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CERVICAL INTRAEPITHELIAL LESIONS AND INTEGRATION OF HUMAN PAPILLOMAVIRUS

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VILNIAUS UNIVERSITETAS

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INTRAEPITELINIAI GIMDOS KAKLELIO POKYČIAI IR ŽMOGAUS PAPILOMOS VIRUSO INTEGRACIJA

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List of abbreviations

AGC – favor neoplasia

AIS – glandular adenocarcinoma *in situ*

ASC–H – atypical squamous cervical cells undermined signification cannot exclude high-grade squamous intraepithelial lesion

ASCUS – atypical squamous cervical cells undermined signification

CIN cervical intraepithelial neoplasia

CIN I – low grade intraepithelial neoplasia

CIN II – moderate interaepithelial neoplasia

CIN III – severe intraepithelial neoplasia

CIS – carcinoma *in situ*

CSS – cervical squamous carcinoma

DNA – deoxyribonucleic acid

HPV – human papilloma virus

HSIL – high grade squamous intraepithelial lesion

LSIL – low grade squamous intraepithelial lesion

mRNR – messenger ribonucleic acid

Pap – Papanicolaou test

PCR – polymerase chain reaction

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1. Introduction

1.1. Relevance of the problem

The human papillomavirus (HPV) infection plays an important role in cervical intraepithelial lesions and cancer pathogenesis. In 2008, Professor Harold zur Hausen was awarded the Nobel Prize for scientific research in this area. This scholar separated HPV from cervical cancer biopsic specimens and detected the causality between HPV and cervical cancer, which gives the grounds for stating that HPV is a key risk factor for cervical intraepithelial lesions and cervical cancer [1].

In research into HPV prevalence, the Institute of Oncology of the University of Catalonia did a thorough study, the general meta-analysis data of which was published by the World Health Organization in 2007 [2]. The study results demonstrated that among the study population of 157 897 women with no cytological lesions detected in *Papanicolaou* (*Pap*) smear test, 10,4% (95% PI: 10,2÷10,7) had HPV DNA in the cervical specimen. High HPV infection rates feature in economically underdeveloped countries. This infection is the most widespread among African women, especially in East Africa, and the least among South Asian women. Similar results were obtained during the study of the International Agency on Research of Cancer. 15, 613 women aged 15–74 in 11 countries were studied, and general meta-analysis was completed. This study was advantageous, as all the countries used the same protocol to select, analyze specimens and assess risk factors. The total age-adjusted HPV prevalence reached 10,5 % [3].

Compared to the global or European average HPV prevalence, a significantly higher HPV prevalence was detected in Lithuania [4-8]. In Northern Europe, the region where Lithuania is located, approximately 8,0% of women in the general population are infected with HPV. Thus, the cervical cancer incidence and mortality rates are much higher compared to the European average. The average of cervical cancer incidence rate is 11,9 cases/100 000 women in European Union countries, whereas in Lithuania it is 20 to 26 cases/100 000 women. By the mortality trend dynamics, Lithuania also stands out against other European countries [9].

Recently when analyzing the role of HPV in cervical carcinogenesis, increasing attention has been focused on molecular markers, such as virus activity markers: the virus integration, HPV mRNA, gene expression (for instance, *p16*) or DNA methylation changes.

Integration of the HPV genome into the host cells, the deregulated E6/E7 gene expression and incrementing other genetic changes cause the development of a distinct cervical intraepithelial neoplasia (CIN) and cancer [10]. It is believed that the detection of integrated HPV forms may be an important marker to identify the progression of the disease. Though integrated forms of HPV are

detected in more than 40% of women with CIN III/CIS lesions, the active transcription of integrated forms is confirmed only in 15% of such cases. The detection of the received active integrated transcripts would have a better forecasting value.

Many discussions are held on the direct link between the virus integration and the high grade dysplasia. It is unclear if the risk of faster progression of CIN depends on the host's susceptibility, or the interaction of various HPV types, or on the individual effect of each type; however, the data received when analyzing links between HPV integration and the progression of CIN is diverse. There is no common opinion on whether HPV is an early or a late event in cervical carcinogenesis.

1.2. Aim and objectives of the study

Aim:

To identify how the HPV16 DNA integration (HPV E2 gene deletion) into the cervical cells genome is related to cervical intraepithelial neoplasia.

Objectives:

1. To detect HPV prevalence among women with different grades of cervical intraepithelial lesions.
2. To identify HPV types distribution.
3. To identify the prevalent degree of HPV 16 integration in cases of different grades cervical intraepithelial neoplasia.
4. To evaluate the virus activity by detecting HPV mRNA (during follow-up monitoring, in approximately 6 to 12 months).

1.3. Novelty of the study

The new data on HPV 16 integration were revealed during the study.

- It was proved that HPV16 integration into the cell genome is an early event in cervical carcinogenesis.
- It was confirmed that in cases of existing cervical intraepithelial neoplasia HPV 16 integration of the degree II was prevalent.
- HPV mRNA expression is associated with the persistency of the virus in cervical cells.

1.4. Practical benefits

The measurement of HPV DNA and other HPV markers (mRNA, HPV 16 integration or E2 gene deletion) would be a helpful complementary tool for cytological evaluation in primary cervical screening to identify those women at risk of developing high grade squamous intraepithelial lesions and cervical cancer.

1.5. Hypotheses

1. The existence of the integrated form of HPV 16 in cases of low or no cervical intraepithelial lesions indicates that the virus integration is an early event in carcinogenesis.

2. The status of the virus integration (degree III or the episomal form) is not related to the grade of cervical intraepithelial neoplasia.

2. Material and methods

The study covered 253 women aged 18–70 who were standard *Pap* smear tested under the screening program and had cervical pathologies diagnosed: atypical squamous cells of undetermined significance (ASCUS), atypical squamous cells – not possible to exclude HSIL (ASC–H), low grade squamous intraepithelial lesion (LSIL), or high grade squamous intraepithelial lesion (HSIL) and atypical glandular cells – favor neoplasia (AGC).

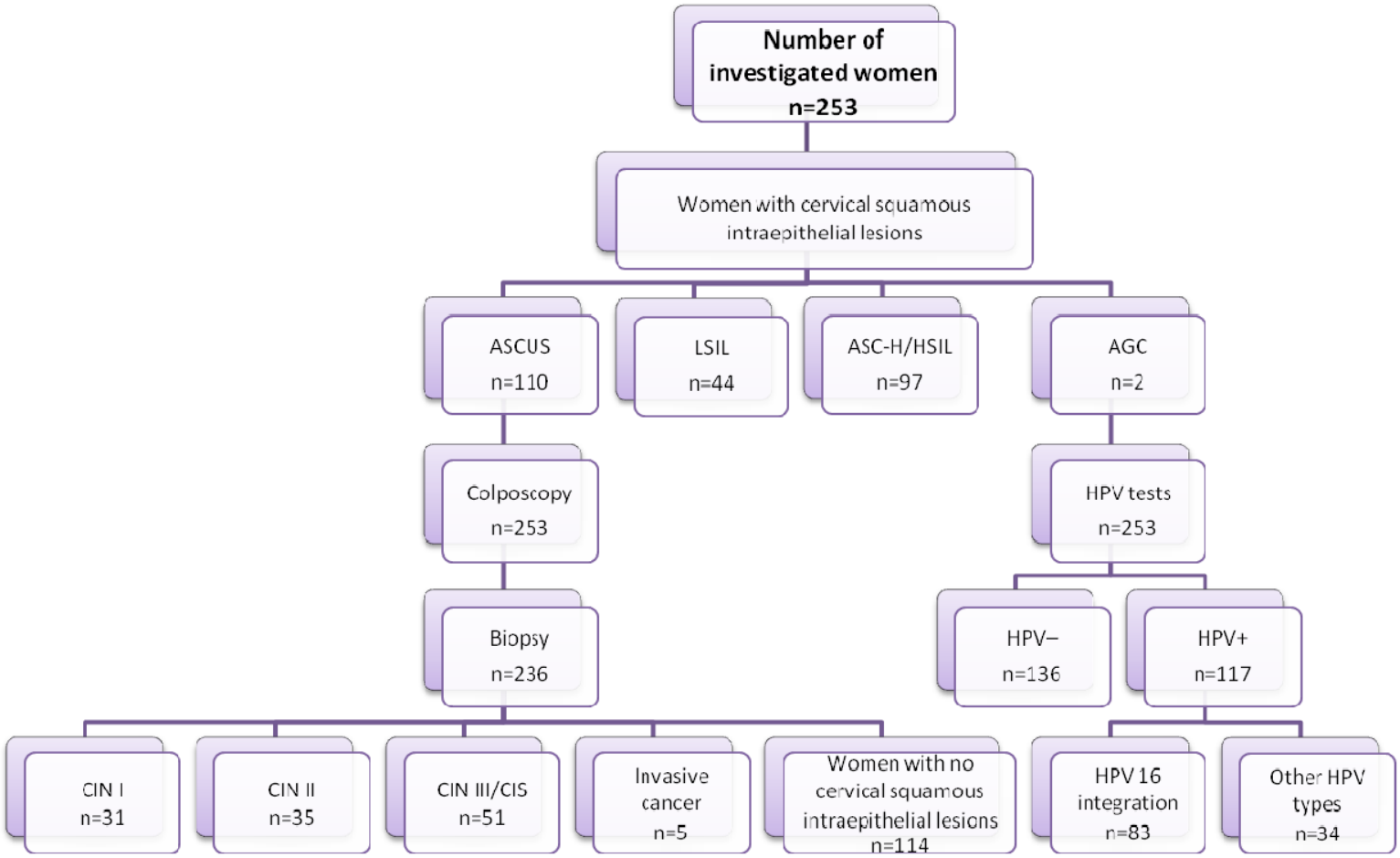
Having agreed to participate in the study, all the women signed an Invitation to participate in the Study and an Informed Consent Forms. The Study Protocol, the Invitation to participate in the Study and the Informed Consent Forms were confirmed by the Lithuanian Bioethics Committee (Permission No.15 to perform Biomedical Research was issued on 02.29.2008). The study did not include any pregnant women. All the studied women were colposcopy-tested and biopsies were performed (17 women refused biopsies).

During the gynecological examination, after mucus was cleaned, a smear was taken from the cervical transformation zone to identify the existence of the HPV infection in general, HPV types, and to assess the degree of integration of the HPV16 E2 gene. Polymerase chain reaction (PCR) for HPV, its types and E2 gene deletion was done at the Institute of Oncology, Vilnius University, and the cervical biopsy materials were tested by the National Centre of Pathology.

Women with CIN II, CIN III/CIS (histological diagnosis after biopsy) were referred to hospital for surgical treatment – cervical conus biopsy (cervical diathermoconisation). Women with no HPV infection diagnosed during the primary screening, and those without cervical intraepithelial lesions in the biopsy, were not invited for a follow-up visit. All other women (i.e., post treatment – cervical diathermoconisation), or those with HPV identified, or light dysplasia (CIN I, detected in the biopsy histology) were invited for a follow-up visit. The duration of the monitoring was 6 to 12 months. During the follow-up visit, a standard *Pap* smear test was done, as well as another test to detect HPV infection. Women with HPV infection diagnosed by the second test were tested for virus activity, i.e., material (cervical cells) was taken to test another HPV integration marker, *messenger* RNA (mRNA). Cytological smear test results were grouped using the Bethesda System 2001.

The detailed testing scheme is provided in Figure 1.

A


Number of investigated women n=253
Women with cervical squamous intraepithelial lesions
ASCUS n=110
LSIL n=44
ASC-H/HSIL n=97
AGC n=2
Colposcopy n=253
HPV tests n=253
Biopsy n=236
HPV– n=136
HPV+ n=117
CIN I n=31
CIN II n=35
CIN III/CIS n=51
Invasive cancer n=5
Women with no cervical squamous intraepithelial lesions n=114
HPV 16 integration n=83
Other HPV types n=34

B

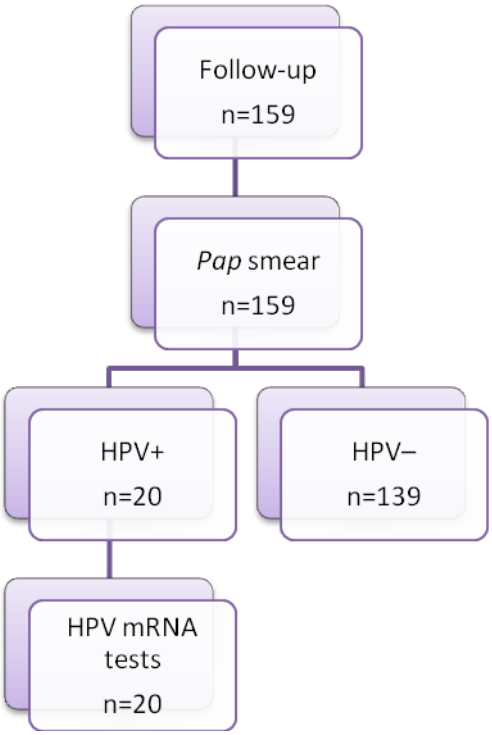
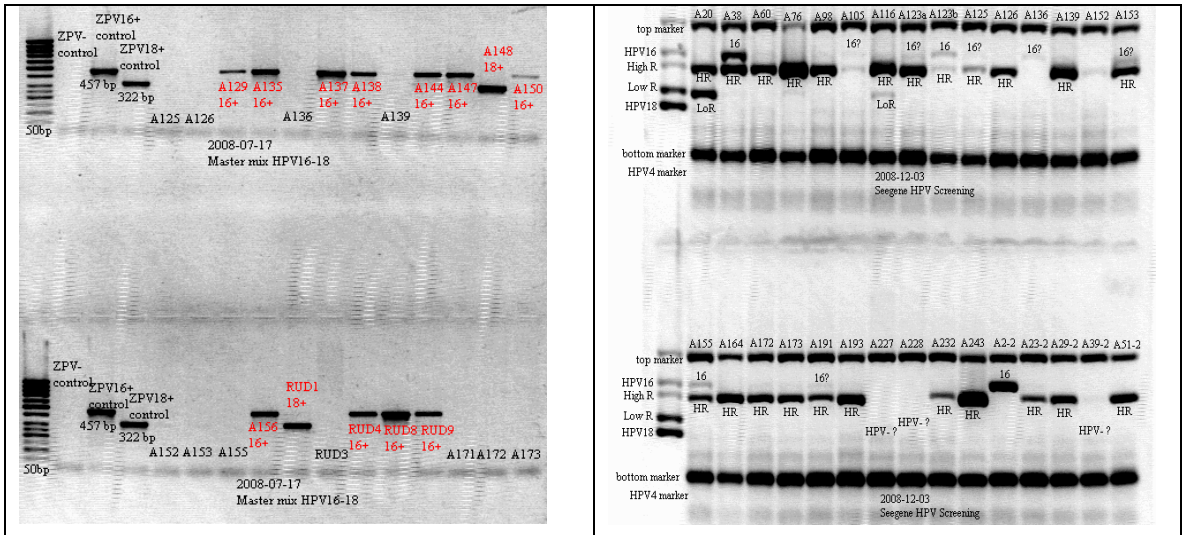


Fig. 1. Testing scheme: A – primary visit, B – monitoring (follow–up)

Note. *ASCUS – atypical squamous cells of undetermined significance, ASC–H – atypical squamous cells of undetermined significance cannot exclude HSIL, LSIL – low grade squamous intraepithelial lesion, HSIL – high grade squamous intraepithelial lesion, AGC – atypical glandular cells, CIN I – light dysplasia, CIN II – average dysplasia, CIN III/CIS – severe dysplasia, HPV+ – HPV positive, HPV- – HPV negative, n – number of cases.*

Identifying HPV and HPV types. The testing substance to identify HPV infection (cervical epithelium cells) was taken with a sterile cervical brush, the end of which was transferred into 2 ml phosphate buffered saline (PBS). DNA was isolated from the cervical cells, and HPV tests were performed. DNA was isolated using the commercial *Sorpoclean* DNA Isolation Kit (*SORPO Diagnostics AB*, Lithuania); DNA was isolated according to the manufacturer's recommendations.

Characteristics of PCA reaction. Commercial kits were used to do PCR (*SORPO Diagnostics AB*, Lithuania). The PCA cycle to detect HPV 6, 11, 16, 18, 31, 33, 45, 59 was performed following the manufacturer's recommendations (Fig. 2).



HPV positive samples – marked in red color

HR – high risk HPV, LR – low risk HPV

Fig. 2. HPV types identification by PCR

Detection of HPV 16 E2 gene deletion. To detect HPV 16 E2 gene deletion, PCA was performed with the use of 3 different primer pairs amplifying 3 gene fragments. Primer sequences are presented in Table 1.

Table 1. Primer sequences used to detect HPV E2 gene deletion

E2 gene fragment	**Primer sequence**	**Nucleotide position**	**Amplified product length**
Amplimer A 1st fragment	A1 5'-AGGACGAGGACAAGGAAAA-3' A2 5'-ACTTGACCCTCTACCACAGTTACT-3'	nt 2735–2753 nt 3187–3210	475 bp
Amplimer B 2nd fragment	B1 5'-TTGTGAAGAAGCATCAGTAACT-3' B2 5'-TAAAGTATTAGCATCACCTT-3'	nt 3172–3193 nt 3630–3649	477 bp
Amplimer C 3dt fragment	C1, 5'-GTAATAGTAACACTACACCCATA-3' C2, 5'-GGATGCAGTATCAAGATTTGTT-3'	nt 3597–3618 nt 3853–3873	276 bp

HPV mRNA detection. To isolate RNA, the commercial kit *All Prep DNA/RNA* (*Qiagen*, Germany) was used. The isolation was done following the manufacturer's recommendations. HPV mRNA expression was detected using Real time PCR.

Statistical methods. The statistical data was analyzed by age group, by diagnosed cervical intraepithelial lesions (cytological and histological diagnosis), HPV infection, infection with various HPV types and HPV16 E2 gene integration status (E2 gene deletion fragments), and mRNA. All the studied features are qualitative; thus, the data was analyzed in frequency tables. These tables present the frequencies of individual features (n) and their respective proportions (%) by categories. χ^2 was used to verify the hypothesis about the independence of the two qualitative features, and the exact Fisher's test was used if the number of observations was small. When qualitative features are ranked, the χ^2 test does not take into account the ranking of categories of variables in the frequency table; thus, the Mantel-Haenszel χ^2 test, also known as the Cochran-Mantel-Haenszel test, was used to evaluate the independence of ranked variables.

The logistic regression model was used to assess if the cytological dynamics depends on HPV16 E2 gene deletion and the patient's age.

The significance level $p<0,05$ was selected to verify the hypotheses. The statistical analysis of the study was done using SPSS 13 and Excel software packages.

3. Results

The study covered 253 women with different intraepithelial lesions in *Pap* smear (from ASCUS to HSIL). All the women were sampled to identify HPV infection, HPV types and HPV 16 E2 gene deletion. The studied women were subjected to colposcopy and biopsy. The women with CIN II–III were referred to hospital for treatment.

3.1. HPV and HPV types prevalence

The age average of the study population was 37,9 (aged 18 to 70 years, SD=10,48). All the women were divided into 5 groups by their age: age group I – aged </=25, age group II – aged 26-35, age group III – aged 36-45, age group IV – aged 46-55, and age group V – aged >56. The largest group was aged 25-36 (74 out of 253). The distribution of women by HPV prevalence according age groups is presented in Figure 3.

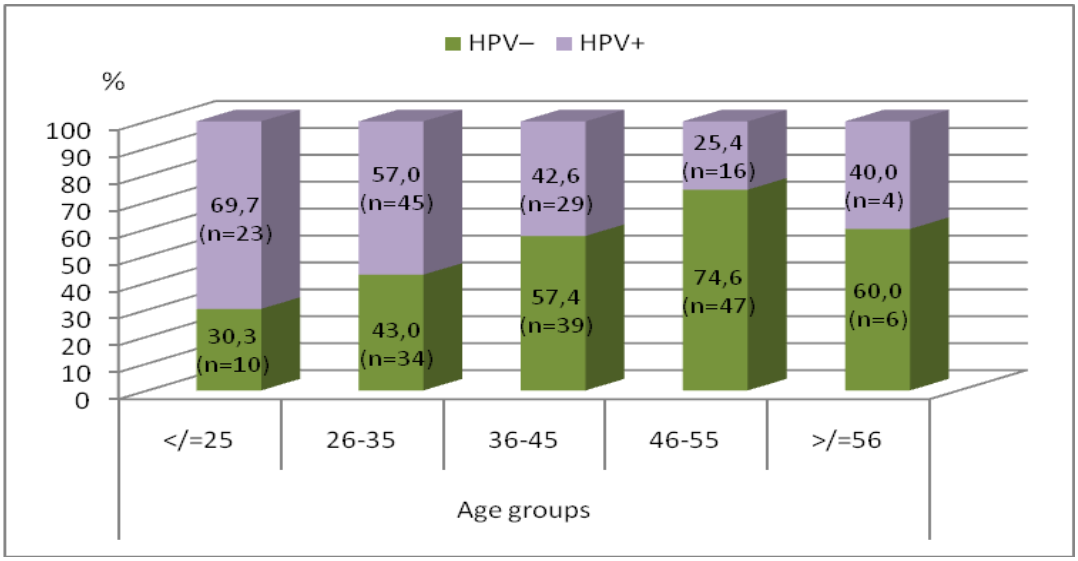


Fig. 3. HPV prevalence by age group

Note. *n – number of cases.*

As has been mentioned above, all the studied women were HPV tested. 117 out of the 253 tested women, i.e., 46,3% (95% PI: 40,10÷52,39) of the study population were HPV positive. Statistically significant differences in HPV prevalence were found when analyzing women by separate age groups (χ^2 test p=0,0002, Mantel-Haenszel χ^2 test p<0,0001). HPV infection was predominantly – at 69,7% – found in women up to 25 years old with cervical intraepithelial lesions. The infection was rather frequent in another age group, in women of 26-35 – 57,0%.

3.1.1. HPV prevalence by cytological diagnosis

Among 253 studied women, 110 women had ASCUS, 97 ASC–H/HSIL, 44 LSIL, and 2 had AGC diagnosed: 43,1; 30,8; 17,4 and 0,8% respectively.

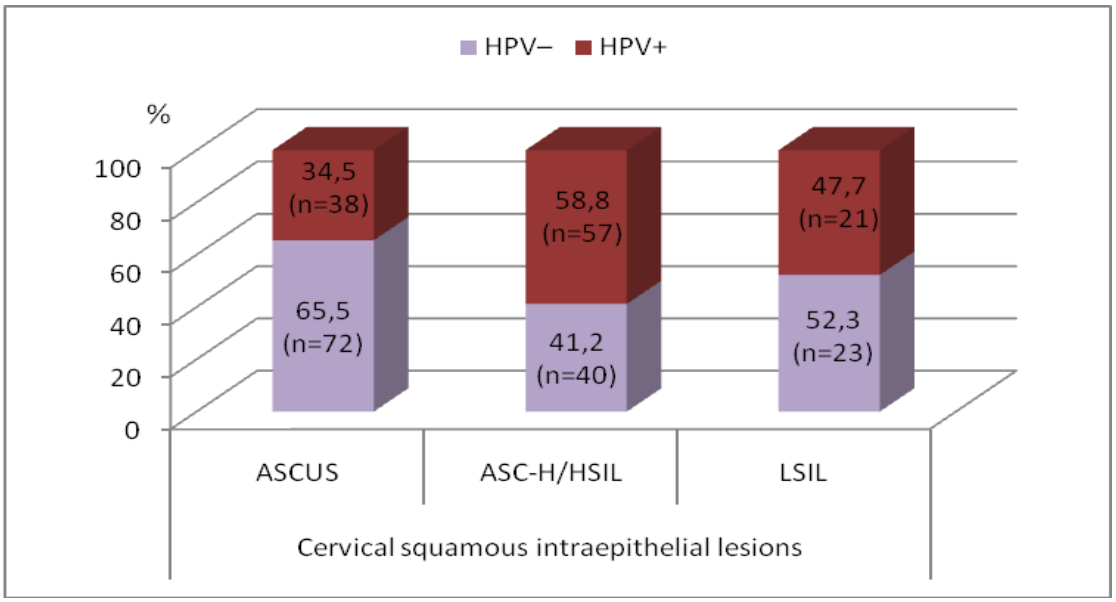


Fig. 4. HPV prevalence by cytological diagnosis

Note. *ASCUS – atypical squamous cells of undetermined significance, LSIL – low grade squamous intraepithelial lesions, ASC-H/HSIL – high grade squamous intraepithelial lesions, n – number of cases.*

The data in Fig. 4 confirms that the highest HPV infection was detected in women who tested positive for ASC-H/HSIL by cytology (58,8%), and the number of confirmed ASCUS infections was half as much (34,5%). On assessing the group differences, statistically significant HPV infection differences were identified subject to cytological diagnosis (p=0,00006).

3.1.2. HPV prevalence by histological diagnosis

Out of the 253 women studied, 236 were subjected to histological tests, as 17 refused a biopsy. Dysplasia was not confirmed by histology for 114 women, 31 women had CIN I diagnosed, 35 women had CIN II, 51 had CIN III/CIS, and 5 women had invasive cancer diagnosed, which is 48,3%, 13,1%, 14,8%, 21,6% and 2,1% respectively.

Table 2. HPV prevalence by histological diagnosis

Histological diagnosis	**HPV infection**				**Total**
	HPV negative		**HPV positive**		
	n	**%**	**n**	**%**	**n**
No dysplasia confirmed by histology	85	74,6	29	25,4	114
CIN I	13	41,9	18	58,1	31
CIN II	4	11,4	31	88,6	35
CIN III/CIS	18	35,3	33	64,7	51
Invasive cancer	3	60,0	2	40,0	5
Total	123	52,1	113	47,9	236

Note. *CIN I – mild dysplasia, CIN II – moderate dysplasia, CIN III/CIS – severe dysplasia.*

The data in Table 2 confirms that the highest HPV prevalence was detected in women who tested positive for CIN II (88,6%) and CIN III/CIS (64,7%).

Prevalence of HPV types. According to HPV type, the majority of women were infected with HPV type 16, i.e., 68,4% of cases (80 out of 117); 5,1% of women (6 cases) were infected with HPV type 18. In one-off cases, HPV types 31, 33 and 45 were detected. Double infections were also confirmed: HPV of types 16 and 18, 16 and 31, 16 and 59, 31 and 33, 31 and 59. Double infections involving HPV type 16 were identified in 70,9% of women (n=83). HPV type was not identified in 12,8% of women (n=15).

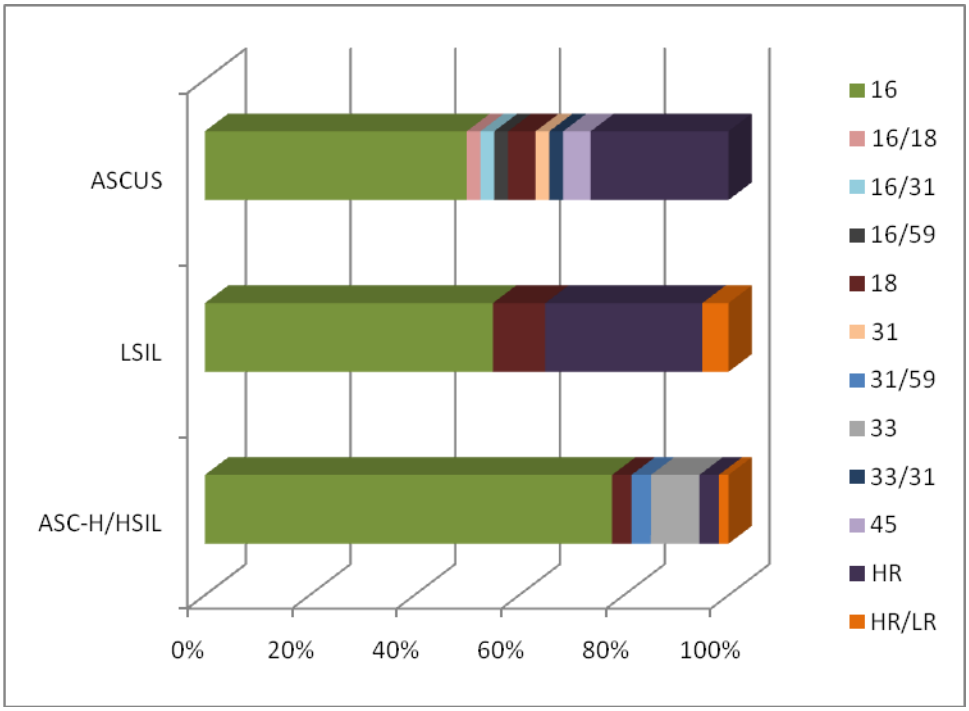


Fig. 5. Identification of HPV types according to cytological diagnosis

Note. *HR – high risk HPV, LR – low risk HPV.*

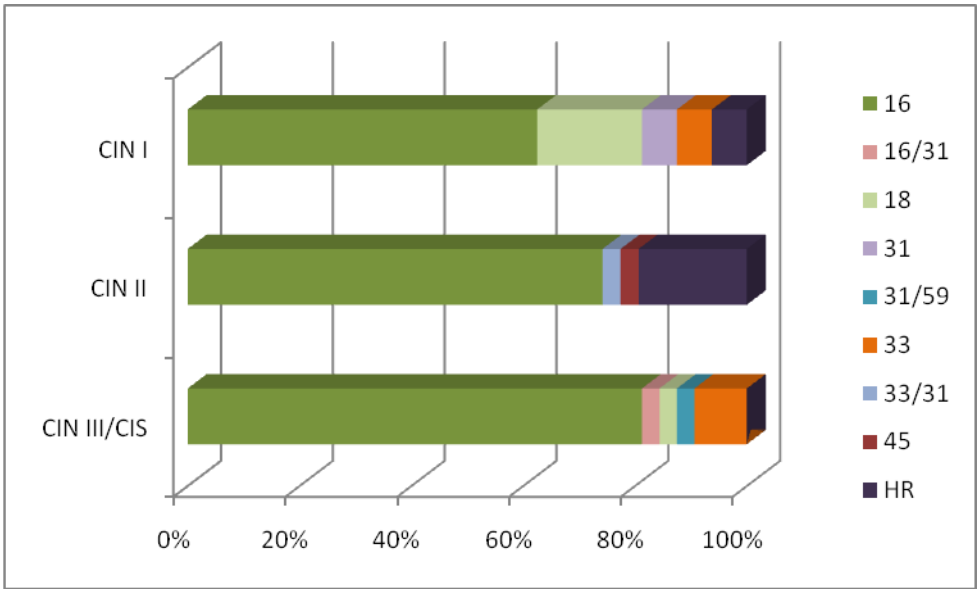


Fig 6. Identification of HPV types according to histological diagnosis

Note. *HR – high risk HPV.*

71,1% (95% PI: 62,13÷78,42) of women were infected with HPV 16. HPV type 16 was identified in all cytological categories: in cases of ASCUS in 68,4%, in cases of LSIL in 66,7%, in cases of HSIL in 79,6% of cases (p=0,0005) (Fig. 5).

In cases of CIN I, HPV 16 was identified in 62,5%; CIN II – 74,2%; and CIN III/CIS – 84,4% of cases. So HPV 16 is less frequently identified in cases of CIN I, and in cases when cytological test results were not confirmed by histology (Fig. 6).

3.2. HPV 16 E2 gene deletion

83 women were screened for HPV 16 E2 gene deletion. The HPV 16 E2 gene integration status was evaluated subject to the level of integration:

- no integration (episomal virus form) – all three HPV 16 E2 gene fragments were amplified;
- degree I integration – deletion of one E2 gene fragment;
- degree II integration – deletion of two E2 gene fragments;
- degree III integration (complete virus integration) – deletion of all three E2 gene fragments.

When testing individual fragments of the HPV 16 E2 gene, degree I integration was identified in 10,8% of cases (9 out of 83), degree II integration was identified in 54,2% of cases (45 out of 83), degree III integration was identified in 26,5% of cases (22 out of 83), and the episomal HPV form in 8,5% of cases (7 out of 83) (p=0,0006) (Fig. 7).

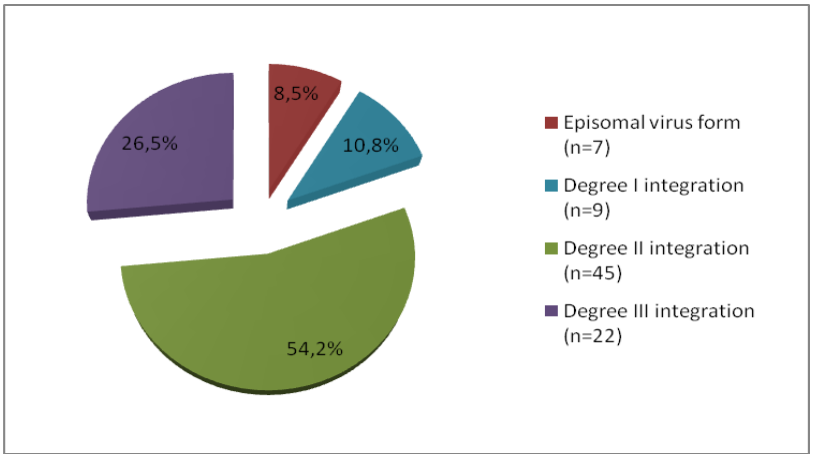


Fig. 7. Distribution of HPV 16 E2 gene deletion by degree of integration

Note. *n – number of cases.*

Analysis by cytological diagnosis. In cases of ASCUS, degree II E2 gene integration was most frequently found, in 50% of cases (13 women out of 26), followed by degree III E2 gene integration, in 42,3% of cases (11 out of 26). The episomal virus form was found only in 1 woman. Similar results were received in the cases of LSIL and HSIL. In cases of LSIL, degree II virus integration was most frequently identified, in 58,3% of cases (7 women out of 12), followed by degree III virus integration, in 33,3% of cases (4 out of 12). No episomal HPV form was identified in LSIL cases. In cases of HSIL, degree II virus integration was most frequently found, in 55,8% of cases (24 women out of 43), followed by degree III virus integration, in 16,2% of cases (7 out of 43). It should be noted that in cases of HSIL, episomal HPV forms were detected in 14,0% of cases (6 out of 43) (Table 3). Statistically significant group differences between the groups were found (χ^2 test p = 0,0295, Mantel-Haenszel χ^2 test p = 0,0086). In ASC-H and AGC cases, only 2 women tested positive for HPV 16 integration of degree I and degree II respectively.

Table 3. Assessment of HPV type 16 integration (E2 gene deletion) status by cytological diagnosis (n=81)

Cytological diagnosis	**HPV 16 integration type**	**Integration**	
		n	**%**
ASCUS	episomal virus form	1	3,9
	degree I integration	1	3,9
	degree II integration	13	50,0
	degree III (complete virus integration)	11	42,3
Total		26	100
LSIL	degree I integration	1	8,4
	degree II integration	7	58,3
	degree III (complete virus integration)	4	33,3
Total		12	100
ASC-H/HSIL	episomal virus form	6	14,0
	degree I integration	6	14,0
	degree II integration	24	55,8
	degree III (complete virus integration)	7	16,2
Total		43	100

Note. *ASCUS – atypical squamous cells of undetermined significance, LSIL – low grade squamous intraepithelial lesions, ASC-H/HSIL – high grade squamous intraepithelial lesions.*

Analysis by histological diagnosis. Irrespective of the small number of cases, degree II integration prevailed in individual histological categories (CIN I, CIN II and CIN III/CIS); however, these differences were not statistically significant (Fisher's exact test, p=0,2521). It should be noted that in cases when cervical intraepithelial lesions were not confirmed by biopsy, degree III integration was also detected in 8 women out of 20. Episomal HPV type 16 forms were detected rarely in cases of CIN I and unidentified dysplasia (Table 4).

Table 4. Assessment of HPV type 16 integration (E2 gene deletion) type by histological diagnosis (n=81*)

Histological diagnosis	**Integration type**	**Integration**	
		n	**%**
No dysplasia detected	episomal virus form	1	5,0
	degree II integration	11	55,0
	degree III (complete virus integration)	8	40,0
Total number		20	100
CIN I	episomal virus form	1	10,0
	degree II integration	6	60,0
	degree III (complete virus integration)	3	30,0
Total number		10	100
CIN II	degree I integration	4	18,2
	degree II integration	12	54,6
	degree III (complete virus integration)	6	27,3
Total number		22	100
CIN III/CIS	episomal virus form	5	19,2
	degree I integration	5	19,2
	degree II integration	14	50,1
	degree III (complete virus integration)	3	11,5
Total number		27	100
Invasive cancer	degree II integration	2	-

Note. *CIN I – mild dysplasia, CIN II – moderate dysplasia, CIN III/CIS – severe dysplasia,* * – 2 patients were not subjected to histological tests.

3.3. Monitoring (follow–up) the study population

3.3.1. HPV infection dynamics

159 out of the 253 women studied arrived for a follow-up examination in 6 to 12 months. It should be noted that only 6 (4,4%) out of the 136 women who tested HPV negative at the beginning of the study became infected with HPV during this time, and in 40 (29,4%) women HPV infection was not found during the follow-up testing. 90 women were not follow-up tested for HPV, as many of them were treated or failed to arrive for the follow-up examination. It should be noted that 23 out of 117 women who tested HPV positive at the beginning of the study (19,7%) retained HPV within this time, as found during the follow-up examination, and in 90 (76%) women, HPV infection was not detected. The majority of these women underwent treatment, and this could have been the cause for finding no HPV infection during the follow-up testing.

A logistic analysis was used to identify the HPV infection progress. The following was found:

- The possibility that HPV infection will be eliminated exceeds the possible HPV retention by 4 times if HPV is found previously. The probability of HPV elimination, if the woman was previously infected, is only 0,7965, and the probability that it will persist is 0,2035.
- The possibility of getting infected with HPV, in cases were no HPV has been detected, is 6,67.

During the follow-up testing, the diagnosis was modified for 50 women out of 110 with ASCUS lesions: cytological lesions were no longer observed in 46 (41,8%) women, and 2 (1,8%) women had ASCUS lesions,1 – LSIL, 3 – ASC – H/HSIL found during the follow-up testing. It should be noted that as many as 58 (52,7%) women did not appear for the follow-up testing.

77 (79,4%) women with a primary cytological diagnosis of ASC–H/HSIL (97 women) had their diagnosis modified. Lesions in the mucous membrane of the cervix were fully eliminated in 75 (77,3%) women, as the majority of them underwent treatment. ASCUS lesions were identified in women probably because the removed tissue edges still had lesions. HSIL was retained in 4 women (one of them was a post-renal transplantation patient and regularly uses immune suppressants and the other one was HIV positive). HSIL persisted in 2 more women probably because they were not referred to hospital after CIN I was found in the biopsy. 16 women (16,5%) were not cytologically examined, as they missed the follow-up visit.

In cases of LSIL lesions (44 women), cytological changes were no longer observed in 26 (40,3%) women, and 3 women had ASCUS diagnosed. It should be noted that the latter refused biopsy

testing during the initial examination. 15 women with LSIL lesions detected missed their follow-up examination.

Analysis by histological test. Based on cytology, 15 women out of 159, who arrived for a follow-up examination after 6 to 12 months, were biopsied and histologically tested. The progression of the disease was diagnosed for 7 women.

3.3.2. Follow-up of cervical intraepithelial lesions based on E2 gene deletion and HPV mRNA expression

The dynamics of cervical intraepithelial lesions was assessed based on HPV 16 E2 gene integrations. The data obtained is presented in Table 5. This data reveals that cervical intraepithelial lesions were eliminated in 7 women found with the episomal virus form.

Table 5. The dynamics of cervical intraepithelial lesions was assessed based on HPV 16 E2 gene integrations

Initial histological test	**HPV 16 gene integration type**	**n**	**Change, n**
No dysplasia	episomal form	1	stabilization
	degree II	11	8 stabilization
			1 progression (CIN I)
			1 progression (CIN II)
			1 progression (CIN III/CIS)
	degree III	8	stabilization
CIN I	episomal form	1	regression
	degree II	6	5 regression
			1 stabilization (CIN I)
	degree III	3	2 regression
			1 stabilization (CIN I)
CIN II	degree I	4	regression
	degree II	12	regression
	degree III	6	regression
CIN III/CIS	episomal form	5	4 regression
			regression/stabilization (CIN II)
	degree I	5	regression
	degree II	14	regression
	degree III	3	regression
Invasive cancer	degree II	2	–
Total number of cases			83

Note. *CIN I – mild dysplasia, CIN II – moderate dysplasia, CIN III/CIS – severe dysplasia.*

Women with HPV diagnosed during the follow-up examination were tested for HPV mRNA. In 10 out of 20 (95% PI:29,93÷70,07) tested women, HPV type 16 and mRNA were found, which indicates persistent active HPV infection (Table 6).

Table 6. Assessment of cervical intraepithelial lesions based on HPV type 16 gene integration and mRNA marker

It. No.	**Initial cytological/ histological test**	**HPV 16 gene integration type**	**Follow-up cytological/histological test**	**Change**
1.	ASCUS/No dysplasia	degree II	LSIL/CIN I	progression/stabi-lisation
2.	LSIL/No dysplasia	degree III	Norm	regression
3.	ASCUS/No dysplasia	episomal form	ASCUS/CIN II	progression
4.	HSIL/CIN III/CIS	degree II	Norm	regression
5.	ASCUS/CIN II	degree I	HSIL/CIN II	stabilization
6.	HSIL/CIN III/CIS	episomal form	HSIL/CIN III/CIS	stabilization
7.	ASCUS/Norm	degree II	LSIL/CIN I	progression
8.	ASCUS/Norm	degree II	HSIL/CIN III/CIS	progression
9.	ASCUS/CIN III/CIS	degree II	Norm	regression
10.	ASCUS/CIS II	degree I	Norm	regression

Note. *ASCUS – atypical squamous cells of undetermined significance, LSIL – low grade squamous intraepithelial lesions, HSIL – high grade squamous intraepithelial lesion, CIN I – mild dysplasia, CIN II – moderate dysplasia, CIN III/CIS – severe dysplasia.*

In summary, the stabilization or regression of disease was confirmed in 7 women (5 of them were treated); the disease progressed in 3 women (these women were not treated according to the monitoring guidelines). It may be assumed that HPV integration and presence of HPV mRNA indicates the trend for progression of cervical intraepithelial lesions; assessment of these additional biomarkers could help to select women for more frequent monitoring and early treatment.

4. Discussion of results

HPV infection plays an important role in cervical intraepithelial lesions and cancer pathogenesis. Prevalence of HPV in cervical cancer patients is approximately 100%, in healthy women – 10,5% worldwide [3]. However, many factors play an important role in cervical carcinogenesis: the most important are virus type, virus load in the cell and virus integration in the cervical cell genome. In the literature, different authors specify different data on the relationship of HPV integration with the grade of cervical neoplasia. Some authors provide data that HPV integration is an early event in carcinogenesis [11-13], others state that it is more frequently detected in cases of high grade intraepithelial lesions [14-17]. However, there is no doubt about the importance of HPV integration to the progression of cervical lesions. Complete virus integration is found in about 90% of cervical cancer cases, integration is more common for the oncogenic HPV type [18]. However, less is known about how the integration degree is related to the grade of cervical neoplasia [19].

According to some authors [11, 20], HPV integration into the cell genome causes selective cell growth, and in cases of HSIL predominantly integrated virus forms are found. According to other authors [13, 21], episomal forms of HPV 16 were exclusively found in normal *Pap* smear or LSIL lesions; they were not identified in cases of HSIL lesions or cancer. However, the research completed by S. Kulmala and co-authors [12] revealed that in cases of normal *Pap* or LSIL, predominantly mixed virus forms were identified, as well as integrated virus forms in a small quantity of specimens; the early integration of HPV 16 DNA was detected in 54% and 46% of cases respectively. Mixed and episomal forms of virus HPV 16 in cases of CIN I were found; and in cases of HSIL or CIN III/CIS or invasive cervical cancer no exclusively episomal virus forms were identified [12, 22, 23].

In our study 46,3% of women with diagnosed cervical intraepithelial lesions were infected by HPV, the majority of them (68,4%) were infected with HPV16. 83 HPV16 positive women were tested for virus E2 gene integration. In general HPV 16 integration was distributed as follows: degree II of HPV16 E2 gene integration was found in 54,2% of cases (45 cases out of 83), degree III - in 26,5% (22 women out of 83), degree I - in 10,8% (9 women out of 83) and episomal forms in 8,5% of cases (7 women out of 83).

According to the cytological diagnosis in cases of LSIL degree I and II virus integration forms were identified in 8 women out of 12, degree III in the other 4 women; episomal virus forms were not found. These results differ from the results of the authors who stated the existence of exclusively episomal forms of HPV16 in cases of normal *Pap* or LSIL [13, 21]. On the other hand, in our study, episomal virus forms were found in 6 women out of 43 (14%) in cases of HSIL. In cases of HSIL degree II integration of HPV16 was predominantly identified, namely in 24 out of 43 (55,8%).

When analyzing data by histological diagnosis degree III of virus was identified in cases of CIN I and when cytological lesions were not confirmed by histology: in 8 women out of 20 (40%), and in 3 women out of 10 (30%) respectively. In cases of CINIII/CIS degree II of virus integration was predominantly identified, namely in 14 out of 27 (50,1%) women. Episomal virus forms were found as well in cases of CIN III/CIS: in 5 women out of 71 (19,2%).

These results correspond to the literature, as many authors disagree about when the virus integration occurs. According to our study, it might occur at a very early stage, when no cervical intraepithelial lesions are observed and the integration process starts very early. According to S. Kulmala and co-authors, in cases of HSIL/CIN III/CIS or cervical cancer, less integrated and more mixed virus forms were found compared to the data of other authors [13, 21]. By the other authors, in cases of HSIL caused by selective cell growth and in cases of CIN III/CIS the exclusively episomal virus forms disappear when intraepithelial lesions progress into cervical cancer [12]. There is data to suggest that HPV DNA integration determines both the faster progression of intraepithelial lesions and the faster re-development of cervical intraepithelial lesions after treatment [20].

The fact that virus integration is already found in some HPV positive women already with cytological lesions, found in a *Pap* smear but not confirmed by histology, while no virus integration is found in other HPV positive women without lesions confirmed by histology, facilitates the thinking that: when the virus integration is found in cases of still low grade cervical intraepithelial lesions, these lesions must progress into significant ones faster than in women with no virus integration detected. Detecting the integration marker (E2 gene deletion) would enable profiling women into an individual risk group with rapid progression of cervical lesions.

5. Conclusions

1. There were 46,3% (95% PI: 40,10÷52,39) women with cervical intraepithelial lesions infected by HPV: in cases of ASC-H/HSIL – 58,8%, in cases of ASCUS – 34,5% (p=0,00006). According the histology women infected by HPV in 88,6% and 64,7% were of moderate and severe dysplasia respectively.

2. 71,1% (95% PI: 62,13÷78,42) of women were infected with HPV 16. This type virus was identified in cases of ASC-H/HSIL – 79,6%, in cases of LSIL – 66,7% (p=0,0005).

3. HPV 16 E2 gene integration degree II was predominantly found in 54,2% of studied women, degree III 26,5% cases, degree I – 10,8% and episomal virus form in 8,5% (p=0,0006).

4. No statistically significant differences were found when analyzing relations between the type of HPV E2 gene integration and the grade of cervical intraepithelial lesions. However, the

presence of integrated virus forms in cases of low grade cervical intraepithelial lesions or with no dysplasia show that HPV16 integration is an early event in cervical carcinogenesis.

5. During the follow-up examination, mRNA expression was found in 50,0% (95% PI:29,93÷70,07) of studied HPV positive women, indicating continuing active HPV infection.

6. Practical recommendations

1. Having identified expression of HPV integration (E2 gene deletion and mRNR) in cervical intraepithelial lesions, it is possible to identify women who are at higher risk of developing cervical carcinoma.

2. Identification of E2 gene deletion, which is significant for foreseeing the progression of the disease, technically is the mostly feasible and easiest way to evaluate virus integration status.

3. Adjustment and combination of separate methods of analyses: from Liquid based cytology (LBC) can be used to identify HPV and its types. In addition, if the same cytological material is used to perform real-time PCR reactions and to identify HPV oncogenes E6, E7 and E2 gene deletions and mRNA, it becomes possible to reduce the number of secondary visits and to separate into specific group the women who are at higher risk of progression of intraepithelial cervical lesions and developing cervical carcinoma.

4. On the grounds of only cytological or histological diagnosis or identification of HPV DNA, it is impossible to distinguish if cervical intraepithelial lesions will regress, persist or progress, which can be the reason of developing cervical carcinoma. The biomarkers showing virus integration into cell genome are significant in both diagnostic and prognostic aspects, evaluating the progression of lesions and choosing the appropriate tactics in treatment and supervision.

7. Reziumė

Įvadas. Žmogaus papilomos viruso (ŽPV) infekcija vaidina svarbų vaidmenį intraepitelinių gimdos kaklelio pokyčių ir vėžio patogenezėje. Už šios krypties mokslinius tyrinėjimus profesoriui Haroldui zur Hausenui (Harold zur Hausen) 2008 metais įteikta Nobelio premija. Šis tyrėjas išskyrė ŽPV iš gimdos kaklelio vėžio biopsijos mėginių ir nustatė priežastinius ryšius tarp ŽPV ir gimdos kaklelio vėžio, kurie duoda pagrindo teigti, kad ŽPV yra vienas pagrindinių intraepitelinių gimdos kaklelio pokyčių ir vėžio rizikos veiksnių [1].

Išsamus ŽPV paplitimo tyrimas (atliktas Katalonijos onkologijos institute), kurio visuminės metaanalizės duomenis 2007 metais paskelbė Pasaulinės sveikatos organizacija [2], parodė, kad tarp 157 897 įtrauktų į tyrimą moterų, kurioms nekonstatuota citologinių pokyčių *Papanicolaou* (*Pap*) tepinėlyje, 10,4 proc. (95% PI: 10,2÷10,7) gimdos kaklelio mėginiuose identifikuota ŽPV DNR. Didelis infekuotumas ŽPV yra ekonomiškai atsilikusiose šalyse. Labiausiai ši infekcija paplitusi tarp Afrikos moterų, ypač Rytų Afrikos, o mažiausiai – tarp Pietryčių Azijos moterų. Panašius rezultatus pateikė Tarptautinio vėžio tyrinėjimų centras. Buvo ištirta 15 613 15–74 metų amžiaus moterų iš 11 šalių ir atlikta visuminė metaanalizė. Šis tyrimas turi privalumų, nes mėginiams atrinkti, ištirti bei rizikos veiksniams įvertinti visose šalyse buvo naudojamas tas pats protokolas. Bendrasis suderintas pagal tiriamųjų amžių ŽPV paplitimas siekė 10,5 proc. [3].

Lietuvoje nustatytas ženkliai didesnis ŽPV paplitimas, palyginti su ŽPV paplitimo vidurkiu pasaulyje ar Europoje [4-8]. Šiaurės Europos regione, kuriam priklauso Lietuva, apie 8 proc. bendrosios populiacijos moterų infekuotos ŽPV. Tad ir sergamumo gimdos kaklelio vėžiu bei mirtingumo nuo jo rodikliai yra didesni, palyginti su Europos rodiklių vidurkiu. Europos Sąjungos šalyse sergamumo gimdos kaklelio vėžiu vidurkis yra 11,9 atvejai 100 000 moterų, Lietuvoje – nuo 20 iki 26 atvejų 100 000 moterų. Lietuva, taip pat ir Latvija bei Estija, pagal mirtingumo pokyčių tendencijas išsiskiria iš kitų Europos šalių [9].

Pastaruoju metu, analizuojant ŽPV vaidmenį gimdos kaklelio kancerogenezėje, vis didesnis dėmesys kreipiamas taip pat į molekulinius žymenis, tokius kaip viruso aktyvumo žymenys: ŽPV mRNR, genų (pvz., *p16*) raiškos ar DNR metilinimo pokyčiai.

ŽPV genomo integracija į šeimininko ląsteles, sutrikusi E6/E7 genų raiškos reguliacija bei tolesnių genetinių pokyčių kaupimasis sąlygoja sunkios intraepitelinės neoplazijos bei gimdos kaklelio vėžio išsivystymą [10]. Manoma, kad nustatytos integruotos ŽPV formos gali būti vertingas žymuo numatant ligos progresiją. Nors integruotos ŽPV formos nustatomos daugiau nei 40 proc. moterų, kurioms yra CIN III/CIS pokyčių, aktyvi integruotų formų transkripcija nustatoma tik 15 proc. tokių moterų, o aktyvūs integruoti transkriptai turėtų didesnę prognozinę vertę.

Daug diskutuojama apie virusų kiekio ir sunkios displazijos tiesioginę sąsają. Nėra aišku, ar padidėjusi intraepitelinės gimdos kaklelio neoplazijos (CIN) progresijos rizika priklauso nuo šeimininko imlumo, ar nuo įvairių tipų ŽPV tarpusavio sąveikos, ar atskirai nuo kiekvieno tipo ŽPV poveikio, o duomenys, gauti tiriant sąsajas tarp ŽPV integracijos ir intraepitelinių gimdos kaklelio pokyčių progresijos, nėra vienareikšmiai. Nėra vieningos nuomonės, ar ŽPV integracija yra ankstyvasis, ar vėlyvasis įvykis gimdos kaklelio kancerogenezėje.

Darbo tikslas – nustatyti, kaip 16 tipo ŽPV DNR integracijos į gimdos kaklelio epitelio ląstelių genomą laipsnis (ŽPV E2 geno iškrita) susijęs su intraepiteliniais gimdos kaklelio pokyčiais.

Šiam tikslui pasiekti buvo sprendžiami **tokie uždaviniai**:

1. Ištirti moterų infekuotumą ŽPV, esant įvairaus laipsnio intraepitelinių gimdos kaklelio pokyčių.
2. Identifikuoti ŽPV tipus.
3. Nustatyti didelės onkogeninės rizikos 16 tipo ŽPV integracijos laipsnį esant intraepitelinių gimdos kaklelio pokyčių.
4. Įvertinti viruso aktyvumą tiriant ŽPV mRNR (stebėsenos metu, t.y. praėjus pusės–vienerių metų laikotarpiui).

Darbo naujumas. Atlikus ŽPV DNR integracijos į gimdos kaklelio epitelio ląsteles tyrimus gauta naujų duomenų.

- Įrodyta, kad 16 tipo ŽPV integracija į gimdos kaklelio epitelio ląstelių genomą yra ankstyvasis gimdos kaklelio kancerogenezės įvykis.
- Nustatyta, kad esant intraepitelinių gimdos kaklelio pokyčių vyravo II laipsnio 16 tipo ŽPV integracija.
- ŽPV mRNR raiška yra susijusi viruso persistencija gimdos kaklelio epitelio ląstelėse.

Praktinė nauda. Atlikti tyrimai svarbūs gerinant patikros dėl gimdos kaklelio patologijos programas. Derinant *Pap* testą ir ŽPV DNR bei kitus biologinius ŽPV žymenis (E2 geno iškrita, mRNR), galima atrinkti infekuotas ŽPV moteris, kurioms dar nėra klinikinių požymių, tačiau kurios priklauso didelės rizikos susirgti gimdos kaklelio vėžiu grupei.

Hipotezės:

1. Integruotos 16 tipo ŽPV formos, esant nežymių arba net nesant intraepitelinių gimdos kaklelio pokyčių rodo, kad viruso integracija yra ankstyvasis kancerogenezės įvykis.

2. Viruso integracijos pobūdis (III laipsnio integracija ar episominė forma) nėra susijęs su intraepitelinių gimdos kaklelio pokyčių laipsniu.

Rezultatai

Į tyrimą įtraukta 253 18–70-ies metų amžiaus moterys, kurioms pirminės patikros metu jau buvo nustatyta gimdos kaklelio patologija tiriant įprastinį *Pap* tepinėlį: atipinės nenustatytos svarbos plokščiojo epitelio ląstelės (ASCUS), atipinės plokščiojo epitelio ląstelės, kai negalima paneigti HSIL (ASC–H), nedidelio laipsnio plokščiojo epitelio intraepiteliniai pokyčiai (LSIL) ar didelio laipsnio plokščiojo epitelio intraepiteliniai pokyčiai (HSIL) bei atipinės liaukinio epitelio ląstelės (AGC).

Sutikusios dalyvauti tyrime moterys pasirašė Asmens informavimo formą (1 priedas) bei Informuotojo asmens sutikimo formą (2 priedas). Tyrimo protokolas, Asmens informavimo forma bei Informuotojo asmens sutikimo forma patvirtinti Lietuvos bioetikos komiteto (gautas leidimas atlikti biomedicininį tyrimą, 2008.02.29 Nr.15; 3 priedas). Nėščios moterys į tyrimą nebuvo įtraukiamos. Visoms į tyrimą įtrauktoms moterims buvo atliekama kolposkopija (t.y. gimdos kaklelio apžiūra optiniu prietaisu – kolposkopu) ir gimdos kaklelio biopsija (17 moterų biopsijos atsisakė). Biopsijos metu nustačius CIN II – CIN III/CIS, moterys pasiųstos gydytis į stacionarą.

Ginekologinės apžiūros metu, nuvalius gleives, iš gimdos kaklelio transformacijos zonos buvo imamas mėginys bendram infekuotumui ŽPV ir jo tipams nustatyti bei 16 tipo ŽPV E2 geno integracijos laipsniui įvertinti. ŽPV infekuotumo, 16 tipo viruso identifikavimo ir E2 geno iškritos tyrimai buvo atliekami Vilniaus universiteto Onkologijos institute, gimdos kaklelio biopsinės medžiagos tyrimai – Valstybiniame patologijos centre.

Infekuotumas ŽPV pagal tirtų moterų amžių. Tirtųjų amžiaus vidurkis yra 37,9 metai (nuo 18 iki 70 metų, SD=10,48). Visos moterys pagal amžių buvo suskirstytos į 5 grupes: I amžiaus grupė – 25 metų ir jaunesnės, II amžiaus grupė – 26–35 metų, III amžiaus grupė – 36–45 metų, IV amžiaus grupė – 46–55 metų, V amžiaus grupė – 56 metų ir vyresnės. Didžiausią grupę sudarė 26–35 metų amžiaus moterys. Dažniausiai ŽPV infekcija, esant intraepitelinių gimdos kaklelio pokyčių, nustatyta 25 metų amžiaus ir jaunesnėms moterims: ŽPV teigiamas testas buvo 23 moterų, ŽPV neigiamas – 10, t.y. atitinkamai 69,7 ir 30,3 proc. atvejų.

Moterų infekuotumo ŽPV analizė pagal citologinę diagnozę. Tarp 253 moterų, įtrauktų į tyrimą, ASCUS diagnozuota 110 moterų, ASC-H/HSIL – 97, LSIL – 44 ir AGC – 2 moterims, atitinkamai 43,1; 30,8; 17,4 ir 0,8 proc.

Tarp tirtų moterų, kurioms nustatyta intraepitelinių gimdos kaklelio pokyčių, ŽPV infekuota 46,3 proc. (95% PI: 40,10÷52,39). Didžiausias infekuotumas ŽPV buvo tų moterų, kurioms citologiniu tyrimu nustatyta ASC-H/HSIL (58,8 proc.), beveik dvigubai mažesnis infekuotumas buvo

ASCUS atveju (34,5 proc.) (16 pav.). AGC nustatyta tik 2 atvejais. Nustatyti statistiškai reikšmingi infekuotumo ŽPV skirtumai priklausomai nuo citologinės diagnozės (p=0,00006).

Moterų infekuotumo ŽPV analizė pagal histologinę diagnozę. Iš 253 įtrauktų į tyrimą moterų histologinis tyrimas atliktas 236-ioms, nes 17 moterų atsisakė biopsijos. Displazija nepatvirtinta histologinio tyrimo metu 48,3 proc. (n=114) atvejų. CIN I, CIN II, CIN III/CIS ir invazinis vėžys nustatyti atitinkamai 13,1 (n=31), 14,8 (n=35), 21,6 (n=51) ir 2,1 proc. (n=5) atvejų.

Didžiausias infekuotumas ŽPV buvo tų moterų, kurioms diagnozuota CIN II (88,6 proc.) bei CIN III/CIS (64,7 proc.).

ŽPV tipų identifikavimas. Identifikuojant ŽPV tipus nustatyta, jog dauguma moterų infekuotos 16 tipo ŽPV – 68,4 proc. (80 moterų iš 117), o 18 tipo ŽPV – tik 5,1 proc. moterų (6 atvejai). Pavieniais atvejais nustatyti 31, 33 ir 45 tipų ŽPV. Konstatuota ir dviguba infekcija: 16 ir 18, 16 ir 31, 16 ir 59, 31 ir 33 bei 31 ir 59 tipų ŽPV. 16 tipo ŽPV kartu su dviguba infekcija identifikuotas 71,1 proc. moterų (n=83). 15,3 proc. (n=18) atvejų ŽPV tipas, panaudojus *SORPO diagnostics* rinkinį, nenustatytas.

16 tipo ŽPV paplitimas esant įvairaus laipsnio intraepitelinių gimdos kaklelio pokyčių. 71,1 proc. (95% PI: 62,13÷78,42) tirtų moterų buvo infekuotos 16 tipo ŽPV.

Analizė pagal citologinę diagnozę. 16 tipo ŽPV buvo identifikuotas esant visų kategorijų citologinių pokyčių. Esant ASCUS šis virusas nustatytas 68,4 proc. atvejų, LSIL – 66,7 proc. HSIL atveju šio tipo virusas buvo identifikuotas dažniau, net 79,6 proc. ASC-H ir AGC atvejais buvo tik 2 infekuotos 16 tipo ŽPV moterys.

Analizė pagal histologinę diagnozę. Esant CIN I pokyčių 16 tipo ŽPV identifikuotas buvo 62,5 proc. (10 moterų iš 16 infekuotų ŽPV) atvejų, esant CIN II pokyčiams – 74,2 proc. (23 moterys iš 31 infekuotų ŽPV), esant CIN III/CIS – 84,4 proc. (27 moterų iš 32 infekuotų ŽPV). Taigi rečiau šio tipo virusas nustatytas esant CIN I ir tais atvejais, kai histologinis tyrimas nepatvirtino citologinio tyrimo rezultatų ir patologijos nenustatyta.

16 tipo ŽPV E2 geno iškritos analizė. Dėl 16 tipo ŽPV E2 geno iškritos tirtos 83 moterys.

Tiriant 16 tipo ŽPV E2 geno atskirus fragmentus 10,8 proc. atvejų (9 iš 83) nustatyta I laipsnio, 54,2 proc. atvejų (45 iš 83) – II laipsnio ir 26,5 proc. (22 iš 83) – III laipsnio integracija, o episominė 16 tipo ŽPV forma – 8,5 proc. (7 moterys iš 83) atvejų (p=0,0006).

Analizė pagal citologinę diagnozę. Esant ASCUS citologinių pokyčių dažniausiai buvo identifikuota II laipsnio – 50 proc. (13 moterų iš 26) bei III laipsnio 16 tipo ŽPV E2 geno integracija – 42,3 proc. (11 moterų iš 26) atvejų. 16 tipo ŽPV episominė forma nustatyta tik 1 moteriai. Panašūs rezultatai gauti ir LSIL bei HSIL atvejais. Esant LSIL dažniausiai buvo identifikuota II laipsnio viruso integracija – 58,3 proc. (7 iš 12); III laipsnio viruso integracija – 33,3 proc. (4 iš 12) atvejų. Episominės

formos ŽPV LSIL atveju nebuvo nustatyta. HSIL atveju dažniausiai buvo identifikuota II laipsnio – 55,8 proc. (24 iš 43); rečiau – III laipsnio viruso integracija – 16,2 proc. atvejų (7 iš 43). Pažymėtina, kad esant HSIL, 14,0 proc. atvejų buvo identifikuotos ir episominės formos ŽPV (6 iš 43) (1 lent.). Nustatyti statistiškai reikšmingi skirtumai grupėse (χ^2 testui p = 0,0295, Mantel-Haenszel χ^2 testui p = 0,0086). ASC-H ir AGC atvejais buvo tik 2 moterys, kurioms 16 tipo ŽPV integracija – atitinkamai I ir II laipsnio.

Analizė pagal histologinę diagnozę. Nepaisant nedidelio tyrimų skaičiaus, atskirų histologinių kategorijų atvejais (CIN I, CIN II ir CIN III/CIS) vyravo II laipsnio viruso integracija, tačiau skirtumai nebuvo statistiškai reikšmingi (Fisherio tikslusis testas p=0,2521). Pažymėtina, kad ir tais atvejais, kai ištyrus biopsijos medžiagą nebuvo patvirtinti intraepiteliniai gimdos kaklelio pokyčiai, 8 moterims iš 20 nustatyta III laipsnio viruso integracija. Episominės 16 tipo ŽPV formos nustatytos pavieniais atvejais esant CIN I bei nenustatytos displazijos atvejais (2 lent.). Pažymėtina, kad atlikus histologinį tyrimą, esant ASCUS citologinei diagnozei, 62,7 proc. atvejų (70 moterų) nebuvo nustatyta jokių pokyčių gimdos kaklelio ląstelėse: 17 jų buvo infekuotos ŽPV, 9 identifikuotas 16 tipo ŽPV. Atlikus histologinį tyrimą, esant LSIL, 12 moterų buvo nustatyta CIN I, 7 – CIN II, 2 – CINIII/CIS. 21 moteriai pokyčių gimdos kaklelyje nenustatyta: 5 jų buvo infekuotos ŽPV, o 3 infekuotos 16 tipo ŽPV. Esant ASC-H/HSIL citologinei diagnozei 46 moterims buvo diagnozuota sunki displazija,t.y. CIN III/CIS, 19 moterų – CIN II. Lengva displazija CIN I nustatyta 6 moterims. 22 moterims pokyčių gimdos kaklelio epitelio ląstelėse iš viso nenustatyta: 7 jų buvo identifikuotas ŽPV ir net 5– 16 tipo ŽPV. Moterys, kurioms buvo identifikuotas ŽPV, bet citologinių radinių nepatvirtino histologinis tyrimas, yra stebimos toliau.

Tiriamųjų stebėsena. Iš 253 į tyrimą įtrauktų moterų 159 moterys atvyko pasitikrinti praėjus pusės-vienerių metų laikotarpiui. Pažymėtina, kad iš 136 moterų, kurios tyrimo pradžioje nebuvo infekuotos ŽPV, per šį laikotarpį ŽPV užsikrėtė tik 6 (4,4 proc.) ir 40 (29,4 proc.) moterų ŽPV infekcijos pakartotinio tyrimo metu nenustatyta. 90 (66,2 proc.) moterų dėl infekuotumo ŽPV pakartotinai tirtos nebuvo, kadangi daugelis jų buvo gydytos arba neatvyko pasitikrinti.

Iš 117 moterų, kurioms tyrimo pradžioje nustatyta ŽPV infekcija, pakartotinio tyrimo metu ŽPV infekcija išliko 23 (19,7 proc.) ir net 90 (76, 9 proc.) moterų ŽPV išnyko. Dauguma šių moterų buvo gydytos, matyt, dėl to joms ŽPV infekcija pakartotinio tyrimo metu nebuvo nustatyta.

Logistinė analizė buvo panaudota siekiant nustatyti ŽPV infekcijos eigą. Nustatyta, kad:

- tikimybė, kad ŽPV išnyks, jei pirmojo tiriamųjų vizito metu buvo nustatyta ŽPV infekcija, yra 4 kartus didesnė nei tikimybė, kad ŽPV išliks. Tikimybė, kad ŽPV išnyks, jei pirmojo vizito metu moteris buvo infekuota, yra 0,80, o tikimybė, kad ŽPV išliks – 0,20.
- tikimybė, nesant infekuotumo ŽPV, užsikrėsti šiuo virusu yra 6,67.

Analizė pagal citologinę diagnozę. Tiriant pakartotinai atvykusias moteris, iš 110, turėjusių ASCUS pokyčių, citologinė diagnozė pasikeitė net 50 moterų: citologiniai pokyčiai išnyko 41,8 proc. (n=46) atvejų ir 2 (1,8 proc.) moterims pakartotinio tyrimo metu nustatyta ASCUS, 1 – LSIL, 3 – ASC–H/HSIL. Pažymėtina, kad net 58 (52,7 proc.) moterys pakartotinai pasitikrinti neatvyko.

Iš 97 moterų, kurių pirminė citologinė diagnozė buvo ASC–H/HSIL, 79,4 proc. (n=77) atvejų diagnozė pasikeitė. Intraepiteliniai gimdos kaklelio gleivinės pokyčiai visai išnyko 77,3 proc. (n=75) atvejų, nes dauguma moterų buvo gydytos; 2 moterims diagnozuota ASCUS, matyt, dėl to, kad pašalinto audinio kraštuose išliko pokyčių; 4 moterims išliko ASC-H/HSIL (viena iš jų po inkstų transplantacijos nuolat vartoja imunosupresantus, kita – infekuota ŽIV, dar dviem minėti pokyčiai išliko, matyt, dėl to, kad tiriant biopsijos medžiagą buvo nustatyta CIN I, o moterys nebuvo pasiųstos gydytis į stacionarą. 16 moterų (16,5 proc.) neatlikti citologiniai tyrimai, kadangi jos neatvyko pakartotinio vizito.

Iš 44 moterų, kurioms pirminė citologinė diagnozė LSIL, 26 moterims (40,3 proc.) citologiniai pokyčiai išnyko, 3 moterims diagnozuota ASCUS. Pažymėtina, kad pastarosios pirminio vizito metu atsisakė biopsijos. Ir net 15 moterų, kurioms buvo nustatyta LSIL, pakartotinio vizito neatvyko.

Analizė pagal histologinę diagnozę. Tarp 159 atvykusiųjų pakartotinai pasitikrinti praėjus pusės–vienerių metų laikotarpiui 15 moterų, remiantis citologine diagnoze, atlikta biopsija ir histologinis tyrimas. 7 moterims konstatuotas ligos progresavimas.

Intraepitelinių gimdos kaklelio pokyčių dinamika pagal 16 tipo ŽPV E2 geno iškritą ir ŽPV mRNR. Įvertinta intraepitelinių gimdos kaklelio pokyčių dinamika pagal 16 tipo ŽPV E2 geno integracijos laipsnį. Pažymėtina, kad daugumai moterų, esant įvairaus laipsnio intraepitelinių gimdos kaklelio pokyčių ir integruotų formų viruso, pokyčiai išnyko. Pažymėtina, kad esant CIN II ir CIN III/CIS, moterys buvo gydytos. Trim atvejais, kai biopsijos metu nebuvo nustatyta displazijos, tačiau konstatuota 16 tipo ŽPV II laipsnio integracija, pokyčiai progresavo.

7 moterims, kurioms buvo konstatuota episominės formos virusas, intraepiteliniai gimdos kaklelio pokyčiai išnyko arba liga stabilizavosi. Pažymėtina, kad 1 moteriai esant CIN I histologinei diagnozei ir episominės formos virusui pokyčiai regresavo savaime.

Moterims, kurioms pakartotinio vizito metu nustatyta ŽPV infekcija, tirtos dėl ŽPV mRNR. Tarp 20 tirtųjų 10 nustatyta (95% PI:29,93÷70,07) 16 tipo ŽPV ir mRNR, kas rodo tebesitęsiančią aktyvią ŽPV infekciją. Įvertinus gautus rezultatus matyti, kad 7 moterims konstatuota ligos stabilizacija ar regresija (5 jų buvo gydytos, 2 – stebėtos, 3 – liga progresavo. Pažymėtina, kad šios trys moterys nebuvo gydytos, nes, vadovaujantis moterų, kurioms aptikta gimdos kaklelio patologinių citologinių ir histologinių pokyčių, priežiūros algoritmais, jų nereikėjo gydyti, todėl paliktos tik stebėti. Galima daryti prielaidą, kad viruso aktyvumo įvertinimas pagal 16 tipo ŽPV integracijos laipsnį ir

mRNR žymenį rodo tendenciją intraepiteliniams gimdos kaklelio pokyčiams progresuoti, kai histologiniu tyrimu nepatvirtinama displazija. Taigi 16 tipo ŽPV integracijos laipsnio ir mRNR įvertinimas gali būti papildomi žymenys siekiant atrinkti moteris, kurias, atliekant stebėseną reikia dažniau tikrinti ir laiku pradėti gydyti. Šią prielaida tikslinga toliau patvirtinti, sukaupus didesnį atvejų skaičių.

Išvados

1. Tarp tirtų moterų, kurioms nustatyta intraepitelinių gimdos kaklelio pokyčių, ŽPV infekuota 46,3 proc. (95% PI: 40,10÷52,39): 58,8 proc. atvejų – esant ASC-H/HSIL (atipinės plokščiojo epitelio ląstelės, kai negalima paneigti HSIL/didelio laipsnio plokščiojo epitelio intraepiteliniai pokyčiai), 34,5 proc. atvejų, esant ASCUS (atipinės nenustatytos svarbos plokščiojo epitelio ląstelės) (p=0,00006); pagal histologinę diagnozę infekuotumas ŽPV esant vidutinei bei sunkiai displazijai – atitinkamai 88,6 ir 64,7 proc. atvejų.

2. 71,1 proc. (95% PI: 62,13÷78,42) tirtų moterų buvo infekuotos 16 tipo ŽPV. Šio tipo ŽPV identifikuotas esant ASC-H/HSIL, 79,6 proc. atvejų, esant LSIL – 66,7 proc. (p=0,0005).

3. Tirtosioms moterims vyravo – 54,2 proc. – II laipsnio 16 tipo ŽPV E2 geno integracija į gimdos kaklelio epitelio ląsteles, III laipsnio integracija buvo 26,5 proc., I laipsnio – 10,8 proc. ir episominės formos ŽPV nustatyta 8,5 proc. atvejų (p=0,0006).

4. Nenustatyta statistiškai reikšmingo skirtumo tarp ŽPV E2 geno integracijos į gimdos kaklelio epitelio ląsteles pobūdžio ir intraepitelinių gimdos kaklelio pokyčių laipsnio tiek analizuojant gautus rezultatus pagal citologinę, tiek pagal histologinę diagnozes. Integruotų formų virusų buvimas net ir nesant intraepitelinių pokyčių ar esant nedidelio laipsnio pokyčiams rodo, kad 16 tipo ŽPV integracija yra ankstyvasis įvykis gimdos kaklelio kancerogenezėje.

5. ŽPV infekuotų moterų pakartotinio tyrimo metu 50 proc. (95% PI:29,93÷70,07) tirtųjų nustatyta mRNR raiška, kas rodo besitęsiančią aktyvią ŽPV infekciją.

Praktinės rekomendacijos

1. Nustačius ŽPV integracijos žymenis (E2 geno iškrita bei mRNR), esant intraepitelinių gimdos kaklelio pokyčių, galima identifikuoti moteris, kurioms gimdos kaklelio vėžio rizika didesnė.

2. E2 geno iškritos nustatymas, kuris svarbus numatyti ligos progresavimo galimybę, yra techniškai lengviausias ir labiausiai prieinamas būdas viruso integracijos laipsniui įvertinti.

3. Atskirų tyrimo metodų derinimas ar sujungimas: skystoji terpė, kurią panaudojus atliekamas *Pap* tyrimas, vėliau gali būti panaudota ŽPV bei jo tipams nustatyti, o atliekant realaus laiko PGR reakcijas ir ŽPV onkogenų E6, E7, E2 iškritai bei mRNR identifikuoti. Taip galima sumažinti moterų

papildomų vizitų skaičių bei išskirti į atskirą grupę moteris, kurių yra didesnė intraepitelinių gimdos kaklelio pokyčių progresijos ir vėžio išsivystymo rizika.

4. Remiantis vien citologinių ir histologinių tyrimų rezultatais ar identifikavus ŽPV DNR, negalima atskirti, ar intraepiteliniai pokyčiai regresuos, persistuos ar progresuos, bei išsivystys gimdos kaklelio invazinis vėžys. Žymenys, rodantys viruso integraciją į ląstelės genomą, yra svarbūs diagnostiniu ir prognoziniu aspektu vertinant intraepitelinių gimdos kaklelio pokyčių progresiją bei parenkant atitinkamą moterų gydymo bei stebėjimo taktiką.

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