

Molecular-Epidemiological Study for 19 High- and Medium- Risk HPV Types in Bulgarian Patients

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Abstract: Cervical cancer ranks as the 4th leading cause of female cancer and cancer deaths in the world. More than 99% of cervical cancer cases are due to HPV infection. Vaccination can significantly reduce the number of HPV-related cancers in the future. The next big step is to change the order of the tests, i.e. cervical cells are first tested for HPV, and then analyzed under a microscope. The objective of our study was to define the incidence of 19 types of HPV in Bulgarian women attended for routine gynecologic examination, in order to obtain the molecular-epidemiological spectrum of this infection in the context of the actual preventive programs. We have used the medium-high risk PapillomaStrip kit, based on the principle of reverse hybridization, in samples of 151 Bulgarian women. We detected the total frequency of 44.4% for HPV infection and higher frequency in younger age group (<30 years – 47.5% and 30-40 years – 50%) compared to > 40 years old women (28%). The most frequent HPV types from our study were HPV 45 and 52 (10.6%), HPV 66 (8.6%) and HPV 31 (8%). In PAPI/II it was HPV52 (11.3%), followed by HPV45 (10.5%) and HPV66 (8.3%). In PAPIII the most common was HPV16 (44.4%), followed by HPV 45, 31, 66 and 59 (each of 22.2%). We emphasized on the high frequency of HPV45 in our study, which is reported as one of the most frequent types in cervical cancer. In conclusion, HPV genotyping provides information on the carriage of high-risk types and increased attention to these patients. This is a valuable information for planning preventive programs in regard to choosing diagnostic tests and vaccination. Our results revealed the wide spectrum of HPV infection in Bulgarian women, as about 70% of infected patients carry high-risk HPV 45, 52, 31, 16 and 18.

Keywords: HPV Types, Molecular Epidemiology, HPV Vaccine

Introduction

Cervical cancer ranks as the 4th leading cause of female cancer and of female cancer deaths in the world. More than 99% of cervical cancer cases are due to HPV infection, highlighting the infectious nature of this cancer type [Munoz N et al, 2006]. The genome of the virus interacts with the genome of the host cell and triggers the disease.

Age-standardized incidence rate of cervical cancer cases attributable to HPV by country in Europe (estimated 2012) showed 3rd place for Bulgaria, after Rumania and Ukraine, with 24.5 affected women per 100 000 [Bruni L et al, 2017]. This is a higher incidence, compared to average numbers per 100 000 in the world (14.0 per 100000), as well as to the number in the less developed (15.7 per 100000) and in the more-developed regions (9.9 per 100000). Detailed sub-regions' statistics revealed the similar incidence in Central America (23.5 per 100000), whereas the average number of affected women per 100 000 is only 7.3 in Western Europe, 8.5 in Southern Europe and 16.3 in Eastern Europe. All over the World, cervical cancer is the 4th of the most

frequent cancers in women; in the developing regions it is the 2nd cancer type, but it is the 6th in the more developed regions. In Bulgaria, it is the 4th most frequent cancers in women after breast, endometrial and colon cancers. This cancer type is the 4th cause for cancer-related death in the world, but 3rd – in the developing countries and 7th – in the more developed regions.

After discovering of HPV 16 и 18 by Harald zur Hausen in more than 50% of cervical cancers in 1984 [Boshart M et al, 1984], in the next 15 years it has been established that 99,7% of cervical cancer cases are due to HPV infection. Cytological analysis was the first screening test introduced in 1988 for prophylaxis of cervical cancer and by this test about 3/4th of cancer cases were prevented [Safaeian M et al, 2007]. In 2008 the first HPV vaccine was developed in the Prof. Lionel Crawford's lab [HPV immunisation uptake statistics, 2018]. Vaccination can significantly reduce the number of HPV-related cancers in the future. But not everybody gets a vaccination, so there are still people who are without protection. In addition, the vaccine does not protect

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against all types of HPV causing cancer. So, there is an unquestionable need of research to give us certainty that we are doing our best to prevent HPV-related cancer. How can the current program be improved? The next big step is to change the order of the tests, ie. cervical cells are first tested for HPV, and then analysed under a microscope. This little change may be of great importance: research shows that it can save more lives than previous programs. This approach is pilot for the moment, but many countries are campaigning to make it a reality in the future. Professors Jack Cuzick and Anne Szarewski have been re-testing HPV cervical samples to shed light on the issue. Women with borderline or very low abnormal cells in the cervix, which are at the same time infected with high-risk HPV types, have an increased risk of cancer and should be referred for further testing and treatment. While the risk for non-infected women is much lower and can make normal screening every 3 years. This so-called 'HPV triage' is now a routine part of cervical screening in many countries [Tota JE et al Canfell K et al, 2017; Lew JB et al, 2017;].

One of the main difficulties in detecting HPV and characterizing tumors is the high polymorphic nature of HPV virus (over 200 genotypes and variants) as well as the sporadic nature of viral integration in the host cell genome. There are at least 12 types of high-risk oncogenic HPVs – 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59; in addition, probable/possible carcinogens and non-oncogenic HPV types exist. Molecular epidemiology is a branch of epidemiology which uses molecular methods in epidemiological studies. It dissolves many issues connected to causation, pathogenesis, transmission and circulation of the infectious agent, its source/reservoir, as well as the basics for vaccine development and drug therapy.

The objective of our study was to define the incidence of 19 types of HPV in Bulgarian women attended for routine gynecologic examination, in order to obtain the molecular-epidemiological spectrum of this infection in the context of the actual preventive programs.

Materials and methods

Subjects

We have collected cervical swabs samples from gynecological patients (n= 151). Incidence and distribution of different HPV types were studied in three age groups (Group 1 - under 30 years; Group 2 - 30-40 years and Group 3 - above 40 years old women) and in different PAP groups (PAP I/II and PAP III).

The participants were recruited at the Medical Complex for Obstetrics and Gynecology “D-r Shterev”, Sofia, Bulgaria. All of them declared their written informed consent and were familiar with the

aims, methods and risks of participating in the study in accordance with the Helsinki Declaration and rules of Good Clinical Practice, as the study was approved by the Ethics Committee of the Medical University, Sofia.

HPV genotyping

The cervical swabs of patients were investigated. Whole genome DNA was extracted using DNeasy Blood & Tissue Kit, Qiagen. Genotyping for HPV was done by using PapillomaStrip test (Operon, S.A). The test is based on the reverse dot blot. It consists of a nylon membrane where a series of DNA probes specific for the following HPV types are attached - 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73 and 82.

We have used the above-mentioned Medium-high risk PapillomaStrip kit, based on the principle of reverse hybridization and allows for the detection and identification of 19 genital HPVs in DNA samples from cervico-uterine smears and biopsies. After DNA extraction/purification, an amplification of regions E6-E7 from the 19 HPVs (using specific primers for each of them) was performed (38 µl of PCR premix + 5 µl of primers + 2 µl of Taq + 5 µl of DNA). The following program was used: 94°C – 5 min; 40 cycles of 94°C – 1 min, 58°C – 1 min, 72°C – 1 min; 72°C – 5 min; 4°C holding. The analysis of the PCRs amplified with medium-high risk primers was conducted using the HIGH PapillomaStrip strip. Probes for each HPV detected are covalently linked to the membrane, along with a probe for the amplification control (GAPDH or β-globine), a probe for the developing control and two or three lines, one black and one or two red, to control the positioning of the strip and as an aid in the interpretation of results. The detection of the fragments hybridized to different probes is performed using a conjugate (streptavidine-peroxidase) that binds to a biotin label that is added to the DNA fragments amplified during the PCR. Following the addition of a substrate for the peroxidase (TMB) a blue precipitate generates where hybridization has occurred. As final result of the test, a band pattern is obtained which is interpreted with the aid of a control strip.

Results

Totally, 151 cervical samples were analysed for the presence of HPV and 67 of them (44.4%) were found HPV positive. There was a tendency for higher frequency of HPV positivity in younger age group (Group 1 – 47.5% and Group 2 – 50%) compared to > 40 years old women (28%), not reaching statistical significance – Figure 1. Totally, 92 HPV infections were detected in the positive cervical samples – the number of HPV infections overload the number of samples, because 17 of the samples (11.2%) had more than one HPV types simultaneously: 11

samples (7.3%) with double infection, 4 samples (2.6%) with triple infection and 2 samples (1.3%) with multiple HPV infection.

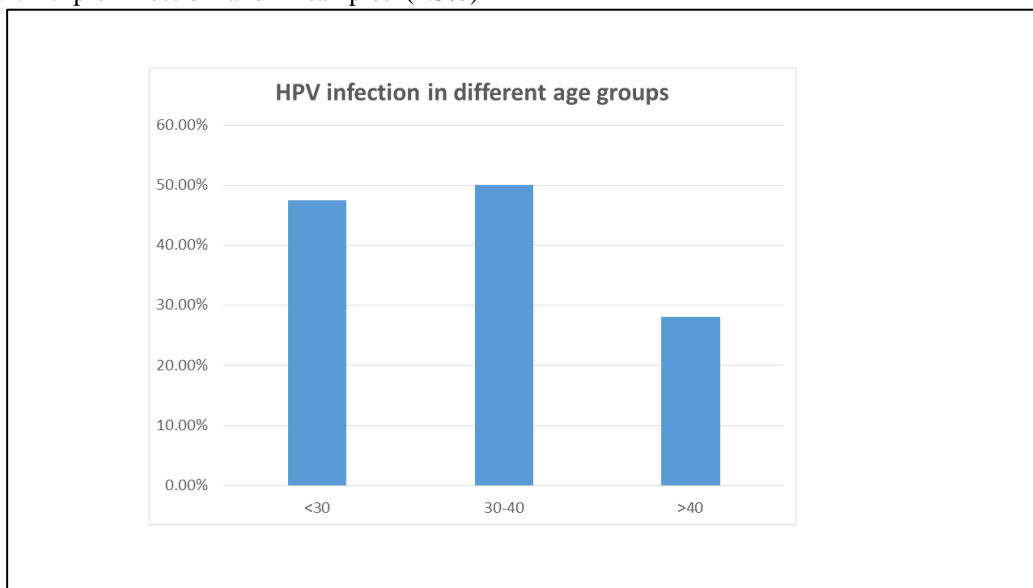


Figure 1. The incidence of HPV infection in different age groups

Figure 2 represents the frequency of different HPV types among all samples. The most prevalent HPV types in all samples were HPV 45 and 52 (10.6%), followed by HPV66 (8.6%) and HPV31 (8 %). From all 15 HPV types, detected in our study, five types are responsible for 70% of positive cases (Figure 3): HPV45 (17%), HPV 52 (17%), HPV66 (14%), HPV31 (13%) and HPV16 (9%).

There was prevalence of HPV 45 in all age groups and slight difference in other HPV incidence. HPV 45 and 66 were the most frequent in Group 1 (12.6%) and HPV 16, 18, 31 and 52 were the second more frequent (8.4%) in this group (Figure 4). In Group 2 the frequency was 14% for HPV 45 and 10% for HPV31 and 66, whereas in women > 40 years only three types of HPV were detected – HPV45 (15%), HPV66 (10%) and HPV58 (5%).

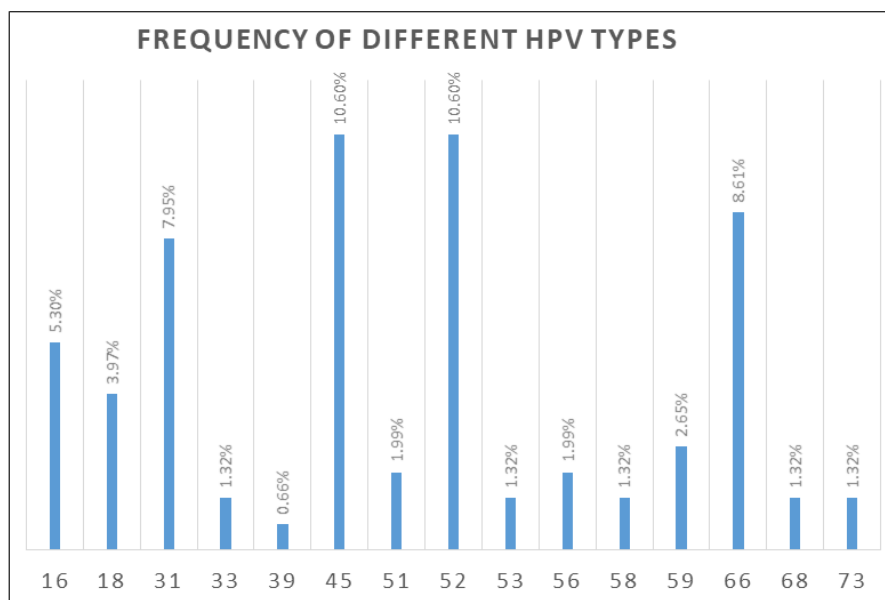


Figure 2. The incidence of different HPV types in our cohort

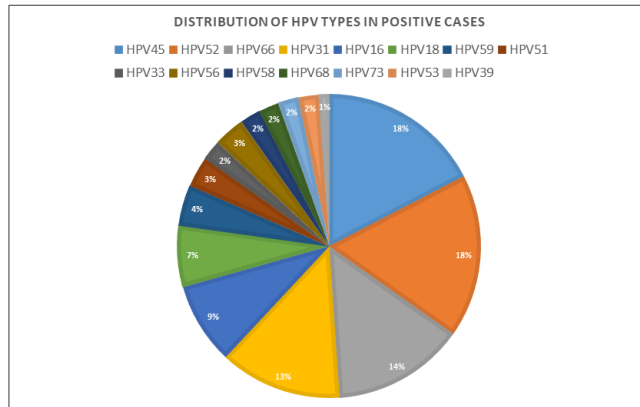


Figure 3. The distribution of different HPV types among all positive cases

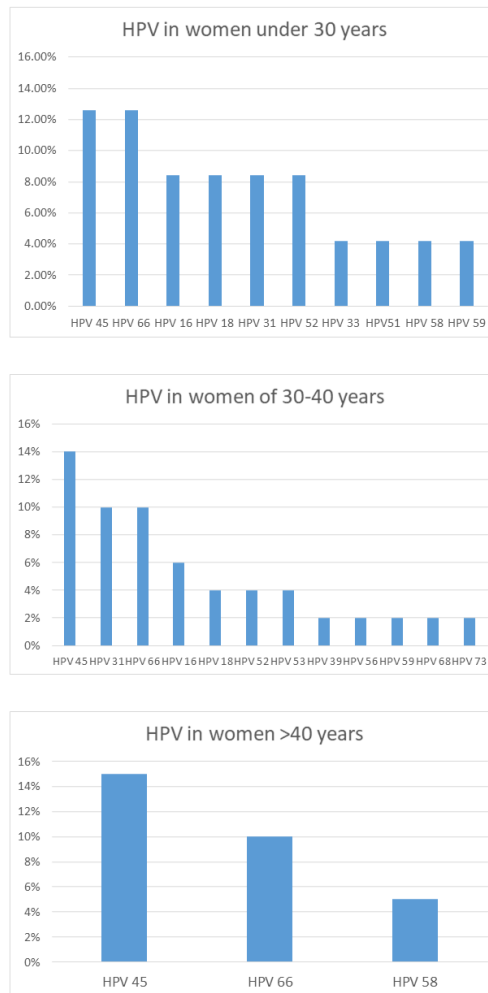


Figure 4. Frequency of different HPV types in different age groups

We analysed the frequency of HPV presence in different PAP groups – PAP I/II and PAP III. There was statistically significant difference in HPV positivity between these groups – 100% of PAP III samples were HPV positive versus 40.6% of PAP I/II followed by HPV 45, 31, 66 and 59 (each of 22.2%) – Figure 5.

samples ($P= 0.05$). The most frequent HPV type in samples of PAPI/II was HPV52 (11.3%), followed by HPV45 (10.5%) and HPV66 (8.3%). In samples of PAPIII the most common was HPV16 (44.4%),

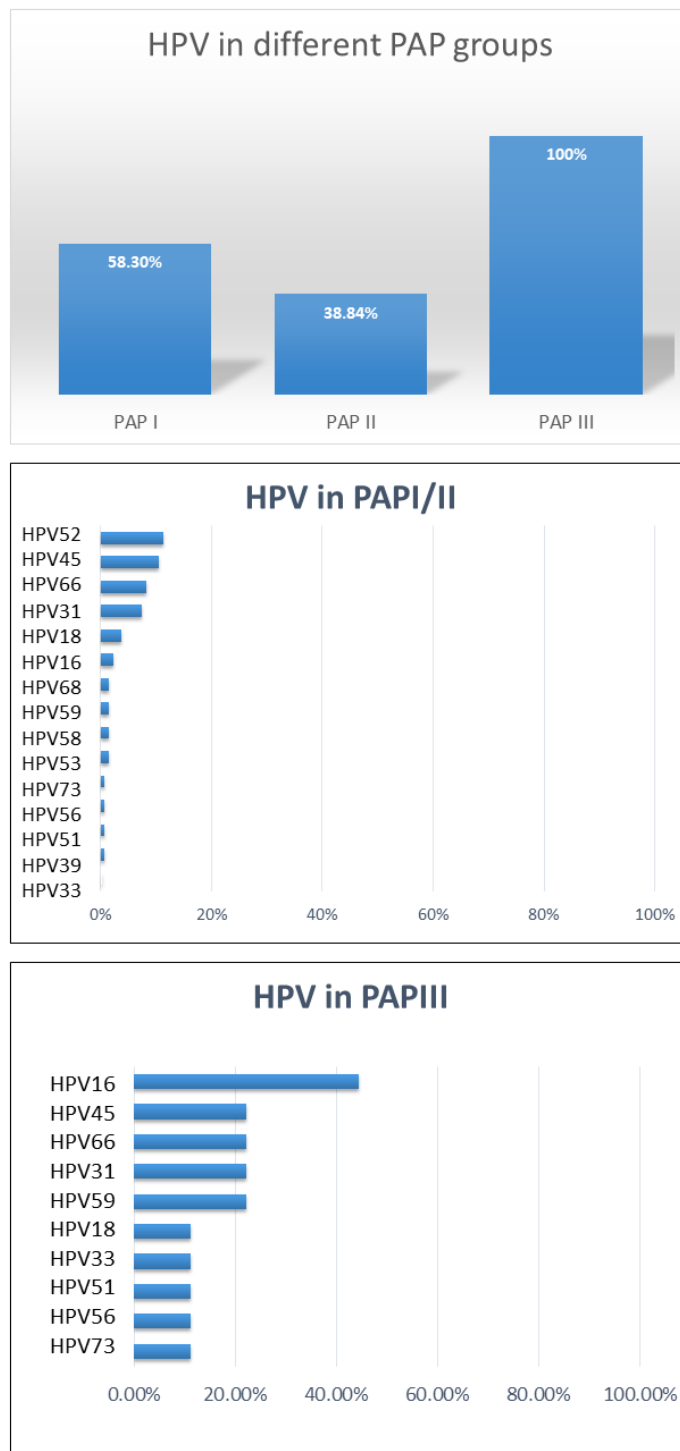


Figure 5. The frequency of different HPV types in PAP groups

Discussion

In our study, we detected the total frequency of 44.4% for HPV infection in women attended gynecologic clinics for routine examination. This is comparable with the first Bulgarian population study of Kovachev et al, which reported a frequency of 38.8% in 1120 patients [Kovachev S et al, 2013]. Recently, the same authors up-dated the survey, by studying 5277 Bulgarian women for HPV infection and found it in 29.8% of women [Kovachev S&Slavov V, 2017]. We found higher frequency of HPV infection in younger women (Group 1 and 2) than in > 40 years old women – 47.5% and 50% versus 28%. It is well known that the young age is one of the main factors for HPV infection – study in more than 2000 women reported a statistically significant trend for increasing HPV prevalence with each year of age from 14 to 24 years, followed by a gradual decline [Dunne EF et al, 2007].

The most frequent HPV types from our study were HPV 45 and 52 (10.6%), HPV 66 (8.6%) and HPV 31 (8%). The most of patients in our study were presented with normal cytology and normal gynecological status. The HPV spectrum is quite different when women from gynecological oncologic units are investigated. Most of the studies are conducted in women with cervical dysplasia, including previous Bulgarian studies. HPV genotyping in 355 women, 288 of which manifesting severe cervical dysplasia or carcinoma in situ, revealed HPV positivity in 61% of women and the highest frequency of HPV16 – 34.6% [Grozdanov P, 2011]. The last study reported the need for developing detection technique with higher sensitivity for HPV45 type, which is the most frequent type from our study. A large Bulgarian study of HPV infection in 227 pregnant women [Georgiev S, 2017] reported HPV positivity in 38% of the women – the most common were HPV16 (17/227, 7.5%), HPV52 (10/227, 4.4%) and HPV 45 and 56 (both in 9/227, 4%). We compared our data with these ones of other Balkan countries. In Macedonia the most common reported types are HPV16 (28.4%), HPV31 (12.1%) and HPV66 (7.9%) [Duvlis S et al, 2001]; in Greece – HPV16 (23.3%) followed by HPV53 (21.6%) [Stamataki P et al, 2010]; in Turkey - HPV16 (32%), HPV18 (8%) and HPV31 (6%) [Dursun P et al, 2013]; in Serbia - HPV16 (37.3%), HPV31 (31.7%) and HPV51 (15.5%) [Kovacevic G et al, 2016]; in Romania – HPV16 (28.1%), HPV53 (14.5%) and HPV51 (13.5%) [Ursu RG et al, 2011]. Obviously, the high incidence of medium-risk HPV66 is characteristic mainly for Bulgarian and Macedonian patients, but it is not observed in other Balkan countries. The analysis of different PAP

groups showed HPV45 is mostly present in normal cervical cytology, whereas HPV16 is definitely involved in dysplasia progression since its frequency in higher PAP grade was statistically higher in our study. The total frequency of this most popular HPV type 16 in our study was 5.3%, but in PAPIII samples – 44.4%. In other Bulgarian studies it was found in 7.5% [Georgiev S, 2017], 13% [Kovachev S&Slavov V, 2017] and 34.6% [Grozdanov P, 2011] – the differences are due to heterogenous cytology and dysplastic lesions in investigated women. HPV16 is definitely the type with the highest impact for cervical dysplasia and cervical cancer, as it is visible from WHO data. According to ICO/IARC Information Centre on HPV and Cancer 2017 [Bruni L et al, 2017], among women with normal cytology the three most frequent HPV types (data are presented per 100 000) in the World are HPV16 (2.9), HPV52 (1.5) and HPV31 (1.3); in the less developed countries – HPV16 (3.0), HPV52 (1.7) and HPV58 (1.4) and in the more developed countries – HPV16 (2.8), HPV53 (1.5) and HPV51 (1.4). Among cervical cancer samples, the most common HPV types are HPV16 (55.2), HPV18 (14.2) and HPV45 (5.0) in the world; in less-developed regions - HPV16 (55.8), HPV18 (13.7) and HPV45 (5.9); in more developed countries - HPV16 (55.7), HPV18 (16.1) and HPV33 (4.7). We emphasized on the high frequency of HPV45 in our study, which is reported as one of the most frequent types in cervical cancer world-widely.

Some studies tried to determine the duration of different HPV infection – Franco [Franco EL et al, 1999] reported median duration of 8.1 months for oncogenic HPV types; Woodman [Woodman CB et al, 2001] estimated 10.3 months duration for HPV16 and 7.8 months for HPV18. There is no consensus for the definition of persistent infection. It requires detection of the same HPV infection more than one time in the interval of 3-6 months; for longer intervals could not exclude re-infections. Latent infection represents positivity of HPV infection in the absence of cervical lesion; it is the equivalent of the occult infection. There is number of co-factors, connected to malignant transformation in the presence of HPV infection. From patient side, these include co-infection (with HIV, Chlamidia trachomatis and Herpes simplex virus 2), immunosuppression, smoking, multiparity, oral contraceptives, and restricted diet. From viral part, the important factors are the viral type, the variant of the viral type, the viral quantity and the viral genomic integrity. Prospective studies have shown that 15 to 28% of women carrying HPV DNA develop cervical intraepithelial lesion up to 2 years, compared with 1

to 3% of non-carriers. A high-risk HPV types infection, lasting for 2 years, creates a 300-fold higher risk of cancer. The sensitivity of the PAP test is about 70%, the inclusion of a HPV screening test and the so called "HPV triage" of the patients reduces cervical cancer cases with another 70%.

Three vaccines that prevent infection with disease-causing HPV types have been approved by The Food and Drug Administration (FDA): Gardasil®, Gardasil® 9, and Cervarix®. All three vaccines prevent infection with HPV types 16 and 18, that cause about 70% of cervical cancers and an even higher percentage of some of the other HPV-caused cancers [Chaturvedi AK et al, 2011; Gillison ML et al, 2008]. Gardasil also prevents infection with HPV types 6 and 11, which cause 90% of genital warts [Koutsky LA et al, 2002]. Gardasil 9 prevents infection with the same four HPV types plus five additional cancer-causing types - 31, 33, 45, 52, and 58. As we can see from our study, these HPV types account for 67% of all positive cases, regarding the total group of patients. In PAPIII group they are present in 90% of patients, whereas HPV types 16 and 18 account for 50% of patients. To date, protection against the targeted HPV types has been found to last for at least 10 years with Gardasil [Kjaer SK et al, 2018], at least 9 years with Cervarix [Naud PS et al, 2014], and at least 6 years with Gardasil 9 [Huh WK et al, 2017]. Long-term studies of vaccine efficacy that are still in progress will help scientists better understand the total duration of protection.

In conclusion, HPV genotyping provides information on the carriage of high-risk types and increased attention to these patients. This is a valuable information for planning preventive programs in regard to choosing diagnostic tests and vaccination. Our results revealed the wide spectrum of HPV infection in Bulgarian women, as about 70% of infected patients carry high-risk HPV 45, 52, 31, 16 and 18.

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