

THESES OF DOCTORAL (PhD) DISSERTATION

**EPIDEMIOLOGY AND IMPORTANCE OF PROGNOSIS  
OF HUMAN PAPILLOMA VIRUS INFECTIONS**

**Dr Tamás Sápy**



Consultant: Dr Zoltán Hernádi

Division of Gynaecologic Oncology  
Department of Obstetrics and Gynaecology  
Medical and Health Science Center  
University of Debrecen

2007

## INTRODUCTION

Cervical cancer is one of the most common gynecological tumors. In women, cervical carcinomas make up 15% of the detected malignomas, belonging to the second most common malignant type of female tumors after breast cancer, as far as their incidence and mortality are regarded.

In Hungary, cervical cancer screening traditionally involves the colposcopic examination of the vaginal portion of the uterine cervix and the microscopic investigation (cytology) of cells exfoliated from that region, following the “German traditions” which played a basic role in the formation of the national school of gynecology. Although in the possession of a reliable screening technique the screening efficiency of this malignoma is 100% and it takes almost a decade for the precancerous stage (cervical intraepithelial neoplasia [CIN]) to develop and malignant transformation to take place, still approximately 2600 new cases of cervical cancer are diagnosed in this country each year. Worldwide, the incidence of cervical cancer is recorded at 500 000 new cases/year. An additional negative detail is a change in age: the median age of patients suffering from this disease was 54 years in the 1970s, while, today, the disease emerges 10 years earlier, morbidity having doubled in the population below 35 years of age.

In addition to well known factors (sexual life started at a young age, promiscuity, multiparity, poor socio-economic background, smoking, use of oral contraceptives, immunosuppression, and lack of antioxidants), studies of the role of etiological factors focused scientific attention to the role of new risk factors. Observations in this field directed attention to the oncogenic role of sexually transmitted viral infections. Initial research was focussed on *Herpes simplex virus*, type two, (HSV-II) while today it is HPV, the *human papillomavirus* that is in the focus of attention.

According to our current knowledge, HPV infection and the aforementioned etiological factors, as co-factors, are responsible for the

development of *cervical carcinoma*. In 1956, Koss and Durfee described koilocytosis to be a pathognomonic change in response to HPV, while in 1977, Meisels and zur Hausen simultaneously suggested that the development of cervical cancer and its precancerous stages might have resulted from HPV infection. Since then, several articles have been published on the effect of the papillomaviruses inducing cervical cancer and the viruses' role played in the progression of the disease.

Human papillomaviruses are basically transmitted via sexual intercourse. According to estimates, 30-70% of sexually active women contract HPV infection. Over 100 genotypes of HPV have been identified by now, of which approximately 30 can be detected in the female genital tract.

From an etiological point of view, i.e. depending on what the likelihood of the development of cervical carcinoma parallel to viral infection is, two groups are distinguished: 1. *Low-risk group* (6, 11, 42,43, and 44), and 2. *High-risk group* (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 58, and 60). Low-risk HPVs usually occur in *condyloma acuminatum*, while high-risk HPVs are often seen in the precancerous stages of cervical cancer (CIN I, II, III, CIS) and even more often in cervical cancer (ICC) itself; in the latter they are found in practically 100% of the cases, although it depends on the sensitivity of the technique of investigation.

The prevalence of HPV infections is widely divided according to geographical location and the patients' age. At the same time, however, it is important to know the geographic, ethnic and age scatter of HPV in order to elaborate effective prevention strategies for screening or vaccination. There are only few studies devoted to the prevalence of HPV in Hungary, but according to several data, HPV infections can be regarded as the most common STDs in this country, too. Since infections are common, but the incidence of cervical carcinoma is relatively lower, the question arises: when is it necessary to classify a cases as a high-risk one in the possession of a positive HPV diagnosis and to what extent does this contribute to the efficiency of screening; also, in what way can it help

with the elaboration of a therapeutic plan in the detected cases? Several authors suggest the inclusion of HPV examinations not only in secondary but also primary screening practice.

Should HPV-typing be included in the screening practice – which is supported by increasingly more data – it should definitely meet the criteria required in screening techniques. As HPV-typing (secondary prevention) is a supplement to classical screening techniques, on the basis of epidemiological data at our disposal, there is a real chance to rationally plan the other alternative for prevention (primary prevention).

The prognostic role of HPV in diagnosed and treated cases of cervical cancer has been evaluated in several studies. However, there are not unanimous data on the HPV type of primary tumors and that of the HPV status of lymph nodes.

## GOALS

- We aimed at determining high and low-risk HPV prevalence among patients with a positive screening test for previous colposcopic and/or cytological screening.
  - What differences in HPV incidence can be registered in the different age groups?
  - What prevalence changes can be noticed by age?
  - Are there any differences in the HPV-clearance data?
  - We have studied if there were any changes in the HPV-clearance details of patients of different ages.
- 
- Determining HPV in the primary tumor and lymph node samples of patients having undergone Wertheim's surgery for operable cervical cancer, we wanted to find an answer if histologically negative but HPV-positive lymph nodes had prognostic importance or not.
- 
- Evaluating the HPV type-specificity of Hybrid Capture test (HCT) using the PCR-RFLP technique, we investigated if HCT, a technique for diagnosing HPV, fulfilled the criteria of screening techniques or not.

## PATIENTS AND METHODS

### *Groups of patients*

I. Routine colposcopic and cytological screening for *cervical cancer* was supplemented with HPV testing in 3480 patients (median and mean ages: 32.00 and 34.00 years, respectively; SD: 10.56) at the Department of Obstetrics and Gynecology of the University of Debrecen in the period between January 1997 and December 2007. In these patients, positive colposcopic findings (Group II, according to the Roman classification, 1990) and/or positive cytological findings (The Bethesda System, 1991: Atypical Squamous Cell of Unknown Significance, ASCUS; Low Grade Squamous Intraepithelial Lesion, LGSIL; High Grade Squamous Intraepithelial Lesion, HGSIL, or suspected invasive cervical carcinoma) were registered.

Native cervical smear was taken immediately after oncocytological sampling, using Digene Hybrid Capture® (HCT) kit, prior to colposcopy requiring the use of 3% acetic acid. According to the recommendations by the manufacturers, the sample was collected from the region known as the *squamocolumnar junction* of the cervical canal and the supposedly suspicious lesion of the *ectocervix*.

Virus typing was performed at the Virus Laboratory of the Department of Microbiology, Medical and Health Science Center of the University of Debrecen.

II. The other group of patients consisted of 47 women with cervical cancer, followed up after radical hysterectomy (Wertheim's operation) in 1992 and 1993. In these patients HPV testing from the primary tumor and nearby lymph nodes was also performed in addition to routine histological evaluation (fixation in formaline and staining with haematoxylin-eosine). Biopsies from the cervical tumor and lymph nodes were placed in dry ice and carried to the Virus Laboratory of the Department of Medical Microbiology, where HPV testing was done.

According to professional recommendations at that time, postoperative intracavitary and external telecobalt irradiations (doses of 400, 1700 and 1700 reu and 20x2 Gy, respectively) were applied among standardized conditions. After combined treatment, the patients were asked to present at 3-monthly follow-up examinations.

*III.* In the third group of patients, using the HCT sampling kit, exfoliated cells were collected from the *squamocolumnar junction* of 570 women who came for secondary screening owing to former colposcopic and/or cytological changes detected at the Department of Obstetrics and Gynecology. The samples were stored at  $-20^{\circ}\text{C}$ . Virus testing was performed at the Department of Microbiology.

#### *HPV genotyping*

*I.* *Digene Hybrid Capture® Tube Test* [HCT I] (Digene Diagnostics, Inc. Beltsville, MD USA) applied in the study involving 3480 patients is an HPV-DNA liquid hybridization technique which makes use of a signal amplifying chemiluminescence detection technique. Cervical samples containing HPV-DNA hybridize with the ‘cocktail’ containing the specific HPV-RNA. The emerging DNA-RNA hybrids are bound to the wall of a test tube coated with anti DNA-RNA monoclonal antibodies. Next, antibodies containing specific alkaline phosphatase (which serves as a conjugate) are added to the preparation. The enzyme substrate of this alkaline phosphatase can be used to measure signal amplifying chemiluminescence. Light emission resulting from the splitting of the substrate can be measured in relative light units (RLU) – compared to a 10 pg/ml HPV-16 DNA positive control (RLU/PC) – using a luminometer. The extent of light emission shows HPV-DNA present in or absent from the sample. Values lower than the “cut off” value refer to the absence of HPV-DNA, while light units equal to or higher than the “cut off” value show the intensity of HPV-DNA presence. This technique (HCT I) allows for the typing of 14 HPV types in the anogenital region. The latest type (HCT II) is capable of detecting the 18 HPV types). Two combined RNA-tests

were applied in the investigations. In Test A, RNA was hybridized with the HPV 6/11/42/43/44 genome of virus strains with low oncogenicity, while Test B was hybridized with the HPV 16/18/31/33/35/45/51/52/56 genome of strains with moderate to high oncogenicity.

In all of the patients in all of the studies we performed test B to detect HR-HPV, but we could perform the LR-RPV test in only 2315 cases.

Based on the RLU/PC value obtained in chemiluminescence investigations, the intensity of infection with HPV-DNA can also be measured.

*II.* In our second study, we determined the HPV status of the primary tumor and regional lymph nodes using the PCR (Polymerase Chain Reaction) technique. The isolation of DNA from the samples obtained from the gynaecological operating theatre and fast-frozen in dry ice, was done according to international standards (DNase and proteinase digestion, phenol-chloroform extraction and ethanol precipitation). Beta-globin, gene specific PCR was used to justify the presence of human DNA in the sample. In the first step, “consensus primer” (group specific) PCR amplified the L1 ORF (open reading frame) region of 250 basic pairs (bp) of HPV types 6, 11, 16, 18, 31, 33, 52 and 58, in 40 cycles in an “automatic thermal cycler”. The characteristics of the individual cycles were as follows: 1.5 min 95°C, 1.5 min. 48°C and 2 min 70°C°. To type the amplified products, Dde I, Hae III, and Pst I and restriction enzymes such as Rsa I and Xba I were used, if necessary.

In the case of samples not amplified by “consensus primer”, amplification was performed using type-specific primers (Evander et al, 1991). The design of primers HPV16 and 18 was made according to the 200 and 310 bp-containing E7 ORFs region. Temperatures and duration of the applied 30 cycles were as follows: 1 min 95 °C, 1.5 min 55 °C and 1.5 min 72 °C. Control examination of each PCR amplification was done using human DNA as the positive and negative control. In

the lymph nodes, HPV-18 E6 mRNA transcripts were detected by PCR following reverse transcription (RT-PCR).

*III.* In the third study, the cervical samples were tested using HCT in the first step. If the hybridization technique yielded positive results, PCR amplification of the samples was done. Fast frozen samples were thawed, DNA was isolated and, using pCO3 and pCO4 primers, we applied beta-globin gene specific PCR. In 16 cases the amplification was unsuccessful – those cases were excluded from further investigations. Amplification was done using consensus primers MY09 and MY11, in 40 cycles. The PCR mixture was made visible by silver staining following PolyAcrylamide (5%; acrylamide/bisacrylamide ratio: 50:1) Gel Electrophoresis (PAGE). After that, PCR products were typed using the Restriction Fragment Length Polymorphism (RFLP) technique. Restriction enzymes such as AluI, BamHI, DdeI, HaeIII, HinfI and PstI were applied. The electrophoresis of simple PCR restriction fragments was carried out on agarose gel, and multiple infections were confirmed by PAGE.

#### *Interpretation of results, statistical analysis*

*I.* The patients in the epidemiological study were divided into six age groups (<25 years, 26-34 years, 35-44 years, 45-54 years, 55-64 years and >64 years). The patients with HR and LR-HPV infections were included in separate tables, and HPV frequency plotted as percentages. Comparing the frequencies, we calculated the odds-ratio and 95% confidence interval. Statistical analysis was performed using SPSS software.

Patients positive for HR-HPV (n=433) who had no histological test or did not disappear from the follow-up program were checked up every six months and repeated HPV typing was done to determine their *HPV clearance*. *Clearance* time was calculated by regarding the time of a patient entering the study and the time of her first negative HPV test. We calculated the so-called “monthly HPV clearance ratio”, in which we regarded the quotient of the number of HPV-negative cases

(patients) of the last visits and all of the followed up women-months and plotted it as percentages. Clearance results were plotted according to age groups and initial cytological findings.

*II.* The data of four patients chosen from among 47 women having undergone Wertheim's surgery were not processed in a separate table. Their histories are outlined in the running text.

*III.* Control of HCT-results: A two-column scoring system using 1-2-4 codes was generated for the fast evaluation of RFLP values in our PCR-RFLP study. The use of the 1-2-4 system made it unnecessary for us to exactly determine the length of restriction fragments. Coding was based on the question whether or not the MY09-MY11 fragment was split owing to the effect of different restriction enzymes. (For example: HPV-33 was split under the influence of DdeI, HinfI and PstI, but it did not split when AluI, BamHI or HaeIII were applied. Thus the score was  $0 \times 1_{(AluI)} + 0 \times 2_{(BamHI)} + 1 \times 4_{(DdeI)} = 4$  and  $0 \times 1_{(HaeIII)} + 1 \times 2_{(HinfI)} + 1 \times 4_{(PstI)} = 6$ ). This way, most of the HPV types have had individual scores; however, identical scores were distinguished on the basis of which enzyme created the greatest difference in the length of the fragments. (For instance, the score for both HPV-18 and 45 was 64 points, but they were distinguished according to their DdeI fragments.)

Next we plotted the comparison of the results for HCT and PCR-RFLP. The correlation between the RFLP and HCT signal results in double positive samples was shown as the quotient of the mean relative luminescence of the three positive control replicates and the relative luminescence of the hybridized sample; HR and LR test mixtures were listed in the first and second places, respectively.

## RESULTS

### I. HPV prevalence in patients with positive colposcopic and/or cytological findings

The *Hybrid Capture* technique detected HPV infections in 1222 among 3480 patients (35.1%) examined due to changes in their colposcopic and/or cytological findings. Among them, single incidence of the low-risk infections was found in 91 cases (2.6%), high-risk infections were detected in 1072 cases (30.8%) while the combination of the two kinds of infection was seen in 59 patients (1.7%).

Studying the quantitative classification and distribution of subjects suffering from infections caused simultaneously by several HPV types we could detect low-risk HPV infections in a total of 150 cases (12.3%). *Condyloma acuminatum*, the clinical change corresponding to this kind of infection was registered in only 38 patients (25.3%), i.e. an infection of latent stage was present in three quarters of the cases at the time of the investigation. High-risk human papillomavirus infection was noted in 1131 cases (32.5%).

Examining the patients' age included in the study we could see that the youngest and oldest of them were 16 and 72 years of age (median and mean ages being 32.00 and 34.00 years, respectively; SD: 10.56). Over 90% of the patients belonged to the 20-50-year-old age group. The median age of those carrying the HR-HPV infection was 29.00 years (mean: 31.36 years; SD: 9.21 years); the majority (68%) were between 20 and 35 years, i.e. in their fertile period. Seventy-four per cent of those carrying the LR-HPV infection came from the under-35 age group. Their median age was 26.50 years (mean: 29.97 years; SD: 10.06) – the data showing a younger population than that of the HR-HPV group. The high proportion of under-35 year olds in both types of HPV infection is not surprising: the infection is spread by sexual intercourse and this is the most active age group as far as their sexual habits are regarded.

Considering the likelihood of incidence between the two groups, high-risk HPV infections were more likely to occur than the low-risk ones ( $p < 0.001$ ), independently of age.

Dividing the patients in the study into six groups (>25, 25-34, 35-44, 45-54, 55-64 and 64< years of age) and examining HR and LR HPV infections separately, we found that HPV prevalence decreased by age in both types of HPV infections.

Carriers of the high risk virus showed statistically significant decrease in prevalence over 34 and 44 years of age ( $p < 0.001$ ), while the difference in older age groups was insignificant. It should be noted, however, that HPV infection was detected in high numbers in older age groups, too (19% and 8%) which could be associated with cases of incidence and prevalence alike.

Although in the case of low-risk infections it was a decrease in prevalence that we observed in older age, this reduction came earlier, after the 25<sup>th</sup> and 34<sup>th</sup> years of age. The early drop in prevalence resulted in a negligible number of persisting HPV infections in older age.

Studying the follow-up and *HPV clearance* details of 433 HR-HPV patients we found that virus clearance was found less often, although the difference in “monthly clearance rates” was insignificant in view of the relationship between the (enclosed) cytological results and age. In the case of HG-SIL, *clearance* rates could not be calculated as the patients underwent surgical treatment – according to the rules of our profession.

## **II. Prognostic role of HPV positive lymph nodes**

The mean age of patients with operable cervical carcinomas was 41.4 years (21-59 years). An interesting difference in the age distribution could be observed in the various, most common HPV types: compared to the infected patients in the HPV-16 group, much lower mean ages were registered in the HPV-18 group, at 43.7 (27-59) years and 33.5 (21-56) years, respectively.

In four of the 47 HPV 18-positive patients, very early recidivation and short survival were observed. Studying their predisposing histories we found that all of them belonged to the high-risk group characterized by sexually active life started at an early age, promiscuity, frequent vaginitis, questionable hygiene, neglecting screening for gynaecological carcinomas, but none of them smoked.

Three of those four patients were in the young age groups (at 21, 33 and 35 years of age) and one of them was a 56-year-old woman in her menopause. Postoperative histological staging confirmed the preoperative one: in two cases, FIGO Ib stage (35 and 56-year old patients), in the two other cases stage IIa (21 and 33-year old patients) were recorded. The histological findings of the sample taken during the operation described low-differentiated (G3) *planocellular/squamous cell carcinoma* in the three young patients, while the older patient had moderately differentiated (G2) *adenocarcinoma*, all of the four primary tumors being 1-2 cm in size. Preparations of the lymph nodes confirmed metastasis in the adenocarcinoma case alone, but, in addition to the primary tumor, HPV-18 was present in the lymph nodes in each of the four patients! Very early recidivation was found in the 21, 33, 35 and 56-year old patients in the 7<sup>th</sup>, 7<sup>th</sup>, 17<sup>th</sup> and 22<sup>nd</sup> postoperative months, respectively, which affected the retroperitoneum in the young patients and the vaginal stump in the older one. Fast progression of the disease and subsequent death were registered after 9, 10, 21 and 24 months of survival.

### **III. Studying the clinical applicability of the HCT test in HPV infections**

The HCT test yielded 145 positive results out of the 570 cervical smears, 15 of which were LR-HPV positive (Test A), 102 were HR-HPV positive (Test B) and double positive (LR+HR HPV) infections were detected in 12 patients. (Sixteen cases were excluded from the study because Beta-globin primers did not amplify them later.) The PCR technique of detection used by us met two requirements: 1) It did not modify the result of HCT as DNA needed for PCR was

acquired after hybridization. 2) Hybridization and polymerase chain reaction were performed in the same sample, thus the techniques were comparable. The application of non-denaturing PAGE allowed for the equally effective investigation of mixed and single infections since the electrophoretic mobility of PCR products (449-458 bp) of approximately the same length showed great differences owing to the different nucleotide sequences.

PCR products were typed using the RFLP technique. The 1-2-4 evaluating system made the exact measurement of the restriction fragments unnecessary, combination with non-denaturing PAGE allowed for the detection of all of the HPV<sub>s</sub> in multiple coinfections. This technique enabled us to justify triple infections, which could be confirmed from another sample of the same patient a month later.

The results of HCT tests – which only reacted with either the low-risk (A) or high-risk (B) tests – were exactly the same as the results obtained by the PCR-RFLP technique.

Of the 102 HCT HR-HPV positive samples, multiple infections were found in 13 cases, but all of the coinfection HPV<sub>s</sub> belonged to the high-risk group.

Further high-risk papillomavirus types were detected, although the HCT did not contain the corresponding testing mixtures. That was how we could detect singular HPV-53 infections (two cases), HPV-58 (two cases), HPV-66 (three cases) HPV-MM4 and HPV-CP8304 infections as well. These types might have undergone cross-hybridization with some HR probes. The new types included HPV-58 for which the type-specific probe was contained in the new generation Hybrid Capture test (HC II). Although we do not know the detection sensitivity of the hybridization technique for these new types, but the infection caused by them may have been detected by the first generation test as well. [Peyton et al (1998) reported their results in detecting new HPV types using HC II (HPV-53, -66, -67, -73, -CP6108 and -CP8061) at the time when we were conducting our study. Of these types, HPV-53 and -66 hybridize with the high-risk probes of HCT I.]

Among the twelve HCT-double-positive samples (“A” and “B”), PCR-RFLP could justify the simultaneous presence of LR and HR-HPV in two cases, and except for one in the other multiple infected samples it justified high-risk HPV alone.

The missed simultaneous detection of LR and HR HPVs in these samples could not be attributed to our method, since sensitive silver staining can easily detect multiple PCR bands even if there is a hundred times difference in the copy number of target sequences in question.

Having established that the PCR-RFLP typing technique was reliable, we re-tested the samples using HCT test, which allowed for the exclusion of false-positive HCT samples. Of the 13 samples, 12 hybridized again with both the high and low risk probes.

In the next step, we examined the significance of the power of the hybridization signal, which is expressed by the luminescence of the sample and the mean luminescence of three positive controls in each test sequence. When the HR signal (“B”) was stronger than the LR one (“A”), dominantly high-risk infections were present. But in the three cases in which LR-HPV was also detected, the LR-signal (“A”) was stronger than the HR one (“B”) in each case. Interestingly enough, a new type, HPV-62, was also present in a dominantly LR positive sample.

The RFLP-typed results of new HPV types (HPV-53, -58, -62, -66, -CP8304, and -MM4) were justified by sequencing. The MY09-MY11 amplicon sets were cloned, sequenced and adjusted to the relevant HPV reference types. The first four clones were sequenced in two ways. As, due to the use of the two-way technique, the majority of the sequences overlapped, the other clones were sequenced in one way only. Compared to the reference sequence, the extent of homology was 96-100%. Amplification was done using Taq polymerase and the difference, amounting to a few nucleotides, was noticeable between the clones and

the reference sequences, owing to an amplification error. However, this had no influence on the justification of the results of PCR-RFLP typing.

## DISCUSSION

In the first part of the current study, the prevalence of HPV infections was assessed in a large population with colposcopically and/or cytologically positive findings in North-east Hungary. The frequency of HPV infections at 35.1% registered in our epidemiologic study is very close to that of the European Region. Of course, these data, suggestive of high infection rates, are the parameters of a subpopulation found positive in primary screening in which cytological and colposcopic investigations were carried out. Similarly high HPV frequencies were only detected in high-risk populations such as students and closed communities, while the highest figures were found among prostitutes.

HR-HPV infections were more common than LR-HPV ones ( $p < 0.001$ ). We could confirm the observation in the Hungarian and international literature according to which HPV prevalence showed a linear decrease with the progression of age. In both the low-risk and high-risk groups, the high incidence of HPV infections between 20 and 35 years of age served as an indirect proof of what we were facing was a sexually transmitted infection (STI), as this age group is regarded to be the sexually most active one.

In the current study, the decrease in HR-HPV prevalence turned out to be significant after 35 and 45 years of age. It should be noted, however, that, despite the decrease in linear frequency, older age groups were also characterized by relatively high rates of infection (17% and 8%), proving that HPV-DNA may persist.

Significant decrease in prevalence in the case of LR-HPV occurred earlier (after the 25<sup>th</sup> and 35<sup>th</sup> years of age) and the disease developed in a negligible number of cases afterwards. Summing up the final observations in this study, we can say that in a large population with *cervical epithelial* changes of different levels, the chances for the virus to persist are greater in HR-HPV than LR-HPV infections. This important feature of this group of viruses may be the reason why they are oncogenic.

A practical conclusion beyond the observations published in the literature earlier is that patients carrying persisting HPV infections should be regarded as a high risk population because of the possible development of cervical cancer in the future. In contrast, the observation according to which the incidence of HPV infections is inversely proportional with age shows that a considerable proportion of HPV infections detected at a young age are transient, therefore their oncological importance is lower.

Therefore it seems worth determining the HPV status of patients in whom primary screening techniques such as colposcopy and cytology yielded positive results. This is how treatment and follow-up can be tailored to the individual. HPV typing may be necessary at certain intervals even if the primary screening tests are negative. Such a supplementary investigation may contribute to the efficiency of screening. Secondary HPV tests following cytological investigations have also proved that their high sensitivity allows for the identification of patients at a potential risk for the infection. It was brought up that negative cytological and HPV test results may not have a protective effect for the future. According to the practice followed in the USA, – referring to the importance of a negative HPV test – in the case of a negative HPV test and subsequent negative cytological findings (2-3 occasions) CINN III or ICC will not develop in 5-10 years, unless women change their sexual partners. According to certain opinions, negative cytology but persistent positive HR-HPV findings may predispose one to having

positive oncocytological results quite soon, therefore such patients are regarded to be in a high-risk group.

As far as the course of cervical cancer is regarded, the presence of metastases in the lymph nodes is one of the most important factors for the prognosis of the disease. To study this field we have extended the investigations to determine the HPV status of the lymph nodes of patients that were removed due to cervical carcinoma. Analyzing the details of 47 patients having undergone surgery we noticed that in histologically metastasis-free patients who carried HPV-DNA in their predilection lymph nodes (their HPV status being the same as in the case of primary tumors), the course of the disease was as fulminant as in patients with histologically tumorous lymph nodes. In four patients, who were HPV-18 positive for primary tumor and lymph nodes alike, early recidivation, unfavorable prognosis and short survival were recorded, although in three of them the lymph nodes were tumor-free.

Based on the above, HPV-specific nucleic acids are supposed to be sensitive indicators of metastases. We have also observed that HPV-18 emerged in consequently less favourable groups, so the presence of this HPV infection can also be taken for a bad prognostic sign if cervical cancer has already developed. Several international authors share the view that the HPV status of lymph nodes does play a role in the outcome of the disease. They emphasize that the HPV-positivity of predilection lymph nodes can be regarded as an independent prognostic parameter for the survival of patients and the risk of their mortality, similarly to the size and FIGO stage of the primary tumor. Considering all of the above, they have concluded that the presence of HPV-DNA in the lymph nodes of the minor pelvis can be regarded as the sign of early metastasis and, also, that this prognostic factor should be given maximum attention in designing the plan of treatment.

At the same time, however, other teams have not found a relationship between the HPV status of the lymph nodes in the minor pelvis and the spread of the tumor.

Presuming that the prognostic potential of various HPV types are different in cervical carcinomas, a Dutch team drew the above conclusions for the case of HPV-16 infections. Further confirming the different behavior of HPV-16 infection from that of HPV-18, in a Chinese study, HPV-DNA in the neighboring lymph nodes was found to be in direct proportion with the viral load of the primary tumor. There is no known explanation for the poor prognosis of HPV-18 infections in cervical cancer. Several molecular theories have been published on (a) a greater integration potential and (b) enhanced E7 phosphorylation capacity. In tumors associated with HPV-18, a shorter preclinical period of detectability was justified, also faster progression between CINs and into ICC. Lately, some authors have published their observations that the shorter precancerous stages found in such cases cannot be attributed to the aggressive effect of HPV-18 infection, but it is due to the less severe cytological changes caused by them.

The above, sometimes contradicting data show that the prognostic role of HPV-DNA detectable in tumor-free lymph nodes is not explicit. However, we should come to the conclusion that in early stage cervical carcinoma, especially when it is associated with HPV-18 infection, the presence of the HPV genome ought to be regarded as the early sign of a metastasis when it is histopathologically detected in the lymph nodes of the pelvis; the patients should be given treatment accordingly.

Several observations have confirmed the supposition that patients screened positive in the routine gynaecological screening tests should be involved in secondary screening/follow-up and their traditional screening for cervical cancer (cytology and colposcopy) ought to be supplemented with HPV investigation. In HPV diagnostics, the techniques used for screening and diagnosing cervical intraepithelial neoplasia cannot be used, therefore demand for a test that can detect HPV-DNA has been worded; such a test should have appropriately reliable scoring efficiency, be cost-effective and easy to use in routine examinations. In the spirit of the above, we tested the capacity of HCT, with special regard to suspicious cases.

The high sensitivity and specificity HCT (I) test can be used to distinguish 14 HPV types (HC II is suitable for distinguishing 18 types), and this technique can undoubtedly make a difference between the most commonly occurring low and high-risk types. The steadily increasing number of newly identified HPV-types gives rise for the opportunity that the HCT test gives cross reactions and interferes with the interpretation of the results.

Supported by the PCR-RFLP and sequencing techniques, it can be concluded that the HTC test is a suitable diagnostic technique to tell low and high risk HPV-types apart and, also, distinguish the low and high-risk populations prone to developing cervical cancer. According to our studies, double positive (LR and HR cases) are to be listed among the highly oncogenic groups and should be treated accordingly. The current study has also confirmed the close correlation between positive colposcopic and SIL cytological findings, and high-risk HPV infections, independently of the fact that double infection is present, i.e. the low-risk type of the virus is also present. This observation has been confirmed by the international literature.

All the above conclusions have been supported by the PCR-RFLP technique; we had a contradictory result in a single case, when we could not detect HR-HPV in the case of a double-positive sample. According to our investigations, HCT type-specificity was found to be 128/129, i.e. 99.2%.

Summing up our observations it can be concluded that the HCT test is a reliable tool to easily screen the high risk population in secondary screening programs.

## SUMMARY

In a prospective clinical epidemiological study, the prevalence of low and high-risk HPV infections was examined in patients with positive colposcopic and/or cytological findings. Using the Hybrid Capture Technique among the 3480 patients in the study, we could detect HPV infection in 1222 cases (35.1%). Single incidence of LR and HR infection, and the combination of the two were found in 91 cases (2.6%) and 1072 cases (30.8%), and 59 patients (1.7%), respectively. The median age of women with HR-HPV infection was 29.00 years, while the carriers of LR-HPV infection were 26.5 years of median age. The likelihood of high-risk HPV infection was higher compared to the low-risk one ( $p < 0.001$ ), independently of age. We could also confirm the fact in the literature that both HR- and LR-HPV prevalence decreased by age. Statistically significant decrease in prevalence was noted in patients over 34 and 44 years of age in HR-HPV infections ( $p < 0.001$ ). At the same time, however, high incidence of HR and LR HPV infection was also found among the elderly (19% and 8%, respectively). A decrease in prevalence came earlier in LR-HPV infections (after 25 and 34 years of age) but HPV-infection persisting into old age could be registered in a negligible number of cases. Analyzing the follow-up and HPV-clearance data of the 433 patients found positive for HR-HPV, we could see that virus clearance was rarer in the older age group, but the differences in monthly clearance-rates were not significant as far as the cytological findings and the patients' age were considered.

Studying the prognostics role of HPV in cervical cancer, in the 47 patients in the sample with operable cervical carcinoma (mean age 41.1 [21-59] years) the primary tumor and lymph nodes of the minor pelvis were used to perform HPV-typing via PCR after radical hysterectomy. Studying the patients' history further, early recidivation and short survival were found in 4 patients. In the lymph node preparations, the presence of HPV-18 coinciding with the primary tumor was found in each of the 4 cases, but, histologically, metastasis could only be detected

in one patient. Based on the above, it might be hypothesized that HPV-specific nucleic acids act as sensitive indicators of metastasis development.

We evaluated the HCT I. HPV-specificity (widely used in clinical practice) in 570 cervical samples applying PCR (MY09-My11)-RFPL technique. The control technique confirmed the HCT results in cases of single infection (15 and 102 samples positive for LR-HPV and HR-HPV). At the same time, however, in double positive samples by HCT we failed to detect HR-HPV using PCR-RFLP in one case only. Based on our investigations, the type specificity of HCT was found to be 99.2%. Summing up our observations we can conclude that the HCT test is an easy and safe method to use in a secondary screening program to highlight the population at high risk.

## PUBLICATIONS

### Publications used in the dissertation

**Sápy T**, Póka R, Szarka K, Kónya J, Huga S, Hernádi Z. Age-specific prevalence of high-risk human papilloma virus infection in a Hungarian female population with positive cytology. *Eur J Obstet Gynecol Reprod Biol.* 2007; (in press)

**IF: 1.273 (JCR 2006.)**

**Sápy T**, Hernádi Z, Kónya J, Lukácskó L. Poor clinical outcome in early stage cervical cancer with human papillomavirus-18 positive lymph nodes. *Eur J Obstet Gynecol Reprod Biol.* 2000; 90(1): 93-95.

**IF: 0.703 (JCR 2000.) CIT: 5**

Kónya J, Veress G, Juhász A, Szarka K, **Sápy T**, Hernádi Z, Gergely L. Additional human papillomavirus types detected by the hybrid capture tube test among samples from women with cytological and colposcopic atypia. *J Clin Microbiol.* 2000; 38(1): 408-411.

**IF: 3.503 (JCR 2000.) CIT: 7**

**Sápy T**, Szikszay Á, Kónya J, Borsos A, Hernádi Z. Human Papillomavírus infekció prevalenciája közel 5 éves anyagunkban. *Orv Hetil.* 2001; 142(24): 1265-1268.

**Sápy T**, Hernádi Z, Kónya J, Veress Gy, Czeglédy J. Korai recidíva és fulmináns kórlefolypás HPV 18-pozitív cervixcarcinómában. *Magy Nőorv L.* 1997; 60(3): 233-235.

**Sápy T**, Hernádi Z, Lukácskó L, Borsos A. The role of high risk HPV lymph node positivity in the surgical staging of cancer of the uterine cervix. *Acta Chir Hung.* 1997; 36(1-4): 313-315.

### **Other publications relating to this field of research**

Hernádi Z, **Sápy T**, Kónya J, Veress G, Czeglédy J. Magas kockázatú Humán Papillomavírus (HPV)- pozitív méhnyakrákos betegek kórlefolrásának követése. *Orv Hetil.* 1997; 138(20): 1249-1253.

Lukácskó L, Hernádi Z, **Sápy T**, Borsos A. The prognostic value of CA-125 in epithelial ovarian cancer patients during and after chemotherapy. *Acta Chir Hung.* 1997; 36(1-4): 213-214.

Hernádi Z, **Sápy T**, Lukácskó L, Borsos A. Second-look surgery (SLO) in the management of carcinoma of the ovary. *Acta Chir Hung.* 1997; 36(1-4): 128-129.

Hernádi Z, Lukácskó L, **Sápy T**, Borsos A. Petefészekrákos betegek cyclophosphamid és cisplatin (CP) kombinált kemoterápiája Amifostine (WR-1065) védelemben. *Magy Nőorv L.* 1998; 61: 489-496.

Szarka K, Veress G, Juhász A, Kónya J, **Sápy T**, Soós G, Hernádi Z, Gergely L. Integration status of virus DNA and p53 codon 72 polymorphism in human papillomavirus type 16 positive cervical cancers. *Anticancer Res.* 2000; 20(3B): 2161-2167.

**IF: 1.331 (JCR 2000.) CIT: 11**

Hernádi Z, Huga S, Lukácskó L, Krasznai Z, **Sápy T**, Borsos A. Second-line Taxol treatment of ovarian cancer patients refractory to first line platinum-based chemotherapy. *Dzem Gin.* 2000; 2: 29-32.

**Sápy T**, Lampé L. Terhességgel szövődött méhnyakrák érdekes esete. *Magy Nőorv L.* 2001; 64: 421-423.

Hernádi Z, Huga S, Lukácskó L, Krasznai Z, **Sápy T**. Rezisztencia a petefészekrákos betegek platina-bázisú kemoterápiája során – a paclitaxel, mint kezelési lehetőség. *Magy Nőorv L.* 2001; 64: 249-254.

Szőke K, **Sápy T**, Krasznai Z, Hernádi Z, Szládek G, Veress G, Dillner J, Gergely L, Konya J. Moderate variation of the oncogenic potential among high-risk human papillomavirus types in gynecologic patients with cervical abnormalities. *J Med Virol.* 2003; 71(4): 585-592.

**IF: 2.371 (JCR 2003.) CIT: 1**

Hernádi Z, Szarka K, **Sápy T**, Krasznai Z, Veress G, Póka R. The prognostic significance of HPV-16 genome status of the lymph nodes, the integration status and p53 genotype in HPV-16 positive cervical cancer: a long term follow up. *BJOG.* 2003; 110(2): 205-209.

**IF: 1.191 (JCR 2003.) CIT: 3**

Hernádi Z, **Sápy T**, Krasznai Z. The prevalence of the HPV 16 genome, integrated viral status and p53 genotype in cervical cancer population of north-eastern Hungary, the correlation with the established markers of tumor progression. *Eur J Obstet Gynecol Reprod Biol.* 2004; 113(1): 83-86.

**IF: 0.955 (JCR 2004.) CIT: 1**

Hernádi Z, Szőke K, **Sápy T**, Krasznai Z, Soós G, Veress G, Gergely L, Kónya J. Role of human papillomavirus (HPV) testing in the follow-up of patients after treatment for cervical precancerous lesions. Eur J Obstet Gynecol Reprod Biol. 2005; 118(2): 229-234.

**IF: 1.141 (JCR 2005.) CIT: 3**

Hernádi Z, Gazdag L, Szőke K, **Sápy T**, Krasznai Z, Kónya J. Duration of HPV-associated risk for high-grade cervical intraepithelial neoplasia. Eur J Obstet Gynecol Reprod Biol. 2006; 125(1):114-119.

**IF: 1.273 (JCR 2006.)**

**Cumulative impact factor: 13,741**

**Independent citations: 31**

### **Lectures related to the topic of the dissertation, presented at conferences and published in journals**

Hernádi Z, **Sápy T**, Czeglédy J. HPV-infekció mint prognosztikai tényező. Magy Onkol. 1995; 115. Suppl.

Hernádi Z, **Sápy T**, Veress Gy, Kónya J, Czeglédy J. Follow-up results of cervical cancer patients with high-risk HPV-positive status. C Eur Assoc Gynec Obstet. 1997; 110. Suppl.

## LECTURES AND POSTERS

**Sápy T.,** Hernádi Z., Czeglédy J.: Méhnyakrákos betegek kórlefordyása különböző HPV típusú infekciók esetén. Fiatal Szülész és Nőgyógyász Orvosok Tudományos Ülése, Miskolc, 1995. szeptember 29-30.

Hernádi Z., **Sápy T.,** Czeglédy J.: HPV infekció mint prognosztikai tényező. Magyar Onkológusok Társasága XXI. Nemzeti Kongresszus, Pécs, 1995. november 9-11.

**Sápy T.:** A HPV infekció prognosztikai szerepe méhnyakrákban. DOTE PhD és TDK Tudományos Diáktalálkozója, Debrecen, 1996. március 21-23.

**Sápy T.:** Sikeres terhesség korai stádiumú méhnyakrák konzervatív terápiaját követően. Magyar Nőorvos Társaság Cervixpatológiai Szekciója XII. Tudományos Ülése, Debrecen, 1996. április 26-27.

**Sápy T.:** Méhnyakrákos betegek hisztológiailag negatív, de Human Papillomavírus-18 pozitív nyirokcsomói rossz prognózisra utalnak. DOTE PhD és TDK Tudományos Diáktalálkozója, Debrecen, 1997. március 6-8.

**Sápy T.:** Videokolposzkópia., Újabb ismeretek a szülészet-nőgyógyászatban – Továbbképző tanfolyam. Debrecen, 1997. március 12.

**Sápy T.:** A méhnyakrákos betegek hisztológiailag negatív, de Human Papillomavírus-18 pozitív nyirokcsomói rossz prognózisra utalnak. (I. helyezés) Korányi Frigyes Tudományos Fórum, Budapest, 1997. április 24.

**Sápy T.,** Hernádi Z., Lukácskó L., Borsos A.: The role of high risk HPV lymph node positivity in the surgical staging of cancer of the uterine cervix. XVI. Kísérletes Sebészeti Kongresszus, Debrecen, 1997. szeptember 25-27.

**Sápy T.,** Hernádi Z., Kónya J., Gergely L.: Human Papillomavírus fertőzés kimutatása nukleinsav hibridizációs technikával. Magyar Nőorvos Társaság Északkelet-magyarországi Szakcsoportjának Tudományos Ülése, Berettyóújfalu, 1999. október 15.

**Sápy T.:** Human Papillomavírus fertőzés kimutatása nukleinsav hibridizációs technikával. Magyar Nőgyógyász Onkológusok Társasága II. Kongresszusa, Budapest, 1999. december 10-11.

**Sápy T.,** Hernádi Z., Kónya J.: Human papillomavírus kimutatása cervikális kenetektől nukleinsav hibridizációs módszerrel. Magyar Nőorvos Társaság Cervixpatológiai Szekciójának XV. Kongresszusa, Hajdúszoboszló, 2000. június 15-17.

**Sápy T.:** A méhnyakrák szűrésének újabb szempontjai – a HPV diagnosztika jelentősége. Nőgyógyászati malignómák korszerű diagnosztikája és terápiája, Szakreferátum, Debrecen, 2000. december 15.

**Sápy T.,** Kónya J., Hernádi Z.: Onkogén HPV típusok előfordulása Klinikánk járóbeteg körében, MOT Nőgyógyászati Szekciója Továbbképző Ülése, Budapest, 2001. október 5.

**Sápy T.,** Kónya J., Hernádi Z.: Humán papillomavírus infectio praevalenciája öt éves anyagunkban. Magyar Nőorvos Társaság XXVII. Nagygyűlése, Budapest, 2002. augusztus 28-31.

**Sápy T.:** A méhnyakrák szűrésének újabb szempontjai, a HPV diagnosztika szerepe. V. Zempléni Őszi Orvosnapok, Sátoraljújhely, 2002. október 4.

Kónya J., **Sápy T.**, Szóke K., Szládek Gy., Hernádi Z., Gergely L.: Predictive value of human papilloma virus (HPV) testing for incident and recurrent cervical intraepithelial neoplasia (CIN). 20th International Papillomavirus Conference, Paris, 2002. október 4-9.

**Sápy T.:** Terhességgel szövődött méhnyakrák érdekes esete. Magyar Nőorvos Társaság Északkelet-magyarországi Szakcsoportjának Kongresszusa, Mátészalka, 2002. október 10-12.

**Sápy T.:** A méhnyakrák szűrésének újabb szempontjai, a HPV diagnosztika szerepe. Szülészeti-Nőgyógyászati Prevenációs Tudományos Társaság Továbbképző, Debrecen, 2003. január 17.

**Sápy T.:** A méhnyakrákszűrés továbbfejlesztése, hatékonyságának növelése – HPV vizsgálatok preventív értéke, Nőgyógyászati Onkológia – Képzés és továbbképzés, a szakterület szerepe a daganatos halálozás csökkentésében, rákszűrés, tudományos ülés, Debrecen, 2003. február 14.

**Sápy T.:** Nőgyógyászati daganatok szűrése, korszerű méhnyakrák szűrés, ÁNTSZ háziorvosi szakmai nap, Debrecen, 2004. október 18.

**Sápy T.:** Genitális HPV infekciók. Cervicalis rák megelőző állapotok diagnosztikája c. továbbképző tanfolyam, Debrecen, 2004. november 8-11.

**Sápy T., Kónya J., Hernádi Z.:** A HPV infekciók epidemiológiája – Hospital based epidemiology. Magyar Nőorvos Társaság Cervixpatológiai Szekció XVI. Tudományos Ülés, Hajdúszoboszló, 2005. október 14-15.

Hernádi Z., **Sápy T.**, Krasznai Z., Kónya J.: A HPV genom azonosítása és annak prognosztikai jelentősége a méhnyakrák kialakulása és progressziója különböző stádiumaiban. Magyar Nőorvos Társaság Cervixpatológiai Szekció XVI. Tudományos Ülés, Hajdúszoboszló, 2005. október 14-15.

Huga S., **Sápy T.**, Krasznai Z., Óvári L., Hernádi Z.: Az előreheladott stádiumú méhnyakrák különböző protokollok szerint végzett radio-kemoterápiájának tapasztalatai. Magyar Nőorvos Társaság Cervixpatológiai Szekció XVI. Tudományos Ülés, Hajdúszoboszló, 2005. október 14-15.

**Sápy T.:** Genitális HPV infekciók. Cervicalis rákmegelőző állapotok diagnosztikája c. továbbképző tanfolyam, Debrecen, 2005. november 28-December 1.

**Sápy T.:** Az onkológia családorvosi vonatkozásai. Családorvosképzés, Debrecen, 2006.

**Sápy T.:** Genitális HPV infekciók. Cervicalis Rákmegelőző Állapotok Diagnosztikája c. Tanfolyam, Debrecen, 2006. november 27-30.

Gazdag L., **Sápy T.**, Hernádi Z.: HPV-pozitív betegek biopsziás mintáinak elemzése. Magyar Nőorvos Társaság Cervixpatológiai Szekció XVII. Tudományos Ülés, Hajdúszoboszló, 2007. március 23-24.

Krasznai Z., Gazdag L., Szőke K., **Sápy T.**, Kónya J., Hernádi Z.: A HPV asszociált kockázat időtartama a magas fokú CIN kialakulására. Magyar Nőorvos Társaság Cervixpatológiai Szekció XVII. Tudományos Ülés, Hajdúszoboszló, 2007. március 23-24.

Hernádi Z.: Krasznai Z., Gazdag L., Szőke K., **Sápy T.**, Kónya J.: A HPV típus-specifikus meghatározásának jelentősége a CIN sebészeti kezelését követően. Magyar Nőorvos Társaság Cervixpatológiai Szekció XVII. Tudományos Ülés, Hajdúszoboszló, 2007. március 23-24.

**Sápy T.**, Hernádi Z.: A magas kockázatú HPV-infekció és a méhnyakrák kapcsolatát alátámasztó epidemiológiai adatok régióinkban. Magyar Nőorvos Társaság Cervixpatológiai Szekció XVII. Tudományos Ülés, Hajdúszoboszló, 2007. március 23-24.