
<td>cervix</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VAIN</td>
<td>cervix</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIN</td>
<td>vulva</td>
<td></td>
</tr>
<tr>
<td>HPV 11</td>
<td>Condyloma acuminatum</td>
<td>vulva cervix</td>
<td>Low risk</td>
</tr>
<tr>
<td></td>
<td>CIN</td>
<td>cervix</td>
<td></td>
</tr>
<tr>
<td>HPV 16</td>
<td>Condyloma acuminatum</td>
<td>vulva cervix</td>
<td>High risk</td>
</tr>
<tr>
<td></td>
<td>CIN</td>
<td>cervix</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VAIN</td>
<td>vagina</td>
<td></td>
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<tr>
<td></td>
<td>VIN</td>
<td>vulva</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Squamous carcinoma</td>
<td>cervix vulva</td>
<td></td>
</tr>
<tr>
<td>HPV 18</td>
<td>CIN</td>
<td>cervix</td>
<td>High risk</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>cervix</td>
<td></td>
</tr>
<tr>
<td>HPV 30</td>
<td>CIN</td>
<td>cervix</td>
<td>Intermediate</td>
</tr>
<tr>
<td>HPV 31</td>
<td>CIN</td>
<td>cervix</td>
<td>High risk?</td>
</tr>
<tr>
<td>HPV 33</td>
<td>Squamous carcinoma</td>
<td>cervix</td>
<td>Intermediate</td>
</tr>
<tr>
<td>HPV 34</td>
<td>Squamous carcinoma</td>
<td>cervix</td>
<td>Intermediate</td>
</tr>
<tr>
<td>HPV 35</td>
<td>CIN</td>
<td>cervix</td>
<td>Intermediate</td>
</tr>
<tr>
<td>HPV 39</td>
<td>adenocarcinoma</td>
<td>cervix</td>
<td>Intermediate</td>
</tr>
<tr>
<td>HPV 40</td>
<td>CIN</td>
<td>cervix</td>
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</tr>
<tr>
<td>HPV 42</td>
<td>Papillomas</td>
<td>vulva cervix</td>
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</tr>
<tr>
<td>HPV 43</td>
<td>Hyperplasia</td>
<td>vulva cervix</td>
<td>Intermediate?</td>
</tr>
<tr>
<td>HPV 44</td>
<td>Condyloma acuminatum</td>
<td>vulva cervix</td>
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</tr>
<tr>
<td>HPV 45</td>
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</tr>
<tr>
<td>HPV 51</td>
<td>CIN</td>
<td>cervix</td>
<td>Intermediate?</td>
</tr>
<tr>
<td>HPV 52</td>
<td>Squamous carcinoma</td>
<td>cervix</td>
<td>Intermediate?</td>
</tr>
<tr>
<td>HPV 53</td>
<td>CIN</td>
<td>cervix</td>
<td>Intermediate?</td>
</tr>
<tr>
<td>HPV 56</td>
<td>Squamous carcinoma</td>
<td>cervix</td>
<td>Intermediate?</td>
</tr>
<tr>
<td>HPV 57</td>
<td>CIN</td>
<td>cervix</td>
<td>Intermediate</td>
</tr>
<tr>
<td>HPV 58</td>
<td>CIN</td>
<td>cervix</td>
<td>Intermediate</td>
</tr>
<tr>
<td>HPV 59</td>
<td>CIN</td>
<td>vulva</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>
Virion structure and genetic function

Virions contain a central core of viral DNA, enclosed within an icosahedral outer capsid of viral protein. The capsid consists of two different structural proteins, a major and a minor protein encoded by the open reading frames L1 and L2, respectively (14). The minor protein appears to be highly type-specific. The double-stranded DNA molecules have a length of about 7,900 base pairs and a molecular weight of about $5.2 \times 10^6$ daltons (15).

The viral genetic information is divided into ‘early’ and ‘late’ regions, Fig. 1. Genes from the early region establish viral control over the infected cell and initiate viral DNA replication and oncogenic transformation. Genes in the late region are involved in capsid protein synthesis. There is also a non-coding segment.

The upstream regulatory region (URR) represents 15% of the viral genome. This region contains the origin of DNA replication and gene expression is controlled from this site.

The early region represents 45% of the genome, it contains at least six open reading frames (ORF) with potential transcriptional capacity. Functions of individual ORFs are as follows (15):

- **E1 ORF** controls the episomal replication of viral DNA (16).
- **E2 ORF** either promotes or inhibits viral transcription.
- **E4 ORF** initiates the onset of koilocytotic changes.
- **E6 ORF** which is studied intensively, is involved in malignant cellular transformation (17).
- **E7 ORF** plays a significant role in malignant transformation (18).

The late region comprises about 40% of the viral genome. The two open reading frames are essential to vegetative viral replication:

- **L1 ORF** encodes the major capsid protein.
- **L2 ORF** encodes the minor capsid protein.

The ORFs are located on one DNA strand indicating that synthesis of mRNA occurs in only one direction.
HPV and cancer of the lower genital tract

Carcinoma of the uterine cervix— is one of the most common cancers of the female and constitutes a major cause of morbidity and mortality among women in various parts of the world.

Cervical cancer has long been associated with sexually transmitted diseases. Sexual behavioral characteristics such as early age of coital debut (19) and several sex partners (20) were early recognized as indicating a ‘high risk female’. The male as a transmitter of the carcinogenic agent to his partner was first discussed by Rotkin in 1967 (21). Herpes simplex virus was at that time suggested to be an etiological agent. However, it has not been possible to obtain evidence that convincingly correlates HSV to cervical cancer. The HSV genome was rarely detected in cervical cancer. Instead, there is now strong evidence implicating HPV as the background factor in the development of genital neoplasia (Table I). The majority of cervical cancers contain the oncogenic HPV 16 and 18 (22). Even though the same HPVs are detected throughout the world, the frequency of the oncogenic types varies in different geographic areas.

Carcinoma of the vulva is a relatively rare form of cancer. Women with vulval neoplasia do run an increased risk of contracting cervical neoplasia, suggesting a common causative agent (23). A small number of studies have been conducted on the prevalence of HPV in vulvar cancer (24).

Vaginal cancer is one of the most uncommon gynecologic malignancies. Vaginal squamous cell carcinoma in situ has been associated with identical lesions elsewhere in the genitalia (25). HPV-DNA has also been detected in vaginal cancers (26).

HPV and genital intra-epithelial neoplasia

The genital intra-epithelial neoplasias are asymptomatic lesions usually brought to the attention of the physician because of an abnormal Pap smear.

The invention of the colposcope made possible the detection of considerable ‘early cancers’. Hinselmann observed that leukoplakia and certain capillary patterns indicated the presence of dysplasia (27). It was not until the nomenclature was simplified, however, and the characteristic pictures illustrated by Coppleson and co-workers (28) and Kolstad and Stafl (29), that the diagnostic value of colposcopy became widely accepted. Nowadays the role of colposcopy is not to predict the histological diagnosis, but rather to delineate the extension of the lesion and to identify the area most suitable for biopsy.

Later it became obvious that many lesions containing patterns such as leukoplakia, punctuation or mosaicism did not carry any premalignant condition (30). Such lesions are probably HPV induced.

Originally it was thought that squamous epithelial neoplasms could arise only from the native squamous epithelium. It was Wespi (31) who emphasized the role of metaplasia in carcinogenesis.
Cytology, introduced in the 1940s by Papanicolaou and Traut, was found to be an excellent technique for detecting precancerous lesions (32). In Sweden, since the introduction of cytology as a screening method in the 1950s, the cervical cancer incidence has decreased by about 40% (33). Several authors concluded that both cytology and colposcopy should be used in combination to achieve early diagnosis (31,34,35).

It was first reported in the mid-1970s that 1–2% of smears from all women with a clinically normal cervix had cytological signs of HPV (36,37). It became obvious that many lesions judged as mild to moderate dysplasia were not neoplastic, but bore instead signs of HPV. These new lesions were named flat or inverted condyloma. Attempts were made to define morphologic criteria for the differentiation of HPV infection from intra-epithelial neoplasia. However, it was soon realized that histological, serological and virological evidence linked both mild and severe degrees of dysplasia with HPV infection (38).

The international histopathological terminology for dysplastic epithelium, Cervical Intra-epithelial Neoplasia (CIN), Vaginal Intra-epithelial Neoplasia (VAIN), Vulvar Intra-epithelial Neoplasia (VIN), and Perineal Intra-epithelial Neoplasia (PIN) was proposed by Richart (39). All these entities were divided into mild (I), moderate (II) and severe (III) degrees of dysplasia.

It has been documented in a number of studies that intra-epithelial neoplastic lesions from the lower genital tract harbour HPV-DNA, especially HPV 16 and 18 (40,41). The HPV-DNA sequences in the intra-epithelial neoplastic lesions are mostly un-integrated, i.e., the viral DNA exists as a free episome (a self-replicating, extrachromosomal, nuclear DNA) (42). This is in contrast to most other DNA-tumour viruses, which integrate with the cellular DNA before transforming a cell.

The progression and regression rates of dysplastic lesions have been studied extensively by many authors. Figures depend to a great extent on the definitions applied and the end point of the studies. The progression rates vary to a great extent in different materials (43,44). In a Finnish follow-up study it has been shown that lesions containing HPV 16 are likely to progress into more severe neoplasia, whereas HPV 6 and 11 lesions are less likely to progress (45).

Cervical papilloma-virus infections represent a heterogeneous group of lesions. The majority of these are caused by low-risk or unknown HPV types. Despite their tendency to give rise to atypical Pap-smears, such lesions do not progress to cervical cancer, whereas the 15–50% of subclinical cervical lesions induced by HPV 16, 18, 31, 33, 35 or 39 do indeed represent the earliest phase of precancerous development (46,47).

Premalignant lesions on the vulva contain a mixture of types 6, 11 and 16, 18. In a study by Gross et al., 17 of 20 patients, both males and females with Bowenoid papulosis, were found to be positive for HPV 16 (48). It is remarkable that the incidence of VIN III has increased and the age at diagnosis has decreased about twenty years (49).

The literature dealing with the vaginal HPV infection is less extensive than that on the vulva and cervix. The first evidence to support the theory of HPV as being a
causative factor in the development of vaginal dysplasia came in 1983. An analysis of vaginal intra-epithelial neoplasia (VAIN) by HPV-DNA hybridization technique showed a high frequency of HPV 6 and 16 in such tissue (50).

In our studies we analysed the presence of HPV in benign and dysplastic tissue in order to elucidate the association between HPV and the development of intra-epithelial neoplasia in the lower genital tract. It should be understood that scientific effort within this field has been very progressive during the last few years. Currently, strong evidence suggests HPV to be the strongest etiologic factor.

**Co-factors**

Whereas HPV is believed to be the necessary factor required for a cell to undergo malignant transformation it is obvious that HPV infection *per se* is not sufficient for the development of genital dysplasia. Experimental data are still lacking. However, suggested co-factors are:

- **Smoking**: which is clearly a risk factor for cervical cancer (51).
- **Hormonal factors**: multiparity and the prolonged use of oral contraceptives have both been associated with an increased risk of cervical cancer (52). Progesterone has been reported to be an accelerating factor in the development of neoplasia in the lower genital tract (53).
- **Immunologic factors**: warts are more frequently seen in immuno-compromised patients, in whom there is also an increased incidence of CIN and cervical cancer (54).
- **Concomitant infection**: with for example Herpes simplex virus (55).

**Vulvovaginitis and HPV**

The problem of vulvar pain was first described a century ago. Due to the absence of abnormal physical findings it was long thought to be a gynecological problem with a psychosomatic origin. It was not until the mid 1970s that scientists tried to elucidate its background. In 1982 a task force was formed to investigate the problem. This group has presented a new terminology (56). In 1983 Friedrich described a syndrome comprising burning and dyspareunia associated with the finding of erythema around the openings of the minor vestibular glands. In consequence the terms 'vestibular adenitis' and 'vulvar vestibulitis' were coined (57,58). Other authors also observed the same condition and described a surgical method for correcting introital dyspareunia (59).

Vulvodynia and pruritus vulvae is a recently recognized symptom complex (60). In women with such symptoms, generally there are no signs of Candida, Gardnerella, Trichomonas, Herpes simplex, Chlamydia or Neisseria gonorrhea. Furthermore there is no visible inflammation as in dermatitis and vestibulitis, and no signs of lichen sclerosus.

Many women who have aceto-white areas and/or papillomatous epithelium, often with a vascular pattern with capillaries extending into individual papillae, present
with symptoms. These women complain of itching, burning, discharge, fissures and dyspareunia. In our first studies we tried to establish whether these symptoms and morphological changes were HPV induced.

Very little attention has been devoted to vaginal HPV infections. The subclinical HPV infection cannot easily be diagnosed with the naked eye, and is not even looked for. Vaginal condylomata are usually asymptomatic. The fact that the changes in the vaginal mucosa coexist with symptoms from the vulva and genital intra-epithelial neoplasia led to our interest in these vaginal manifestations.
OBJECTIVES OF THE STUDY

➢ To elucidate the association between certain symptoms and morphologic (colposcopic and histopathologic) changes in the vulvovaginal mucosa and HPV infection.

➢ To analyse the prevalence of HPV-DNA among women with an atypical Pap-smear, and to correlate HPV type with the degree of intra-epithelial neoplasia.

➢ To compare the efficacy of various diagnostic methods for the detection of HPV.

➢ To determine the extent of HPV-DNA persistence in women following laser surgery of genital intra-epithelial neoplasia.
MATERIAL

The patient materials are described for the various studies performed:

Clinical characteristics of papillomavirus-vulvovaginitis (I)

The study group comprised 74 consecutive women (mean age 29 years) with morphologic and histopathologic signs of papillomavirus vulvovaginitis.

Papilloma virus infection of the vulva (II) and detection of minimal copy numbers of HPV-DNA by polymerase chain reaction in women with vulvo-vaginitis (V)

A total of 78 women (mean age 28 years) with colposcopic and histopathologic signs of vulvovaginal HPV infection were included. Twenty women, comparable in age, with colposcopically and morphologically normal vulvovaginal mucosa served as controls.

Detection of human papilloma virus in women referred for colposcopy (III)

During the period of 1986–1987 all women referred to the gynecological department for colposcopy due to an atypical Pap smear or clinically suspected intra-epithelial neoplasia were included. The material consisted of 168 women (mean age 28 years). A group of 119 apparently gynecologically healthy women comparable in age served as controls.

Follow-up of HPV-DNA positive women after laser-removal of lower genital intra-epithelial neoplasia (IV)

Forty-five women with cervical and/or vulvovaginal neoplasia, all of whom were HPV-DNA positive, were examined before and following laser treatment. Their mean age was 32 years.
METHODS

The following methods were applied:

Interviews (I–V)
All the women were interviewed using a standardized questionnaire, regarding coital debut, contraception, number of partners, present symptoms, partner infection, earlier infections or atypical Pap-smears.

Clinical examination (I–V)
Gynecological examination including light microscopic examination of vaginal secretion was performed in all cases.

Cultures (I, II)
Cultures for chlamydia, gonorrhea and herpes genitalis were performed.

Colposcopy (I–V)
All women were examined by colposcopy (Zeiss Photocolposcope). The colposcopic pattern was described after the application of 5% acetic acid and sometimes iodine. The following criteria for HPV infection were used: aceto-white flat lesions, sometimes with fissures, satellite lesions beyond the transformation zone on the cervix, papillomatous mucosa with granulation and filaments, and spikes (papillae with sharp ends). Mostly, the lesions, were hyperkeratinized (Table II).

Cytology (I–IV)
Pap-smears were obtained from all the women. The presence of koilocytosis, signifying the cytopathic effect of HPV infection, is the diagnostic hallmark in the light microscopic evaluation, Fig. 2 (61,62). The koilocyte is characterized by nuclear atypia, caused by degenerative clumping of host cell chromatin. It also has a peripheral

<table>
<thead>
<tr>
<th>Table II. Predilection site for morphological changes in the genital mucosa revealed by colposcopy in women with suspected HPV-infection.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aceto-white flat lesions</td>
</tr>
<tr>
<td>Papillomatosis/granulation</td>
</tr>
<tr>
<td>Filaments</td>
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<tr>
<td>Fissures</td>
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<tr>
<td>Spikes</td>
</tr>
</tbody>
</table>
condensation of cytoplasm giving rise to an empty zone around the nucleus. However, in some cases — especially in vulvar lesions — koilocytes may either be scanty or completely absent. Since viral cytopathic effects occur only in dying or dead cells, koilocytes can never undergo malignant transformation. On the other hand koilocytes represent the contagious stage in the HPV life-cycle. Dyskeratosis, cells loaded with keratin and with a hyperchromatic nucleus, are frequently present in HPV-infected epithelium.

**Histopathology (I–V)**

Biopsy samples were obtained for histopathological examination.

Histopathological criteria for HPV-infection were those published by Meisels et al., i.e. koilocytosis, hyperchromasia, binucleation, dyskeratosis, parakeratosis, acanthosis and papillomatosis (63).

**Immunohistochemistry (I)**

Identification of viral capsid antigen was performed by using an immunoperoxidase staining kit (DAKO Corp., Santa Barbara, CA, USA), Fig. 3. With this technique, the test is performed against group-specific antigens and the presence of HPV-antigens is confined exclusively to the nuclei of the koilocytes and/or superficial dyskeratotic cells. Thus it is not possible to identify specific HPV types (64).

**Dot blot hybridization on biopsies (II, III, IV)**

Cellular DNA is denatured by alkaline treatment and spotted on to a filter membrane. Viral genomes present therein are detected by hybridization using a $^{32}$p-labelled probe followed by autoradiography. As samples are analysed in batches of 20, this is quite a quick method. However, the dot blot method cannot be performed at low stringency, to probe for related HPV types (65).
Southern blot hybridization on biopsies (II, III, IV)

Cellular DNA is extracted from biopsy specimens. The DNA is digested with specific restriction endonucleases, separated by gel electrophoresis, denatured and transferred (‘blotted’) on to a filter. Hybridization and autoradiography indicate the cleavage pattern of the viral DNA and allow the identification of minor changes within the genome (virus subtype) or the detection of different HPV types, Fig. 4. It is also possible to determine whether the HPV-DNA is episomal or integrated into the cellular DNA (65).

Filter in situ hybridization (FISH) on cell samples (III, IV)

This is a simplification of the dot blot technique with hybridization of exfoliated cells. Cell samples are filtered directly on to a nitrocellulose filter. The proteins are destroyed by alkaline treatment, which also leads to the denaturation of the DNA. After hybridization with labelled probes, cells containing virus DNA can be revealed as spots on an X-ray film. This method is simple, rapid and allows the screening of a large number of samples. However, stringent conditions are required, which means that one cannot deduce whether more unknown HPV types are present (66).

Polymerase chain reaction (PCR) on biopsies and cell samples (V)

This method is theoretically $10^4$–$10^5$ times more sensitive than the Southern blot technique. It allows the amplification of DNA by enzymatic synthesis of a predetermined stretch of DNA. The native double-stranded DNA is denatured, liberating a single DNA strand. Two specific oligonucleotides, primers of DNA synthesis, anneal to specific sequences on the denatured DNA. The synthesis of a complementary second strand of new DNA occurs by the extension of each annealed primer by heat-stable DNA polymerase in the presence of excess dNTPs. A new single strand of DNA is synthesized for each annealed primer. The cycle of denaturation, annealing and extension is repeated 30–40 times (67,68,69).
RESULTS

Evidence of HPV as an etiologic factor for vulvovaginitis and the clinical characteristics of this disease (I, II, V)

The clinical symptoms and signs of HPV suspected vulvovaginitis were described. Discharge, itching, burning, dryness and fissures often causing dyspareunia were typical symptoms. Discharge was more frequent when the lesions were present in the vagina, whereas itching and burning were frequently associated with vulvar lesions. However, many women with typical lesions were asymptomatic. Most of the latter had been referred to the gynecological department because of an atypical Pap-smear.

Colposcopy after application of acetic acid distinguished between the following patterns: 1. white epithelium with or without fissures; 2. papillomatous mucosa with granulation and/or filaments; 3. spikes.

The lesions were often hyperkeratinized and multifocal. Filamental lesions were most frequently located in the middle and upper part of labiae minora, whereas flat lesions were most prominent in the posterior part of the vulva.

In many cases with atypical Pap-smear there was no evidence of cervical HPV infection or intra-epithelial neoplasia, but there were colposcopic HPV signs present in the vagina and/or vulva.

Histopathology of colposcopically directed biopsies showed signs of genital intra-epithelial neoplasia in 23% of 124 women with vulvovaginitis.

Viral structural antigen was demonstrated by immuno-histochemistry in 50% of 54 biopsies from women with vulvovaginitis.

HPV-DNA analysis by dot and/or Southern blot demonstrated HPV-DNA in 50% of analysed samples from 50 women with vulvovaginitis. Twenty-six percent of the women harboured HPV 16. HPV-DNA was detected in 55% of the papillomatous (filamental) and in 43% of flat lesions. HPV-DNA positivity was more often found especially for HPV 16, in women with intra-epithelial neoplasia, compared with those without.

By PCR technique, HPV-DNA was demonstrated in 64% of women with suspected HPV vulvovaginitis without signs of intra-epithelial neoplasia. HPV 16 was present in 57% of these women. Eight out of twelve biopsies earlier deemed HPV-DNA negative by Southern blot technique proved positive by PCR.

Detection of HPV in women referred for colposcopy. A comparison between different diagnostic methods (III)

Out of 168 women referred for colposcopy mainly because of atypical Pap-smear the colposcopic examination revealed evidence of genital HPV infection in 58%.
Cytology indicated HPV infection in 21% of the women. Of these, one woman only had CIN III according to histopathology, while 34% of women with atypical Pap-smears without signs of HPV had CIN III lesions according to histopathology.

Histopathology revealed signs of HPV infection in 53% out of 155 women. Histological signs of HPV were more common in biopsies with mild or moderate neoplasmia than in those with severe neoplasmia (59% vs. 35%).

HPV-DNA analysis by Southern blot revealed that 39% of the women harboured HPV-DNA. Twenty-five percent were HPV 16 positive. HPV-DNA positivity was more common in biopsies with severe dysplasia than in those with mild to moderate dysplasia (CIN III lesions were positive in 66%). Also in cell samples the HPV-DNA positivity was more common in women with severe atypias than in those with mild to moderate atypia.

By the FISH assay, 27% of the cell samples from 105 women with atypical Pap-smear were found to be HPV-DNA positive. The FISH technique on cell samples and Southern blot on biopsy samples were equally sensitive in detecting HPV-DNA in women with benign and mild–moderate dysplastic epithelium, while SB was more sensitive in detecting HPV-DNA in severe dysplasia. FISH analysis on cell samples from 119 women with normal Pap-smears proved positive in 11%.

Altogether 46% of the women were HPV-DNA positive.

In those women positive for HPV 16/18, colposcopy revealed signs of HPV and/or genital intra-epithelial neoplasia in 96%.

Follow-up of HPV-DNA-positive women after laser treatment due to genital intra-epithelial neoplasia (IV)

Forty-five women with cervical and vulvovaginal intra-epithelial neoplasia, all HPV-DNA positive by Southern or dot blot technique, were examined before and following laser surgery.

Before treatment, colposcopy revealed signs of HPV in 76% and histopathology in 67% of the women. According to HPV-DNA hybridization technique, 82% of the women had lesions positive for HPV 16.

Following treatment, colposcopic signs suggested HPV infection in 64% of the women. Histopathology revealed signs of HPV in 43% of directed biopsies from treated and healed areas.

HPV-DNA was present in the new epithelium covering the treated areas in 38%; HPV 16 was found in 24% of the women.

In 18% of the patients, residual neoplasia was found. Of 21 biopsies without histological signs of either intra-epithelial neoplasia or of HPV infection, 5 were positive for HPV-DNA.
DISCUSSION

The results of this study support the view of HPV as an etiologic factor in the development of genital intra-epithelial neoplasia. The association between HPV and certain symptoms and morphological changes in the vulvovaginal mucosa has been emphasized.

**Vulvo-vaginal HPV infection**

Within the heterogeneous group of patients with vulvodynia and pruritus vulvae, HPV vulvovaginitis has been defined as a clinically distinct entity. In our studies a meticulous examination of the vulvovaginal mucosa by colposcopy revealed characteristic patterns such as acetowhite flat lesions and hyperplastic mucosa forming granulation and filaments. Some lesions were solitary, while the majority were multifocal. Of the women with suspected HPV vulvovaginitis according to colposcopy and histopathology, 40-50% had symptoms such as itching, burning, discharge and fissures. It was remarkable that similar morphologic changes in the mucosa were seen in women both with and without symptoms. When specimens from the affected areas were analysed by HPV-DNA hybridization techniques, there was evidently no correlation between specific HPV types and various symptoms or certain colposcopic patterns.

Other authors have also reported evidence showing HPV to be an etiological factor in pruritic vulvar squamous papillomatosis (70,71). In one recent study 7 women with longstanding introital dyspareunia and burning proved positive for HPV-DNA (72).

The various manifestations and symptoms of HPV infection may be a reflection of inter- and intra-individual differences in the immunological response to HPV infection. The local symptoms might result from the presence of one or more specific mediator, such as biogenic amines (prostaglandins, histamines) and polypeptides (bradykinin, serotonin). Sensitization of nerves in the affected area might cause persistent symptoms. Due to increased exfoliation of cells caused by a relative progesterone preponderance during oral contraceptive use the epithelium may become thin and vulnerable, allowing the HPV to more easily invade the deep cell layers (73). One of our hypotheses was that women using oral contraceptives ought to present symptoms more often than women not using them. However, no correlation was found between oral contraceptive use and symptomatic HPV vulvovaginitis.

Biopsies and cell samples from vulvovaginal lesions with characteristic morphological changes suspected of being HPV-induced were analysed by DNA hybridization techniques. The PCR technique showed 64% of such lesions to contain HPV. Hypertrophic epithelium — especially that forming filaments — has been suggested to be a physiological manifestation (74,75). Our results show however that 55% of biopsies from such areas contained HPV-DNA. This should be compared with the
controls having normal vulvovaginal mucosa, all of whom were HPV-DNA negative. These findings support the hypothesis that HPV may be the cause of these hyperplastic changes in the mucosa.

According to our results obtained with immunohistochemical technique (PAP), lesions having a flat appearance had areas with large numbers of cells containing antigens. However, HPV-DNA was more often detected in filamentous lesions than in aceto-white flat lesions. One possible reason could be that the latter harboured HPV types we did not test for. Another explanation could be that acetowhite flat lesions may also be caused by agents other than HPV, while a third explanation might be that samples from papillomatous lesions contain more cells, since a larger area is collected.

There is firm evidence that HPV infections are involved in the development of vulvovaginal intra-epithelial neoplasia (76). Singer & Mc Cance reported that by careful colposcopic examination one could detect subclinical HPV infection. Such lesions were associated with CIN in about 50% of cases (77). Our results also indicate HPV to be a common causative agent in the development of genital intra-epithelial neoplasia. We found that 23% of those with clinically suspected HPV vulvo-vaginitis had concomitant intra-epithelial neoplasia of the lower genital tract. All women with vulvovaginal dysplasia analysed by Southern or dot blot proved positive for HPV-DNA, most of them for HPV 16. These findings support the assumption that the reason for the small proportion of HPV-positive benign lesions is that such tissue harbours fewer HPV-infected cells or perhaps few DNA-copies in each cell, compared with precancerous epithelium. This hypothesis is supported by the result obtained when using the PCR technique, which detected HPV-DNA in samples with suspected HPV infection but without intra-epithelial neoplasia, earlier deemed negative by Southern blot.

The fact that HPV 16 was demonstrated in half of the investigated women, underlines the importance of taking a Pap-smear in women with suspected HPV-vulvo-vaginitis.

**HPV as an oncogenic marker**

Genital infections of various kinds have long been suspected to be involved in the development of cervical cancer. The spectrum of co-factors deemed necessary for oncogenic expression has also been modified to conform with increased knowledge.

During the last decade the human papillomavirus — with its manifold types — has come under suspicion as being the most likely agent underlying neoplastic development in the lower genital tract. The evident oncogenic potential of HPV has emphasized the need to evaluate the sensitivity of various techniques to achieve reliable HPV detection. Against this background it has been hoped to devise a more sensitive screening tool for the identification of the women at high risk to develop carcinoma of the lower genital tract.

Considerable medical resources are allocated to the treatment of women having different forms of atypical Pap-smears. Although general cytologic screening has
been routine for more than two decades, the decrease in the incidence of cervical cancer in Sweden has not yet reached even 50% (34). It has been estimated that only 15% of women with intra-epithelial neoplasia would have had lesions progressing to invasive cancer if left untreated (78). It is also apparent that at least 10% of the Pap-smears examined are diagnosed false-negative. At the same time as there is an ‘overtreatment’, we are still missing cases that should have been detected and treated (79). A more specific technique for evaluating detected cytological abnormalities is evidently needed. Women developing cancer shortly after a normal Pap-smear has been taken tend to be comparatively younger than women with cervical cancer in general. Ashley proposed in 1966 that there might be two different forms of cervical cancer, one progressing rapidly in younger women, the other progressing more slowly in older women (80). It is the more aggressive forms that are difficult to prevent.

In our studies, different methods have therefore been compared concerning their ability to detect HPV. Our ultimate goal was to find a method that would identify high-risk patients harbouring oncogenic viral types among the increasing number of women with an atypical Pap-smear whatever its cause.

In women with an atypical Pap-smear, the various methods gave varying results with respect to the overall frequency of HPV among those women investigated. There was also an overlap between positive and negative cases. Signs of HPV were observed by colposcopy in 58%, by histopathology in 53%, by cytology in 21% and by DNA hybridization techniques in 46%. By colposcopy the signs of HPV infection are easily recognized in benign conditions. In severe dysplasia it is more difficult, however, since growth and dilatation of capillaries may obscure the picture. In severe lesions, koilocytes have been replaced by other abnormal nuclear manifestations. By cytology and histopathology it is therefore more difficult to identify HPV in cell samples and biopsies from precancerous lesions. In contrast, Southern blot technique increases the detection rate of HPV in proportion to the severity of the disease. These results are in agreement with those of other authors (24).

It is important to point out that the various results of HPV-DNA detection in different materials are dependent on the types tested for and the methods used.

The recommendation based on our results must therefore be that the various diagnostic methods should be used to complement each other, so as to achieve optimal detectability. According to our results, SB on biopsies and FISH assays were equally sensitive when analysing benign lesions. However, FISH on cell samples was the most appropriate for detection of HPV in benign lesions because it is simpler and more rapid than SB. The PCR technique was not, it must be remembered, available at the time when the different techniques were compared.

The influence of one or several co-factors in addition to the HPV infection, such as smoking, other infections, and vulnerable mucosa caused for instance by oral contraceptives, must be borne in mind, even though prolonged use of OCs might reflect other facets of sexual behavioural characteristics in women with a high risk of developing cervical cancer. Smoking seems to be a significant factor — of the women using oral contraceptives, 70% of the smokers and 50% of the non-smo-
ers had histopathological evidence of an intra-epithelial neoplasia.

Recently, investigators have started to explore the immune-response to HPV. Profiles of serological reactivity to HPV 6 and HPV 16 have been devised (81,82) and it has been possible to distinguish between reactivity to the open reading frames of different HPV types. Immunological studies may prove more sensitive than HPV-DNA testing. However, the specific site at which the immune response is to be measured may be important as well. HPV infection can occur in mucosal sites other than the genital tract (83). It is an interesting suggestion, that the induction of a vaccine-induced immune response may prevent initial infection with oncogenic viruses.

**Spread of HPV throughout the genital mucosa**

Women with HPV-DNA positive CIN who had been treated by laser surgery were included in the study. The aim was to determine whether HPV-DNA persisted following laser treatment and, if so, whether also intra-epithelial neoplasia was present in the newly formed epithelium covering the treated areas. Before therapy, colposcopie signs suggested genital HPV in 76%. After treatment, 38% still harboured HPV-DNA in treated areas and 18% had residual genital intra-epithelial neoplasia. However, colposcopy revealed signs of genital HPV infection in 64% after treatment, thus supporting the view that the virus spreads throughout the genital mucosa.

At present there is a worldwide epidemic of HPV infections. The prevalence in the general population seems to be as high as 10–15%. According to our results the prevalence of HPV infection, determined by FISH, was 11% in apparently healthy women with normal Pap-smears. It is obvious that the examination and treatment of all these women is a daunting task and it is not yet clear whether this should be our goal. The lack of specific therapy must also be taken into consideration. However, women with subjective symptoms and/or concomitant dysplasia should be treated according to common procedures. Until we have an effective antiviral therapy, we must leave the asymptomatic woman without offering any therapeutic regimen. In consequence, many of these untreated women who have contagious lesions will continue to spread the HPV infection.

**Future prospects**

A future task is to elucidate whether HPV is a ‘passenger’ or a ‘driver’. By using PCR, it may be possible to, retrospectively, examine cancer specimens and vaginal smears from patients for the presence of HPV-DNA. It would then be possible to evaluate whether women with ‘missed’ invasive cancer had evidence of HPV in their smears previously, in contrast to a normal population.

It is evident that the HPV infection *per se* is not sufficient to cause cancer and that other factors may be essential for cancer to occur. It is still unknown why HPV-DNA remains episomal in precancerous lesions and how and why truncated sequences of open reading frames E6 and E7 become integrated to the cellular genome. The fact that the protein products of E7 of HPV 16 may interact with pro-
ducts of the RB gene suggests a common pathway in the malignant transformation of human cells (84). Another issue of concern is the possibility that invasive cancer of the cervix may gain a foothold by more than one route. There may exist two forms of cervical cancer, the one evolving from a longstanding precancerous lesion, while the other develops rapidly without a precursor state. Evidently we still need to learn more about the natural history of this viral infection.
CONCLUSIONS

- Approximately two-thirds of vulvo-vaginal aceto-white flat lesions and diffuse hyperplastic mucosa with a granulated or filamental surface were found to contain HPV-DNA by the PCR technique. All samples obtained from 'normal' vulvo-vaginal mucosa were HPV-DNA negative by the same technique.

- Women with hyperkeratotic and papillomatous vulvovaginal mucosal lesions — judged to be HPV-induced by colposcopy and histopathology and/or confirmed to be HPV-induced by DNA-hybridisation techniques — often have symptoms such as itching, burning, discharge, and fissures, sometimes causing dyspareunia.

- HPV-vulvovaginitis may give rise to atypical Pap smears even in the absence of dysplasia. However, women with genital HPV infection often have intra-epithelial neoplasia.

- In almost half of the women with atypical Pap-smear, directed cervical biopsies proved HPV-DNA-positive by the SB technique. CIN III lesions were positive in two-thirds and HPV 16 was detected in more than half of the biopsies.

- The FISH technique on cell samples was as sensitive as the SB technique on directed biopsies in detecting HPV-DNA in women with benign epithelium and/or mild to moderate dysplasia. The SB technique, however, was more sensitive in detecting HPV-DNA in women with severe dysplasia. It is noteworthy that about 10% of cytologically normal cell samples were HPV-positive according to FISH analysis.

- The PCR technique was more sensitive in detecting HPV-DNA in benign tissue than the SB technique.

- Following laser treatment of HPV-DNA positive genital intra-epithelial neoplasia, HPV could still be detected in more than one third of the biopsies from the epithelium covering the treated areas.
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