# The role of Human papilloma virus (HPV) typing and newly developed HPV detection methods and study of new potentially applicable biomarkers in HPV triage in the diagnosis of cervical lesions

Ph.D. thesis

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# Introduction

The role of human papilloma virus (HPV) in the etiology of cervical cancer has been proven time and time again over many decades. According to case-control and prevalence studies, HPV is the hereditary material in 96.6% of planocellular carcinoma (squamous cell carcinoma, SCC) and adenocarcinoma (AC) and can be detected in mixed-type carcinomas (SCC+AC) in 91.9%.

HPV infection is mostly transient and asymptomatic, but it may remain persistent in a small percentage, with cervical cancer occurring over the years. Proven condition and risk factor for development of cervical carcinoma a durable, persisting high-risk HPV (hrHPV) infection, classified by the risk for development of cervical cancer into a group of high-risk HPV genotypes. Most of the hrHPV tests detect the following 14 hrHPV genotypes: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

The hrHPV test is widely used in cytology-based screening as a triage test for uncertain cytology diagnoses and is a recommended method for postoperative surveillance. Individual determination of hrHPV genotypes (HPV genotyping) also provides an opportunity for accurate patient tracking and risk assessment of HPV positive women. Before vaccination performed HPV genotyping of women who have already started a sexual life, gives information about the possible current infection of HPV vaccine genotypes.

The results of various clinical studies have shown that the hrHPV test further improves the effectiveness of primary screening compared to cytology. The latest European and American guidelines recommend the use of the HPV test as a primary cervical screening tool. There is a need for high capacity hrHPV detection technologies to meet the above-mentioned clinical applications for hrHPV testing.

A further testing of hrHPV positive women (HPV triage) is needed to increase the specificity of hrHPV-based screening, as the one-time HPV test does not provide information on whether cell transformation

has been initiated or if there is only a temporary infection. The proposed protocol for HPV triage is differs worldwide and is the subject of ongoing trials. In addition to the cytology used in HPV triage, biomarkers are also potential candidates, because of their capability to identify the biological processes underlying the progression of HPV infection with specific molecular technologies.

## Objectives

Our aim was to study cervical cancer and premalignant lesions, which we examined by developing molecular diagnostic methods, in the light of clinical data.

The following questions were formulated:

1. To study the epidemiological questions of HPV infections in the light of HPV typing results, on samples from Hungarian institutes in the Genoid Laboratory (1), and on cervical, pharynx and anal samples collected in a high risk group of female sex workers (2).
2. To determine the clinical performance of a newly developed real-time PCR based HPV test using a clinically validated HPV test (3, 4 ).
3. To investigate new potential biomarkers with immunochemical staining of Claudin1 and p16INK4a (5).
4. To research new molecular biomarkers by mapping the role of micro-RNA expression pattern changes in cervical cancers, and its relations to cervical pathology (6).

## Methods

*Objective 1*: In the GenoID Molecular Diagnostic Laboratory, in 2005/2006, **6447 HPV genotyping was performed for cervical, urethral, seminal, anus, condyloma, anogenital scraping, vaginal samples (total number of samples n=12345).** HPV DNA detection was performed by the laboratory's Full Spectrum HPV Amplification and

Detection Test, and the PCR products of hrHPV (high risk) positive samples were individually genotyped. The GenoID HPV detection and genotyping system is referred to as Full Spectrum L1F/L1R-HPV test (Genoid, Hungary, Budapest) in my dissertation. **The Full Spectrum L1F / L1R-HPV test individually genotypes the 14 hrHPV types**: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68; **and detects the following groups**: (1) the **5 lrHPV** (lr, low-risk) types 6, 11, 42, 43, 44; (2) the **29 NA-HPV** (NA, unclassified) genotypes: 2a, 3, 7, 10, 13, 26, 27, 28, 29, 30, 34, 40, 53, 54, 57, 67, 70, 72, 73, 74, 81, 82, 83, 84, 85, 89, 90 and 91.

In the comparative study of **female sex workers (FSW)** and control group we studied the **relationship between sexual habits and the occurrence of cervical, anal and pharyngeal HPV infection.** The collection of samples was done at the Faculty of Medicine of the University of Pécs. A sample of all three anatomical regions of each study subject (FSW) (n = 34) and control group (n = 52) was collected and questionnaires were submitted for sexual and other patterns. HPV genotyping was performed by the Full Spectrum L1F/L1R-HPV test at the Genoid Laboratory.

*Objective 2*: **Development and clinical validation of a high throughput HPV detection method to meet increased HPV diagnostic capacity needs.** In my paper, **two newly developed real-time PCR-based HPV tests** using multiplex single-step HPV type-specific "molecular beacon" probes were tested **developed by the Genoid Laboratory**: **(1) MBRT-HPV** (Genoid, Hungary, Budapest) and **(2) MBRT-HPV-ABI** (Genoid, Hungary, Budapest) adapted to the ABI7900 platform, in a comparison with a clinically validated HPV test. The MBRT-HPV test detects 14+1 hrHPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and the probably high risk HPV26) and group detects 5 lrHPV types (6, 11, 42, 43, 44). The MBRT-HPV-ABI test detects 3 groups: (1) HPV16/18 (HPV16, 18); (2) other 12hrHPV group (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68); (3) lrHPV group (HPV6, 11).

The following populations were enrolled the clinical studies of the new HPV tests. First, the residual samples (n=161) arriving to the Genoid Laboratory for the Full Spectrum L1F / L1R-HPV assay were used for the MBRT-HPV test evaluation. Second, in the frame of a collaboration with the CERVIVA study in Ireland (Irish Cervical Screening Research Consortium) LBC (liquid-based cytology) cervix samples were collected from colposcopy clinic patients (n = 241), with cytological abnormalities and the residual samples after cytology and HPV testing were used for the MBRT-HPV-ABI assay evaluation. **The MBRT HPV test was compared with the Full Spectrum L1F/L1R-HPV test, whereas for MBRT-HPV ABI test the HC2 (Qiagen, Hilden, Germany) and the Full Spectrum L1F/L1R-HPV test was used for comparison,** the differences were investigated with the Linear Array-HPV (Roche, Mannheim, Germany) test for these CERVIVA study samples and genotyped. The results were analyzed where possible, depending on the clinical endpoints (cytology, histology) as well.

*Objective 3*: The third objective of the thesis was to **investigate immunochemical biomarkers in cytology and HPV triage.** With my co-authors, we evaluated the data of the already published biomarker, p16INK4a and a new biomarker, claudin1 (CLDN1) immunohistochemistry/immunocytochemistry tests, compared to the gold standard histology in a case control study on traditional cytology and LBC cytology samples (n=502). Evaluation of immunostaining was in some cases combined with morphology reading, by using the immunostainings in cytology and HPV triage settings. Full Spectrum L1F/L1R-HPV assay was evaluated at the Genoid Laboratory, routine cytology. Histology evaluation was performed by the co-authors. Immunostainings were evaluated by my co-authors according to commonly agreed criteria.

*Objective 4*: The fourth objective of my dissertation was a smaller study, wherein the molecular biomarker assays were performed in a retrospective manner of the University of Pecs archived cervical cancer samples. **Our primary aim was to map the role of changes in the microRNA (miR) expression pattern in cervical pathology and as a**

**secondary target to explore its correlation with the HPV infection and with different types of cancerous tissue samples.**

Formal-fixed paraffin-embedded (FFPE) tissue samples (AC=22, SCC=25) of human cervical cancer were selected randomly from the archives of the Department of Pathology of the Faculty of Medicine of the University of Pécs collected at the Department of Obstetrics and Gynecology in 2007-2010. The expression profiles of miR of the cervical cancers were performed at the University of Pécs, and the Full Spectrum L1F/L1R-HPV assay was performed in the Genoid Laboratory.

## Results

*Objective 1*: The GenoID laboratory received 12354 samples in two years, of which **6447 HPV genotypes were performed**. In 2006, most of the examined samples were cervical samples (n=8033), 41% of which gave HPV-DNA positive results. The number of other types of samples was significantly lower (n=475) and their positivity rates were on a wide scale. The HPV positivity rate was highest in condyloma/tissue (73-74%) and anal (67%), while lowest in semen (10%). Based on the distribution of HPV-DNA positive samples according to risk groups, **HPV infections were mostly high-risk (67%) genotypes whereas low-risk genotypes were in 8.6% involved. Unclassified risk types (NA-HPV) were 14.3% the second most common group in positive samples.**

In 2005/06, the hrHPV typing results were aggregated by genotype, with the highest percentage being HPV16 (in 2005: 15.9%, in 2006: 20.5%) among the HPV-DNA positives. Then, in 2006, the most common HPV types were HPV31, 51, 66, 56, 58, 33, 39, and 18. In both years, the proportion of monovalent infections was high (75%), bivalent infections accounted for 18-20% of the samples and around 5% of the samples were more than two types.

In our second epidemiology study, **high prevalence of HPV infection was observed in cervical, pharynx and anal models of sex**

**workers having high risk of infection**. Of the FSW samples, in most cases (82.4%) at least one HPV-DNA was positive, while less than half (46.2%) of control women were HPV infected. Nearly half of the FSWs (44%) and less than ten percent of the controls were HPV positive in more than one region. No statistically significant correlation was found between the number of sexual partners and the number of HPV types combined by the infections. **Significant correlation was observed between FSW's promiscuity rate and the higher prevalence of HPV-DNA (hrHPV + lrHPV + NA-HPV), hrHPV and multiple HPV infections.** FSW cervical and anal samples showed a significantly higher rate of HPV infection than the control group (64.7% vs. 34.6% $p<0.05$ and 50.0% versus 15.4%, $p<0.05$). The higher HPV prevalence (20.6% versus 7.7%, $p=0.10$) found in the FSW pharyngeal samples did not show any significant correlation with oral sex. HrHPV prevalence was also significantly higher in FSWs (55.9% vs. 25.0%, P <0.05). **In hrHPV positive samples HPV31 was the most common (19.3%), especially in FWS samples, followed by 16, 66, 18, 51, 58 and 56 (14%, 10.5%, 8.8 %, 8.8%, 8.8%, 7.0%).** HPV31, 16, 18, 58, 66, and 33 were mainly positive in FSW samples, while HPV 51 was mostly present in the control group, although differences in the genotype distribution in the two groups were statistically not significant. The FSWs had a significantly higher proportion of genital warts in the past (26.5% versus 3.8%, $p<0.05$).

*Objective 2*: In clinical trials in the Genoid laboratory, comparing the **Full Spectrum L1F / L1R-HPV and MBRT-HPV test, the match was 89.44%** for the detection of hrHPV infections. Based on the comparative study, the **estimated sensitivity of the MBRT-HPV test was 95.45% (63/66) with an estimated specificity of 91.57% (87/95).** Results of the MBRT-HPV test hrHPVtypes resulted in cross-reactivity with some lrHPV types. In a comparative study of the MBRT-HPV-ABI test performed in the Irish laboratory, the prevalence of hrHPV of the three HPV DNA tests showed the following values: 78.8 (186/236) Full Spectrum L1F/L1R-HPV, 83.3% (195/234) HC2 , 78.8% (166/211) MBRT-HPV-ABI. The tests agreement with the HC2 test were: Full

Spectrum L1F / L1R-HPV 94.6% (κ=0.792), MBRT-HPV-ABI 87.4% (κ=0.532). **There was no significant difference between Full Spectrum L1F/L1R-HPV and HC2 test in hrHPV detection.** There was a significant difference between MBRT-HPV-ABI and HC2 test. The hrHPV prevalence of the three HPV tests summarized by the cytological categories, showed that Full Spectrum L1F/L1R-HPV and HC2 were similar in every case, while the detection rate of MBRT-HPV-ABI HPV was lower in each cytology category. **The sensitivity of HC2, Full Spectrum L1F/L1R-HPV and MBRT-HPV-ABI was 98.3%, 97.4% and 93.9%, while PPV was 94.1%, 94.1% and 97.3% , respectively for CIN2+ histological diagnosis.** HPV genotyping with LA-HPV assay was used to dissolve different hrHPV results of the three HPV DNA assays tested. The most common false negative types were HPV16 (9/16) and HPV51 (4/16) for the MBRT-HPV-ABI test. False positive samples contained lrHPV genotypes (HPV53, 73, 42, 54)

*Objective 3*: **Comparing of the performance of cytology (ASCUS or greater), hrHPV and immunochemistry** (immunocytochemistry-IC and immunohistochemistry-IH) we found that the **biomarkers were more specific, in particular with the evaluation of the morphological adjusted scoring method (MASM**), which exceeded the specificity of cytology or HPV test (77.0% (69.6-83.6) of IC-CLDN1, 85.1% (78-90.8) of IC-p16$^{INK4a}$, 69.3% ( 63.6-74.8) of IH-CLDN1, 91.2% (86.4-94.4) of IH-p16$^{INK4a}$ vs. 66.5% (61.1-71.2) of cytology and 61.4% (57.4-63.6) of HPV). **Combinations of both IC and IH tests showed greater sensitivity, with a moderate specificity decrease**. In the triage setting the IC-p16$^{INK4a}$ specificity was higher (81-86% depending on the evaluation and triage algorithm), but its sensitivity was lower (58-76%) than IC-CLDN1. IC test combinations were tested in cytology and HPV triage according to MASM evaluation. As a result of the combined evaluation, their specificity increased significantly with a weak sensitivity decrease. **IC-CLDN1-p16$^{INK4a}$ combined evaluation in cytology triage gave the highest values: sensitivity 70.5% (62.4-76.9), specificity 72.7% (57.7-84.7).**

*Objective 4*: According to our analysis, the **targeted miRNA assays showed a significant difference between the two examined cervical cancers (SCC, AC) in miR21, miR-27a, miR-34a, miR-155, miR-196a, miR-203 and miR-221 expression profiles. The HPV positive SCC, AC samples** also showed significant differences in the miR-21, miR-27a, miR-34a, miR-196a and miR-221 expression, **but this was not confirmed for miR-155 and miR-203**.

**Among the cervical cancer stages** of the International Federation of Obstetrics and Gynecology (FIGO), **a statistically significant difference in certain miR expression was explored**.

## Conclusions

*Objective 1*: Our HPV genotyping results **provide epidemiological information on the distribution of HPV genotypes in domestic populations for different samples types in year of 2005 and 2006** in Hungary. During these two years the GenoID laboratory performed a **large number (n= 6447) of HPV genotyping** with the Full-spectrum L1F/L1R-HPV test, the **with highest number published from Hungary**. The incidence of HPV genotypes, and the frequency of multivalent infections was also determined. Like in the other countries **the HPV16 showed the highest percentage in HPV-DNA positive samples in both years. Thereafter, the most common HPV types in 2006 were HPV31, 51, 66, 56, 58, 33, 39, and 18.** HPV 16 and HPV18 genotypes that can be prevented by the bivalent and quadrivalent HPV preventive vaccines were detected in 25.2% among the HPV-positive DNA samples.

**High prevalence of HPV infection was observed in cervical, pharyngeal and anal samples of sex workers** compared to the control group samples. Our results confirm that promiscuous sexual behavior is **the dominant risk factor for genital HPV infection and the effect of promiscuity on the frequency of increased oropharyngeal HPV infection.** Studying the sexual habits of the two groups, we found that

**there is no correlation between the practice of anal sex and the HPV infection of rectal mucous membranes**. **The results of this study are the first in Hungary to provide insights into the prevalence of HPV infection and HPV genotypes of sex workers**. These findings can be useful for planning further studies to investigate a larger, more comprehensive analysis of sex workers in Central and Eastern Europe.

*Objective 2*: According to European recommendations, the clinical performance of new HPV tests should be assessed against the HC2 test or another clinically validated test. The newly developed MBRT-HPV test of the Genoid Laboratory was compared to the Full Spectrum L1F/L1R-HPV test, which is known to exhibit similar performance to the HC2 test. The MBRT-HPV test on the ABI7900 platform (MBRT-HPV-ABI) and the Full Spectrum L1F/L1R-HPV test was compared to the HC2 test in collaboration with an Irish laboratory. Compared to the HC2 test, the results of Full Spectrum L1F/L1R-HPV and MBRT-HPV-ABI tests for detecting hrHPV genotypes, Full Spectrum L1F/L1R-HPV and HC2 showed greater concordance than MBRT-HPV-ABI and HC2 test and there was no statistical difference between HC2 and Full Spectrum L1F/L1R-HPV tests.

**Overall, the MBRT-HPV-ABI test was somewhat less sensitive compared to either the HC2 or the Full Spectrum L1F/L1R-HPV test**. Limits of MBRT-HPV and MBRT-HPV-ABI test are that **some rare low-risk genotypes cross-reacted with the high-risk HPV** and **MBRT-HPV-ABI test showed weaker sensitivity to HPV16 detection**. **Therefore, further development of the MBRT-HPV test may be necessary.**

*Objective 3*: A new potential biomarker of cervical lesions, the CLDN1 immunostaining was evaluated in a clinical study compared with the previously used $p16^{INK4a}$ immunostaining. **CLDN1 showed similar performance as $p16^{INK4a}$, but it was less specific.** At the evaluation of the immunostainings in triage settings the performance of cytology triage with IC-CLDN1-p16INK4a test with MASM scoring was comparable to HPV triage with IC-p16INK4a test. The IC-CLDN1-p16INK4a MASM scoring used in cytology triage showed similar performance compared to

IC-p16INK4a tested in HPV triage. **Our results show that cytology performance can be improved by using biomarkers** to keep with HPV-based screening technologies competitive. Although we have found that certain benefits are provided by the combination of morphology with immunocytochemistry, neither morphology nor biomarkers alone or in combinations have been able to provide outstanding test performance. Further research on biomarkers can lead to a better understanding of the cervical carcinogenesis process and ultimately result in improved diagnostic performance of cervical diagnostic tests.

*Objective 4*: In our study, we have demonstrated that **miRNA profiles can be used to distinguish the two most common histological types of cervical cancer (SCC, ACC)**. Significant difference between the HPV positive groups of the two histological types was demonstrated in miR-21, miR-27a, miR-34a, miR-196a, and miR-221 expression. The miR-146a expression was also cervical-specific but has been shown to be independent of the cancer tissue type or HPV infection.

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