ASSOCIATION BETWEEN HUMAN PAPILLOMAVIRUS INFECTION AND ATYPICAL CERVICAL SQUAMOUS CELLS

UDRUŽENOST INFEKCIJE HUMANIM PAPILOMA VIRUSOM I ABNORMALNOSTI SKVAMOZNIH ĆELIJA GRLIĆA MATERICE

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Summary
Introduction. The aim of the study was to confirm the association between human papillomavirus infection and atypical cervical squamous cells. Material and Methods. This cross-sectional study, conducted in the period from January 2016 to June 2017, included 128 sexually active women, aged 20 to 59 years with squamous cell abnormalities of the cervical cytology, who came to their annual gynecological exam at the University Clinic of Gynecology and Obstetrics in Skopje. All patients underwent human papillomavirus testing and colposcopic cervical biopsy with endocervical curettage for histopathological analysis. Results. Data analysis showed an increase in the human papillomavirus infection alongside with cytological (p = 0.029296) and histopathological (p = 0.029443) increasing grades of cervical lesions. It showed an association between the oncogenic potential of the virus and the cytological (p = 0.000086) and histopathological (p = 0.00001) grades of cervical lesions. A human papillomavirus infection was detected in 75.00% of the examined women. The relationship between the prevalence of high-risk and low-risk human papillomavirus genotypes was 56.25%: 10.94%. Mixed human papillomavirus infection was detected in 32.03% of all patients, in 42.71% of human papillomavirus positive patients. The most common human papillomavirus genotypes, in descending order, were human papillomavirus-16 (43.75%), human papillomavirus-31 (15.62%), human papillomavirus-18 (10.4%), human papillomavirus-45 (9.37%), human papillomavirus-33 (7.29%), etc. Conclusion. This study has confirmed an association between human papillomavirus infection and squamous cell abnormalities of the uterine cervix. Young women under 30 years of age were the most affected group.

Key words: Papillomavirus Infections; Uterine Cervical Dysplasia; Squamous Intraepithelial Lesions of the Cervix; Polymerase Chain Reaction; Human Papillomavirus DNA Tests; Colposcopy

Sažetak
Uvod. Cilj studije bio je da se potvrdi postojanje udruženosti između infekcije humanim papiloma virusom i abnormalnosti skvamoznih čelija grlića materice. Materijal i metode. Studija preseka, sprovedena u periodu od januara 2016. do juna 2017. godine na 128 seksualno aktivnih žena, starosti od 20 do 59 godina, sa abnormalnostima skvamoznih čelija na cervikalnoj citologiji, koje su došle na godišnji gineko-losoški pregled na Univerzitetsku kliniku za ginekologiju i akušerstvo u Skoplju. Kod svih žena je urađeno testiranje infekcije humanim papiloma virusom i kolposkopska biopsija grlića materice sa endocervikalnom kiretažom za histopatološku analizu. Rezultati. Analiza podataka pokazala je povećanje prisustva infekcije humanim papiloma virusom paralelno sa povećanjem citopatološkog (p = 0.029296) i histopatološkog (p = 0.029443) stepena lezije grlića materice. Analiza podataka pokazala je udruženost između onkogenog potencijala virusa i citopatološkog (p = 0.000086) i histopatološkog (p = 0.00001) stepena lezije grlića materice. Infekcija humanim papiloma virusom otkrivena je kod 75% ispitanih žena. Odnos između prevalencije visokorizičnih i niskorizičnih genotipova humanih papiloma virusa iznosio je 56,25: 10,94%. Mesovita infekcija humanim papiloma virusom otkrivena je kod 32,03% od svih žena, odnosno 42,71% kod žena pozitivnih na humani papiloma virus. Najčešći genotipovi infekcije humanim papiloma virusom u opadajućem redosledu bili su: humani papiloma virus-16 (43,75%), humani papiloma virus-31 (15,62%), humani papiloma virus-18 (10,4%), humani papiloma virus-45 (9,37%), humani papiloma virus-33 (7,29%) itd. Zaključak. Postoji udruženost između infekcije humanim papiloma virusom i abnormalnosti skvamoznih čelija grlića materice. Žene mlađe od 30 godina bile su najugroženija starosna grupa.

Ključne reči: papiloma virusne infekcije; displazija grlića materice; skvamozne intraepitelijalne lezije grlića materice; PCR; HPV-DNK testiranje; kolposkopija

Introduction
Invasive cervical cancer is the fourth most common cancer in women and seventh most common cancer in general, with 527,624 new cases and 265,672 deaths in 2012, accounting for 7.5% of all cancer deaths in women [1]. According to the latest data from Global Cancer Observatory, Macedonia has an estimated incidence of
Cervical cancer of 12.4 per 100,000 inhabitants and it ranks 17th in Europe, which is close to the European average of 11.4 per 100,000 [2]. Squamous cell carcinoma of the cervix is the most common histological subtype of cervical cancer. About 90% of cervical cancer cases are squamous carcinomas, 10% are adenocarcinomas, and a small number are other types [3]. The occurrence of cervical cancer is preceded by various types of intraepithelial lesions including a series of progressive morphological changes, from productive human papillomavirus (HPV) infection/mild dysplasia to in situ carcinoma [4]. The most common risk factor for squamous cells abnormalities of the uterine cervix is HPV infection, especially with high-risk HPV genotypes. Only persistent, high-risk HPV infection represents a major risk factor for squamous cell abnormalities of the uterine cervix [5]. Deoxyribonucleic acid (DNA) from HPV has been found in 99.7% of cases of cervical carcinoma [6]. There are different classifications of HPV: by genetic similarity, by oncogenic potential and by affinity for certain tissues. According to their oncogenic potential, they are divided into high-risk and low-risk [7]. The prevalence of HPV genotypes varies by geographical regions. In Europe and North America, HPV-16 is still the most common high-risk genotype [8]. The population of young women, from 18 to 25, has the highest rates of HPV infection. After the age of 25 years, the incidence of HPV infection is reduced to 0.03% [9]. Detection of HPV can be done using two methods; the first one is direct hybridization or in situ hybridization, and the other is amplification or polymerase chain reaction (PCR) [10]. The aims of the study were to confirm the association between HPV infection and squamous cells abnormalities of the uterine cervix, detection and typing HPV genotypes, which are the most common causes of intraepithelial lesions and cervical cancer, to determine the prevalence of HPV infections and the most affected age groups.

**Material and Methods**

This cross-sectional study included 128 sexually active women aged 20 to 59 years, with abnormal cervical cytological findings, i.e. a finding of Panicolaou (PAP) test showing a squamous intraepithelial lesion or invasive squamous cervical cancer. The study did not include pregnant women, women with previous surgery of the uterine cervix (cervical conization, carbon dioxide laser vaporization and total abdominal hysterectomy) as well as previous abnormal cytological and histopathological findings of the uterine cervix.

The study was conducted in the period from January 2016 to June 2017.

The study was conducted at the University Clinics of Gynecology and Obstetrics and Radiotherapy and Oncology in Skopje, Republic of Macedonia.

All women underwent HPV testing and colposcopic cervical biopsy with endocervical curettage for histopathological analysis.

All samples for cytological analysis were taken using the ThinPrep PAP test and were analyzed in the cytological laboratory at the University Clinic of Gynecology and Obstetrics in Skopje by a cytopathologist. Cytological results were classified according to the revised Bethesda classification [11, 12] including atypical squamous cells of undetermined significance (ASC-US); atypical squamous cells, cannot exclude a high-grade squamous intraepithelial lesion (ASC-H); low grade squamous intraepithelial lesion (LSIL), cervical intraepithelial neoplasia grade 1 (CIN1); high grade squamous intraepithelial lesion (HSIL), cervical intraepithelial neoplasia grade 2 (CIN2), cervical intraepithelial neoplasia grade 3 (CIN3), carcinoma in situ (CIS) and invasive squamous cell carcinoma.

Samples for histopathological analysis were taken at the University Clinic of Gynecology and Obstetrics in Skopje and were analyzed at the University Clinic of Radiotherapy and Oncology in Skopje, at the Department of Histopathology and Clinical Cytology by an experienced histopathologist. According to the morphology determined in biotic samples, cervical findings were characterized as normal findings (non-specific cervicitis); LSIL (cervicitis chronica virosa, flat condyloma, mild dysplasia); HSIL (moderate dysplasia, severe dysplasia, in situ squamous cell carcinoma) and invasive squamous cell carcinoma [13].

Cervical biopsy samples were taken for HPV testing and analyzed at the University Clinic of Gynecology and Obstetrics in Skopje, at the Laboratory for HPV testing. HPV detection and typing were done using multiple polymerase chain reaction (Multiplex PCR) and reverse hybridization. The results of the HPV test were analyzed and demonstrated based on the presence or absence of DNA from HPV and the specified genotype [14]. The first step in HPV testing was the isolation of DNA from the collected cells from cervical biopsies. For isolation of DNA series, three paraffin cuts were prepared. The cuts were incubated in 1 ml xylene for 5 minutes at 55°C and centrifuged at 10,000 G for 5 minutes at room temperature. The same procedure was repeated two more times. After careful removal of the remains of xylene, the samples were briefly incubated twice in 1 ml of 100% ethanol, and centrifuged for 5 minutes at room temperature. After removal of ethanol and a short air dry, the cuts were incubated overnight in a buffer with freshly added protease K at 55°C. The second step was the detection of DNA in HPV by using
PCR. To verify the quality and integrity of the isolated DNA, actually of a present inhibitor, a reaction of multiplication of primers for beta globin PC04 and GH20 was first made for each sample. Three pairs of primers were used, common to a larger number of HPV types: degenerate beginners My09/My11 and CPI/CPII G and Gp5/6+. The samples were carried through all reactions with primers specific to high-risk and low-risk HPV genotypes. The third step was genotyping by using reverse hybridization. It is a method that is based on the hybridization of specific DNA probes that are immobilized on nitrocellulose or nylon tapes. It is a set of beginners (SPF 10) with aim-propagation of the L1 gene on the viral DNA. The product of amplification with SPF beginners of 65 bp allows detection of 25 new genotypes. Denatured biotinylated PCR products are hybridized with specific oligonucleotide probes that are immobilized on nitrocellulose or nylon tapes. It is a set of beginners of 65 bp allows detection of 25 new genotypes. Denatured biotinylated PCR products are hybridized with specific oligonucleotide probes that are immobilized as parallel lines on membrane strips. After hybridization and washing with streptavidin, alkaline phosphatase is added, which binds to the biotinylated hybrids formed previously. Incubation with BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/NBT (nitroblue tetrazolium) chromogens give purple precipitate (5-bromo-4-chloro-3-indolyl-phosphate)/NBT (nitroblue tetrazolium) chromogens give purple precipitate.

Data analysis was done using the Excel database. Statistical analysis of the established statistical series was done by the statistical package for the social sciences, version 23.0. The structure of numerical signs was done by the statistical package for the social sciences, version 23.0. The structure of numerical signs was analyzed by determining the measures of central tendency (arithmetic mean) and measures of dispersion (standard deviation).

The analysis of the relationship (existence of association) between two sets of attribute variables was performed using the Chi-square test. Statistical significance was defined as p value <0.05.

Results

Of the 128 examined patients, aged 20 to 59 years (40.50 ± 10.85), 28 (21.87%) were aged 20 – 29; 38 (29.69%) 30 – 39 years; 30 (23.44%) 40 – 49 years, and 32 (25.00%) were aged 50 – 59 years.

The distribution of HPV infection in 128 patients, correlated with cytopathological diagnosis, is shown in Table 1.

Data analysis showed an increase of HPV infection with an increase in the cytopathological grade of the cervical lesion. There were 46.15% (6/13) of samples with ASC-US, 57.14% (4/7) with ASC-H, 70.97% (22/31) with LSIL, 80.36% (45/56) with HSIL, and 90.48% (19/21) with invasive squamous cell carcinoma (chi-square test = 10.7682, p = 0.029296, p < 0.05).

Data analysis showed an association between the oncogenic potential of the virus and the cytopathological grade of cervical lesions (chi-square test = 23.8298, p = 0.000086, p < 0.05).

Distribution of HPV infection in 128 patients in correlation with histopathological diagnosis is shown in Table 2.

HPV infection was detected in 75.00% (96/128) of studied patients. The lowest percentage was observed in LSIL – 63.41% (26/41), with an increase to 83.33% (45/54) in HSIL and 87.50% (21/24) in invasive squa-

Table 1. Correlation between the HPV infection and cytopathologic diagnosis

| HPV infection | Cytopathologic diagnosis/Citopatološka dijagnoza | H | LSIL | HSIL | In situ squamous cell carcinoma | Total |
|---------------|-----------------------------------------------|---|------|------|---------------------------------|-------|
| HPV negative  | (n = 31)                                       |   |      |      |                                |       |
| ASC-US        | 7 (58.05)                                      | 3 | 9 (30.00) | 4 (44.44) | 3 (33.33) | 2 (18.18) | 32 (25.00) |
| (n = 13)      |                                              |   |      |      |                                |       |
| HPV negative  | (n = 31)                                       |   |      |      |                                |       |
| ASC-H         | 6 (46.15)                                      | 4 | 22 (70.97) | 16 (51.61) | 17 (58.97) | 12 (40.62) | 19 (61.29) |
| (n = 7)       |                                              |   |      |      |                                |       |
| HPV positive  | (n = 31)                                       |   |      |      |                                |       |
| H-R HPV positive/Visko rizične HPV pozitivne | 0 (0.00) | 3 (10.00) | 12 (38.71) | 11 (35.48) | 17 (54.84) | 12 (39.39) | 48 (37.50) |
| (n = 20)      |                                              |   |      |      |                                |       |
| L-R HPV positive/Niskovizične HPV pozitivne | 4 (13.33) | 1 (3.23) | 2 (6.45) | 0 (0.00) | 0 (0.00) | 0 (0.00) | 11 (8.57) |
| (n = 21)      |                                              |   |      |      |                                |       |
| H-R and L-R HPV positive/Visko rizične i niskovizične HPV pozitivne | 2 (6.67) | 0 (0.00) | 6 (19.35) | 3 (9.68) | 0 (0.00) | 1 (3.23) | 12 (9.37) |
| (n = 21)      |                                              |   |      |      |                                |       |

Legend: n - number; ASC-US - atypical squamous cells of undetermined significance; ASC-H - atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion; LSIL - low-grade squamous intraepithelial lesion; HSIL - high-grade squamous intraepithelial lesion; CIN - cervical intraepithelial neoplasia; HPV - human papillomavirus; H-R - high-risk; L-R - low-risk; SPF - single primer; CPI/CPII - degenerate primers; My09/My11 - degenerate primers; CIPI/CII - degenerate primers; Gp5/6 - degenerate primers; In situ squamous cell carcinoma - in situ squamous cell carcinoma; Total - total number of patients; CYT-05 - cytology; HPV - human papillomavirus; H-R - high-risk; L-R - low-risk; SPF - single primer; CPI/CPII - degenerate primers; My09/My11 - degenerate primers; CIPI/CII - degenerate primers; Gp5/6 - degenerate primers; in situ squamous cell carcinoma - in situ squamous cell carcinoma; Total - total number of patients.
Table 2. Correlation between the HPV infections and histopathological diagnosis

| HPV infection | Histopathological diagnosis | Total |
|---------------|-----------------------------|-------|
|               | Normal finding              |       |
|               | HPV negativne (n = 9)        |       |
|               | Cervicitis                  |       |
|               | Flat condyloma/disparadavica|       |
|               | Mild dysplasia/Blaga         |       |
|               | Moderate dysplasia/Umerekena|       |
|               | Severe dysplasia/Teška dysplazija|   |
|               | In situ squamous cell/carcinoma/In situ skvamozni karcinom (n = 24) |       |
|               | HPV pozitivne (n = 72)       |       |
|               | HPV negative (n = 32)        |       |
|               | HPV positive (n = 32)        |       |
|               | H-R HPV positive (n = 20)    |       |
|               | L-R HPV positive (n = 16)    |       |
|               | Mixed HPV infection (n = 23) |       |
|               | H-R and L-R HPV positive (n = 12) |       |
|               | Visokorizične i niskorizične HPV pozitivne (n = 20) |       |

Legend: n - number; LSIL - low-grade squamous intraepithelial lesion; HSIL - high-grade squamous intraepithelial lesion; HPV - human papillomavirus; H-R - high-risk; L-R - low-risk

Data analysis showed an association between the HPV infection and the appearance of squamous cell abnormalities of the uterine cervix (chi-square test = 4.8204, p = 0.028125, p < 0.05). The prevalence of high-risk and low-risk HPV positive tests was 56.25%: 10.94% (i.e. 75.00%: 14.58% among HPV positive patients).

The analysis also showed an association between the oncogenic potential of the virus and the histopathological grade of cervical lesion (chi-square test = 31.5089, p = 0.00001, p <0.05). The incidence of HPV infection was 85.71% (24/28) in patients aged 20 – 29 years; 65.79% (25/38) in 30 – 39 years; 73.33% (22/30) in 40 – 49 years, and 78.12% (25/32) in patients aged 50 – 59 years (Table 3).

A single HPV infection was detected in 42.97% (55/128) of all patients (i.e. in 57.29% of HPV positive patients). The most common single HPV infection was high-risk HPV in 53.12% (51/96) (Table 4).

Mixed HPV infection was detected in 32.03% (41/128) of all patients (i.e. 42.71% of HPV positive patients). The most common co-infection was high-risk HPV: 22.45% (22/96). Co-infection with high-risk and low-risk HPV was found in 11.46% (11/96), and co-infection with high-risk and low-risk HPV in 53.12% (51/96).

Table 3. Age group distribution of HPV infection in 128 patients

| HPV infection | Age groups/Starosne grupe |
|---------------|---------------------------|
|               | 20–29 | 30–39 | 40–49 | 50–59 |
| HPV positive/HPV pozitivne | 24 (85.71) | 25 (65.79) | 22 (73.33) | 25 (78.12) |
| HPV negative/HPV negativne | 4 (14.29) | 13 (34.21) | 8 (26.67) | 7 (21.88) |
| Total/UKupno | 28 (100) | 38 (100) | 30 (100) | 32 (100) |

Legend: n - number; HPV - human papillomavirus/Legend: n - broj; HPV - humani papiloma virus
Table 4. Correlation between single and mixed HPV infections and histopathological diagnosis in 96 HPV positive patients

| Type of HPV infection | Histopathological diagnosis | Invasive squamous cell carcinoma | Total (
| | Normal finding | LSIL (n = 26) | HSIL (n = 45) | Ukupno (n = 96) |
| --- | --- | --- | --- | --- |
| Single infection | | | | |
| Poredinačna infekcija | Cervicitis niskorizična (n = 13) | 1 (21.05) | 1 (21.43) | 2 (21.43) |
| | Flat condyloma dysplasia niskorizična (n = 2) | 0 (18.18) | 0 (18.18) | 0 (18.18) |
| | Mild dysplasia niskorizična (n = 6) | 2 (7.00) | 3 (21.43) | 5 (21.43) |
| | Moderate dysplasia niskorizična (n = 13) | 3 (23.08) | 13 (8.33) | 16 (8.33) |
| | Severe dysplasia niskorizična (n = 19) | 3 (16.67) | 3 (16.67) | 6 (16.67) |
| | In situ squamous cell carcinoma niskorizična (n = 14) | 6 (28.57) | 11 (78.57) | 17 (78.57) |
| | Mixed infection | | | |
| Mešovita infekcija | Cervicitis niskorizična (n = 4) | 1 (100) | 1 (100) | 2 (100) |
| | Flat condyloma dysplasia niskorizična (n = 2) | 0 (0) | 0 (0) | 0 (0) |
| | Mild dysplasia niskorizična (n = 6) | 0 (0) | 0 (0) | 0 (0) |
| | Moderate dysplasia niskorizična (n = 13) | 3 (23.08) | 3 (23.08) | 6 (23.08) |
| | Severe dysplasia niskorizična (n = 19) | 0 (0) | 0 (0) | 0 (0) |
| | In situ squamous cell carcinoma niskorizična (n = 14) | 11 (78.57) | 11 (78.57) | 22 (78.57) |

Legend: n - number; LSIL - low-grade squamous intraepithelial lesion; HSIL - high-grade squamous intraepithelial lesion; HPV - human papillomavirus; H-R - high-risk; L-R - low-risk

HPV was detected in 8.16% (8/96). In correlation with histopathological diagnosis, the prevalence of mixed HPV infections was 75.00% (3/4) in normal findings, 61.54% (16/26) in LSIL, 33.33% (15/45) in HSIL and 33.33% (7/21) in invasive squamous cell carcinoma (Table 4).

Data analysis showed that mixed HPV infections are the most frequent in patients under 30 years of age (58.33%; 14/24) (Table 5).

Table 5. Age group distribution of single and mixed HPV infections in 96 HPV positive patients

| HPV infection | Age groups/Starosne grupe |
| --- | --- |
| | 20–29 | 30–39 | 40–49 | 50–59 |
| Single/Poredinačna | n (%) | n (%) | n (%) | n (%) |
| Mixed/Mehanja | n (%) | n (%) | n (%) | n (%) |
| Total/Ukupno | n (%) | n (%) | n (%) | n (%) |

Legend: n - number; HPV - human papillomavirus; H-R - high-risk; L-R - low-risk

Table 6. HPV genotypes in single and mixed HPV infections in correlation with histopathological diagnosis is shown in Table 5.

Among high-risk HPV genotypes, HPV-16 was the most common (43.75%; 42/96), followed by (in descending order) HPV-31 (15.62%; 15/96), HPV-18 (10.4%, 10/96), HPV-45 (9.37%, 9/96), HPV-33 (7.29%, 7/96), HPV-35, -52 and -56 (5.21%; 5/96), etc. Among the low-risk HPV genotypes, the most common was...
HPV-6 (14.58%; 14/96), followed by HPV-11 (9.37%; 9/96). HPV-16 was most common in patients with HSIL and invasive squamous cell carcinoma, while HPV-6 in patients with LSIL (Table 6).

**Discussion**

In 1976, Harald zur Hausen published his hypothesis about the probable association of cervical cancer

| HPV genotype | Type of HPV infection | Histopathological diagnosis | Total |
|--------------|-----------------------|-----------------------------|-------|
| Normal | Cervicitis | LSIL (n = 26) | HSIL (n = 45) | Invasive squamous cell carcinoma | Total |

**Table 6.** Prevalence of HPV genotypes in regard to histopathological diagnosis

Legend: n - number; LSIL - low-grade squamous intraepithelial lesion; HSIL - high-grade squamous intraepithelial lesion; HPV - human papillomavirus

Legend: n - broj; LSIL - skvamozna intraepitelijalna lezija niskog stepena; HSIL - skvamozna intraepitelijalna lezija visokog stepena; HPV - humani papiloma virus
and intraepithelial lesions with identical cause (HPV), which also causes hyperproliferative changes in the genital tract [15]. In 1996, the World Health Association recognized the importance of HPV for cervical cancer [6]. Early detection and treatment of squamous cell abnormalities of the uterine cervix can be crucial in the prevention of cervical cancer [16]. About 75% of the sexually active population has been in contact with one or more HPV genotypes in the course of their lives [17]. Depending on the geographical region, the study population and the method used, the frequency of HPV genotypes varies considerably in various cervical lesions. In this study, HPV infection was detected in 75% of the examined women. This relatively high percentage of HPV infection in women with squamous cell abnormalities of the uterine cervix corresponds with some previously published studies; in the study of Mazarico et al. (2012), HPV infection was detected in 73.20% of women with squamous cell abnormalities of the uterine cervix [18], while in the study of Pista et al. (2013), HPV infection was detected in 77.4% of studied women [19]. HPV-16 was the most common genotype accounting for 43.75%. In addition to HPV-16, the most common genotypes were HPV-31 (15.62%), HPV-6 (14.58%), HPV-18 (10.41%), HPV-45 and HPV-11 (9.37%), HPV-33 (7.29%) and HPV-35, -52, -56 (5.21%). The retrospective study of Andonovska J. (2014) that included 7,411 women, detected the following distribution of the most common genotypes: HPV-16 (23.39%), HPV-31 (10.68%), HPV-53 (10.60%) and HPV-18 (6.19%) [20]. The study of Stojanovska V. et al. (2009) included 6,988 patients and established the following distribution of the most common genotypes: HPV-16 (32.1%), HPV-31 (14%), HPV-53 (12.6%), HPV-18 (9.9%), HPV-58 (5%), etc. [21], whereas the study of Duvlis S. (2000) included patients from Macedonia and detected the following distribution of the most common genotypes: HPV-16 (27.5%), HPV-31 (13.1%), HPV-66 (10.3%), HPV-6 (9.4%), HPV-18 (8.4%), etc. [22]. In these four studies, the most common HPV genotypes were HPV-16 and HPV-31, but there were discrepancies in the distribution of other most common HPV genotypes. In this study, a significant association was found between the HPV infection and the incidence of squamous intraepithelial lesions and squamous invasive cervical cancer (p = 0.028125). High percentage of high-risk HPV genotypes found in severe dysplasia (73.91%), in in situ squamous cell carcinoma (87.50%) and invasive squamous cell carcinoma (83.33%) once again confirmed a strong relationship between the oncogenic potential of the virus, the development of squamous intraepithelial lesions, and squamous invasive carcinoma of the cervix (p < 0.00001). The relationship between high-risk and low-risk HPV-genotypes in HPV positive patients was 75.00%:14.58%. In the Spanish study conducted by Garcia-Garcia in 2010, the ratio was 79.80%:19.70% [23]. The high percentage of HPV-16 in severe dysplasia (63.16%), in in situ squamous cell carcinoma (57.14%) and invasive squamous carcinoma (47.62%), distinguishes HPV-16 as a high risk genotype with the highest oncogenic potential. In this study, mixed HPV infection was found in 32.03% of patients, i.e. in 42.71% of HPV-positive DNA. This prevalence of mixed HPV infections corresponds with previously published studies of Sandri et al., 2009 (43.00%) [24], Cuschieri et al., 2001 (43.30%) [25], and Vujošević et al., 2012 (42.00%) [26]. The highest prevalence of HPV infection was found in patients under 30 years of age (85.71%), and the lowest in patients aged 30 to 39 (65.79%). Our results are in agreement with the results of some previously published studies: Italian study of Agodi et al., 2009, where the highest prevalence of HPV infection was detected in patients under the age of 25 (73.2%) [27]; American study of Evans, 2006, showed the highest prevalence of HPV infection in patients under the age of 30 (96.00%; 458/475) [28]; the same percentage of HPV infection (96.00%) was also found by Hariri et al., 2012, in a study that included 3,058 American women aged 18 to 39 [29]. The highest prevalence of mixed HPV infection was detected (58.33%) in the group of patients under 30 years of age. A high prevalence in the young population can be explained by their sexual behavior, that is, promiscuity. A Serbian study by Kovačević G. et al., 2016, including students from the University of Novi Sad found mixed HPV infection in 49.4% [30]. Relatively high rates of mixed HPV infection among the young population were also detected in the Bulgarian studies of Grozdanov P., 2014 (63.00%) [31] and Kovachev S., 2013 (53.00%) [32], as well as in the Romanian study of Moga M., 2014 (54.93%) [33].

**Conclusion**

This study has confirmed that there is an association between human papillomavirus deoxyribonucleic acid infection and atypical cervical squamous cells; the young population under 30 years of age is the most affected, and human papillomavirus-16 is the most common genotype in our environment.

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