

## THESIS TITLE:

# **"Correlation of Human Papillomavirus Infection in Preinvasive and Invasive Skin and Ectocervical Tumors of Squamous Cell Origin with Expression of the Transcription Factor FOXP3"**

## INTRODUCTION

The first description of Human Papillomavirus (HPV) was provided by Strauss and colleagues in 1949, using electron microscopy to examine tissue extracts from skin warts (1). Several decades later, Professor Harald zur Hausen proved the connection between HPV infection and cervical cancer and received the Nobel Prize for his discoveries in 2008 (2). Today's knowledge about the various types of this virus increasingly highlights its importance in the development of squamous cell carcinoma of the skin (3).

HPV infection is considered the most common sexually transmitted infection. However, HPV is also a normal component of the skin flora in immunocompetent individuals. HPV belongs to the *Papillomaviridae* family and to date, 441 HPV types have been identified in humans (4), of which 231 have been fully sequenced (5). All HPV types are grouped into five genera based on homology of their *L1* gene sequences, which encode the L1 capsid protein: alpha (66 types), beta (67 types), gamma (302 types), mu (5 types), and nu (1 type). Alpha HPV types cause infections of both the skin and mucosa. It has been determined that HPV types 2, 27, and 57 exhibit a viral tropism for the skin and are associated with the development of cutaneous warts, while types 6 and 11 show mucosal tropism and cause genital warts (6). These HPV types are classified as low-risk types based on their oncogenic potential. In contrast, alpha HPV types are high-risk for the development of anogenital and oropharyngeal cancers and, in some cases skin cancers. The highest-risk types are 16 and 18, but types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 are also notable (7).

Cervical cancer is one of the most common malignancy among women worldwide, with approximately 660,000 new cases diagnosed annually. In Europe, approximately 58,000 women (10.7 per 100,000) are diagnosed with this tumor, and approximately 26,000 women (3.76 per 100,000) die from this disease annually (8,9). Squamous cell carcinoma of the cervix is caused by HPV infection in about 90% of cases, with HPV types 16 and 18 being the most prevalent. Approximately 10% of infected women develop a persistent infection, and 1-4% of these women subsequently develop preinvasive or invasive tumor within a few years. In addition to HPV type, immunodeficiency, post-transplant immunosuppression, oral contraceptives use, smoking, and Chlamydia trachomatis infection are important factors in cervical carcinogenesis (11). Efforts to reduce the incidence of cervical cancer focus on cervical cancer screening programs and the administration of prophylactic HPV vaccine (12).

During or shortly after birth, children may be exposed to HPV beta types through parental contact. These HPV beta types have also been associated with carcinogenesis and the development of non-melanoma skin cancer (13). Immunosuppression following transplantation, sunburn, and advanced age have been reported as additional risk factors (14). HPV types 5 and 8 are consistently detected in patients with epidermodysplasia verruciformis, a condition in which the disease may progress from verrucous lesions to squamous cell carcinoma in sun-exposed skin areas. However, these HPV types may also be associated with actinic keratosis and squamous cell carcinoma in the general population (15), whereas data on lesion development and the underlying mechanisms in sun-protected skin areas are scarce. Squamous cell carcinoma of the skin accounts for 20-50% of all skin cancers, and the number of newly diagnosed cases continues to increase. Although the mortality rate of this cancer is relatively low, the large number of affected patients and the range of required treatment procedures results in a substantial economic burden on both patients and the healthcare system (4).

Dysregulation of regulatory T lymphocytes has been described in various inflammatory and autoimmune diseases, as well as in multiple cancer types. However, there is limited data in the literature regarding the role and significance of T regulatory lymphocytes in the development and progression of keratinocyte tumors of the ectocervix, skin, and vulva, particularly in relation to HPV infection. Forkhead box P3 (FOXP3) is a transcription factor and an indicator of T regulatory lymphocyte identity. FOXP3 expression was first described in pancreatic cancer and was subsequently observed in breast, prostate, gastric, thyroid, melanoma, hepatocellular, and colorectal carcinoma, as well as in non-small cell lung carcinoma (16,17). The FOXP3 protein is encoded by the gene of the same name, which in humans is located on the short arm of the X chromosome (Xp 11.2). Several polymorphisms exist within the *FOXP3* gene sequence, representing normal genetic variation. These polymorphisms are primarily single-nucleotide polymorphisms. The polymorphic loci rs2280883 (IVS9+459 T/C) and rs3761548 (-3279 A/C), located in the regulatory regions of the *FOXP3* gene, affect transcription activity, consequently, specific alleles and genotypes are associated with increased or decreased gene expression and protein synthesis (18,19). Numerous association studies have investigated these polymorphisms as potential predisposition factors for various diseases, including malignancies (19,20,21). However, the results of these studies are often contradictory, which can be explained by differences in the frequency of individual genotypes within polymorphisms in different populations, differences in the size and consistency of the tested sample, among other factors. It has been suggested that gene polymorphisms may serve as predisposing factors for the development of preinvasive and invasive lesions in the skin and ectocervix (21,22).

## **WORKING HYPOTHESIS**

**Infection with different types of human papillomavirus (HPV) in preinvasive and invasive tumors of the skin and ectocervix is associated with increased FOXP3 protein expression and polymorphisms within the *FOXP3* gene.**

## RESEARCH AIMS

1. To examine the presence and determine the genotypes of HPV infection in cervical smear samples of preinvasive ectocervical tumors (low-grade squamous intraepithelial lesion, i.e. L-SIL and high-grade squamous intraepithelial lesion, i.e. H-SIL) and invasive ectocervical tumors (squamous cell carcinoma), as well as biopsies of preinvasive skin tumors (actinic keratosis and Bowen's disease) and invasive skin tumors (squamous cell carcinoma) and preinvasive vulvar skin tumors (low-grade squamous intraepithelial lesion, i.e. VIN-1, high-grade squamous intraepithelial lesion, i.e. VIN-2 and VIN-3) and invasive vulvar skin tumors (squamous cell carcinoma).
2. To determine FOXP3 protein expression in biopsies of preinvasive ectocervical tumors (low-grade squamous intraepithelial lesion i.e. L-SIL and high-grade squamous intraepithelial lesion i.e. H-SIL) and invasive ectocervical tumors (squamous cell carcinoma), as well as in biopsies of preinvasive skin tumors (actinic keratosis and Bowen's disease) and invasive skin tumors (squamous cell carcinoma) and preinvasive vulvar skin tumors (low-grade squamous intraepithelial lesion i.e. VIN-1, high-grade squamous intraepithelial lesion i.e. VIN-2 and VIN-3) and invasive vulvar skin tumors (squamous cell carcinoma).
3. To determine the genotype and allele frequencies of the rs2280883 and rs3761548 loci of the *FOXP3* gene in blood samples from subjects with preinvasive ectocervical tumors (low-grade squamous intraepithelial lesion, i.e. L-SIL and high-grade squamous intraepithelial lesion, i.e. H-SIL) and invasive ectocervical tumors (squamous cell carcinoma), as well as preinvasive skin tumors (actinic keratosis and Bowen's disease) and invasive skin tumors (squamous cell carcinoma) and preinvasive vulvar skin tumors (low-grade squamous intraepithelial lesion, i.e. VIN-1, high-grade squamous intraepithelial lesion, i.e. VIN-2 and VIN-3) and invasive vulvar skin tumors (squamous cell carcinoma) and their association with FOXP3 protein expression in the aforementioned preinvasive and invasive tumor tissues of the ectocervix, skin and vulvar skin.

## MATERIAL AND METHODS

### STUDY DESIGN:

A prospective, multicenter cohort study.

### PLACE AND PERIOD:

The study is conducted at the following institutions: the Clinic for Gynecology and Obstetrics, University Clinical Center of Serbia (UCCS), Clinic for Burns, Plastic and Reconstructive Surgery UCCS, Clinic for Otorhinolaryngology and Maxillofacial Surgery UCCS, Institute for Oncology and Radiology of Serbia, Clinic for Gynecology and Obstetrics “Narodni front”, Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade (FMUB), Institute of Pathology FMUB and Institute of Human Genetics FMUB.

The research period is from 2025 to 2026. Ethical approval was obtained from the Ethics Committee of the UCCS (decision number 1128/23 of July 3, 2025, amendment number 1500/48 of September 29, 2025), the Ethics Committee of the Institute of Oncology and Radiology of Serbia (decision number 01-1/25/2106 of July 11, 2025) and the Ethics Committee of the Gynecological and Obstetric Clinic "Narodni Front" (decision number 2208/2025/021590 of October 9, 2025). Additional consents were also obtained from: Clinic for Gynecology and Obstetrics UCCS (number 2380 dated June 9, 2025), Clinic for Burns, Plastic and Reconstructive Surgery UCCS (number 600 dated June 4, 2025), Clinic for Otorhinolaryngology and Maxillofacial Surgery UCCS (number 965 dated September 22, 2025), Institute of Oncology and Radiology of Serbia (number 01-1/25/2017 dated July 4, 2025), Gynecological and Obstetric Clinic "Narodni Front" (number 29023-2025-011731 dated June 12, 2025), Institute of Microbiology and Immunology FMUB (number 130 dated June 5, 2025), Institute of Pathology of the FMUB (number 02-582/25 of June 2, 2025) and the Institute of Human Genetics of the FMUB (number 108 of June 5, 2025).

#### SUBJECTS AND METHODS:

The study will include women aged 18 years and older who have undergone surgery for preinvasive (L-SIL and H-SIL) and invasive ectocervical tumors (squamous cell carcinoma); preinvasive (actinic keratosis and Bowen's disease) and invasive skin tumors (squamous cell carcinoma); as well as preinvasive (VIN-1, VIN-2, and VIN-3) and invasive vulvar tumors (squamous cell carcinoma). Written informed consent will be obtained from all study participants. The following data will be collected: 1) demographic data (age, occupation, place of residence, body mass index, smoking status and alcohol consumption); 2) anamnestic data (symptoms, duration of symptoms, previous therapies and interventions, first menstrual period, regularity of menstrual cycles, childbirth, miscarriages, sun exposure habits, surgical history, injuries, allergies, chronic diseases, family history of skin and gynecological tumors and chronic diseases); 3) dermatological and dermoscopy examination data (hair, skin, genital and oropharyngeal mucosa, and nail plates of hands and feet); 4) gynecological examination data (vulva inspection, speculum examination, bimanual and ultrasound examination, and Papanicolaou smear results); and 5) histopathological findings.

HPV detection and genotyping in cervical swab samples and biopsies of vulvar and skin tumors will be performed at the Virology Laboratory, Institute of Microbiology and Immunology, FMUB. After collection, swab and biopsy samples will be placed in sterile tubes containing saline solution. The extracted DNA will be stored at -20°C until further analysis. Following DNA isolation, HPV DNA will be amplified by real-time polymerase chain reaction (qPCR) using primers specific to the *E1* and *L1* viral genes. QPCR products will be analyzed by electrophoresis on a 2% agarose gel, using a DNA ladder as a molecular size standard and ethidium bromide for DNA visualization. HPV genotyping will be performed using direct Sanger sequencing. This process includes purification of qPCR products using the MiniElute PCR Purification Kit (QIAGEN, Germany) according to the manufacturer's instructions, followed by bidirectional cycle

sequencing using the ABI BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, USA), with the same primers used for qPCR amplification. Following purification with isopropanol and denaturation with formamide, the sequencing reaction products will be loaded onto an automated DNA sequencer (ABI PRISM 3500 Genetic Analyzer; Applied Biosystems, Foster City, USA). The sequences will be analyzed using Sequence Analysis 5.1 and SeqScape 2.5 software packages (Applied Biosystems, Foster City, CA, USA). HPV genotyping for each isolate will be conducted by comparing obtained sequences with reference sequences in the NCBI database (National Center for Biotechnology Information, USA) using the BLAST algorithm.

FOXP3 protein expression will be analyzed at the Institute of Pathology, FMUB, using immunohistochemical staining on formalin-fixed, paraffin-embedded tissue sections. Staining will be performed using anti-FOXP3 antibody (recombinant mouse monoclonal antibody, clone 236A/E7, Abcam, Chambridge, UK, USA), at a dilution of 1:100 and incubated at room temperature for 60 minutes. The reaction will be detected using the UltraVision Quanto HRP Detection System (Epredia, USA) with 3,3'-diaminobenzidine (DAB) as the chromogen. Immunohistochemically stained tissue sections will be evaluated using a light microscope by counting FOXP3-positive cells in five high-power fields (400x magnification). FOXP3 expressions will be quantified as the average number of FOXP3-positive cells per field of view.

For the analysis of *FOXP3* gene polymorphisms, whole blood samples (10 mL) anticoagulated with EDTA will be collected from all subjects by peripheral venipuncture and transported to the laboratory of the Institute of Human Genetics, FMUB. Genomic DNA will be isolated using the Miller salting-out method (23). Genotyping of the FOXP3 polymorphic loci rs2280883 and rs3761548 will be performed using qPCR, with commercially available TaqMan SNP Genotyping assays (Thermo Fisher Scientific, USA). This method uses allele-specific oligonucleotide probes labeled with distinct fluorophores, together with sequence-specific qPCR primers. Fluorescence emission from each probe indicates the presence of the corresponding allele in the sample, with results automatically recorded by the software. The analysis will be performed on an ABI 7500 RT PCR System (Life Technologies, USA), with primary data processing conducted by the integrated 7500 Software.

#### STATISTICAL ANALYSIS:

For an alpha significance level of 0.05 and a statistical power of the 1-beta test of 80%, the required total sample size was calculated to be 168 patients, of whom 25 were planned to have preinvasive or invasive vulvar skin tumors over the entire study period. As these samples are collected from two national reference centers for the surgical treatment of these tumors in Serbia, the study population is expected to include nearly all patients with preinvasive and invasive vulvar skin tumors. The sample was calculated based on data from previously published studies where HPV genotype frequency by localization was about 43% from cervical smears, 9% from skin biopsies and 43-60% from vulvar skin biopsies (7,24,25).

Data will be described using measures of central tendency (arithmetic mean and median), measures of variability (variation interval and standard deviation), relative numbers, and tabular and graphical presentations, considering data type and distribution. Normality of data distribution will be assessed using statistical (Shapiro-Willk test) and graphical methods (histogram and box plot). For comparisons between two independent groups, Student's *t*-test or the Mann–Whitney *U* test will be used for numerical variables, depending on data distribution, while the chi-square test or Fisher's exact test will be applied for categorical variables. Comparisons involving three or more independent groups will be conducted using the chi-square test, one-way analysis of variance (ANOVA) and Kruskal-Wallis test. Binary logistic regression analysis will be used to estimate the probability of belonging to one of the two comparison groups based on the examined factors. All statistical hypotheses will be tested at a significance level of 0.05, with results considered statistically significant if the p-value is less than 0.05.

Statistical analyses will be performed using IBM SPSS Statistics, version 31 (IBM Corporation, Armonk, NY, USA).

### **Candidate's assessment of potential scientific contribution**

#### **Assessment of the Candidate's Potential Scientific Contribution**

The planned research aims to analyze the presence of Human Papillomavirus infection in preinvasive and invasive tumors of the skin, vulva and ectocervix, with precise HPV genotyping performed through viral DNA sequencing. To investigate the role of regulatory T lymphocytes in the development and progression of these tumors, FOXP3 protein expression, a key marker of regulatory T-cell identity, will be analyzed in affected tissue. The study will also explore genetic determinants of FOXP3 expression, with the aim of identifying potential predisposing factors for tumor development. This research is significant not only due to its interdisciplinary nature, but also because it integrates virological, immunohistochemical, and genetic data with clinical and pathological findings. The collected data are expected to contribute significantly to understanding the etiology and pathogenesis of keratinocyte tumors of the skin and ectocervix, thereby enabling the development of more effective preventive and therapeutic strategies. In addition to its general scientific value, a key contribution of this research will be the identification of HPV types and *FOXP3* genotypes prevalent in our population. If an association between FOXP3 expression and *FOXP3* gene polymorphism is established, this finding could suggest novel therapeutic approaches for the treatment of these tumors. Identification of specific molecular profiles may facilitate the development of personalized treatment algorithms, providing both scientific and practical significance.

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MENTORS SIGNATURES

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