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Original Article

High-risk human papillomaviruses distribution in Romanian women with negative cytology

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Abstract

Introduction: Romania has the highest incidence and mortality rate of cervical cancer in Europe. The objective was to estimate the prevalence of high-risk human papillomavirus (hrHPV) genotypes and to evaluate the role of certain socio-behavioral factors in acquiring viral infection, in a cohort of Romanian women with negative Pap.

Methodology: In a prevalence study 611 women (aged 17-58 years) with negative Pap, with no known history of atypical cytology and valid HPV test were included. Each participant completed a questionnaire containing data on socio-behavioral factors. From 344 women aged between 30-58 years, 63 were randomly selected for a second examination (conventional cytology and HPV detection and genotyping) after twelve months.

Results: Of the 611 women, 19.80% were HPV positive, 14.73% infected with hrHPV. Differences in the prevalence of hrHPV (17.60% versus 12.50%) as single (13.01% *vs* 9.01%) and multiple infections (9.71% vs 3.49%) were noted between women under the age of 30 and above. Among socio-behavioral factors, marital status and multiple sexual partners correlate with HPV and hrHPV infection. At follow-up, from 34 HPV negative cases, 10 changed to positive (5 hrHPV), while 2 developed abnormal cytology. Out of the 29 HPV positive cases, 12 cleared the HPV infection and 17 retested positive of which 4 worsened their cytology.

Conclusions: In Romania, HPV infection is common in women with negative cytology. HPV genotyping is of epidemiological importance because the distribution of hrHPV types can determine the impact of prophylactic vaccines and the necessity of HPV testing as screening method.

Key words: hrHPV; NILM; behavioral factors; screening.

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Introduction

According to the Global Cancer Observatory (GLOBOCAN) from the World Health Organization, cervical cancer is the fourth type of cancer in women worldwide [1], with variable prevalence in different populations due to access to screening programs [2]. From near 200 HPV genotypes identified so far, near 40 target mucosal epithelium [3]. HPV genotypes 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59 and 68 demonstrated increased oncogenic potential and are classified as high-risk HPVs; among them, HPV 16 and HPV 18 account for approximately 70% of total cases of this cancer [4]. Phylogenetic analysis revealed that HPV genotypes associated with cervical carcinogenesis belong to high-risk clade of the alpha genus, alpha-7 (HPV 18, 39, 45, 59 and 68) and alpha-9 (HPV 16, 31, 33, 35, 52 and 58) species groups being most common found in cancers [5]. It is estimated that about 50-75% of sexually active women have acquired HPV infection(s) during their lifetime [6] but few of these infections persist and lead to cervical lesions and cancer. In fact, most of them, mainly infections in women younger than 30-35 years, clear spontaneously due to intervention of the host immune system [7]. The risks of persistence are related to hrHPV genotypes [8] viral variants [9], genetic, immune and socio-demographic factors. Virtually all cases of cervical carcinoma are HPV positive, but the distribution of HPV genotypes varies by geographical area and age [10].

Despite immunization against HPV, GLOBOCAN estimate a significant increase of cervical cancer by 2035, Pap screening remaining the best option for preventing this malignancy. Data show that Pap test's

sensitivity varies between 30% and 87%, although it has an increased specificity (86 - 100%) [11]. Accurate diagnosis of HPV infection is performed by molecular methods (HPV DNA identification in biological sample). Based on several observations suggesting that the detection of HPV DNA precedes abnormal cytology and women who tested negative for hrHPV have a very low risk of developing premalignant lesions over the next 10 years [5] new guidelines recommend HPV test as primary screening. Thus, detecting hrHPV in women with subclinical or asymptomatic infections can identify subjects at risk and refer them to follow-up according to European Guidelines in cervical cancer screening [12].

Romania records the highest incidence and mortality rate of cervical cancer in Europe. The crude incidence rate (CIR) varies between regions: 17.8 for Bucharest and 31.3 for Moldavia while the crude mortality rate (CMR) is 16.7 for Bucharest region and 12.4 for Moldavia region [13,14]. Overall, the mortality rate in this disease is six times higher than the average for the other countries in European Union, this type of cancer being the third most common cancer in women. After 20 years of absence, Romania has reimplemented in 2012 the national cytology-based screening, targeting women aged 24-65 years. The national vaccination program was unsuccessful mainly due to a weak information campaign that failed to explain the advantages of anti-HPV immunization.

The objective of the study was to estimate the prevalence of hrHPV genotypes and the role of certain socio-behavioral factors in acquiring the viral infection in a cohort of Romanian women with negative cytology.

Methodology

Samples collection

This prevalence study was conducted between 2010 and 2015 in Romania and included sexually active women aged 17-58 years. Conventional cytology was performed by trained cytologists according to screening practice and Bethesda System was used for reporting Pap smear results. Inclusion criteria were: women with current or past sexual activity, presenting NILM (*Negative for Intraepithelial Lesion or Malignancy*) cytological diagnosis and willing to participate in the study. Exclusion criteria: current or previous history of abnormal cytology, current pregnancy and hysterectomy. Of the 1726 investigated women, only 611 met the enrollment criteria and were included in the study. Each woman signed an informed consent and filled out a short questionnaire consisting of socio-behavioral data. For viral testing, specimens from ectocervix and endocervix of each woman were collected by gynecologists using a cytobrush. The samples were preserved in Copan medium, transported to Stefan S. Nicolau Institute of Virology and stored at 4°C until DNA extraction (maximum 2 days).

Based on the fact that a large number of transient HPV infections is found in young women and the spontaneous clearance of HPV infection significantly diminishes in women over 30 years old, we decided to focus on the older group. Twelve months after the first investigation, from the 344 subjects aged between 30-58 years, we randomly selected 63 women for a second examination (cytology investigation and viral test).

DNA isolation

DNA was isolated from cervical smears using High Pure PCR Template kit (Roche Molecular Biochemicals, Mannheim, Germany), within 2 days from sample collection. DNA extraction was performed from 1 mL of cell suspension, according to manufacturer's recommendations. Isolated DNA was subsequently used for HPV detection and genotyping.

HPV DNA genotyping

Human papillomavirus detection and genotyping was performed with commercially available Linear Array HPV Genotyping Test (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions. A pool of biotinylated primers amplifies a sequence of nearly 450 base pairs within L1 gene of 37 HPV genotypes stratified into high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68), possibly high-risk HPV types (HPV 26, 53, 66, 73 and 82), low-risk HPV types (HPV 6, 11, 40, 42, 54, 61, 70, 72, 81 and CP6108), and undetermined risk types (ur-HPV) (HPV 55, 62, 64, 67, 69, 71, 83, 84 and IS39). Primers targeting the human β-globin gene for control of cell adequacy, extraction and amplification were included.

Statistical analysis

The statistical calculations were performed using GraphPad Prism v5.0. The association between HPV infection, genotype and social-behavioral factors was analyzed using Fisher's exact test with 95% confidence interval for computing Relative Risk (RR) and Odd Ratio (OR). As reference the condition less risky was chosen. A value above 1 for RR and OR indicates a risk factor for HPV infection while a sub-unitary value suggests respective conditions as a protective factor.

Results

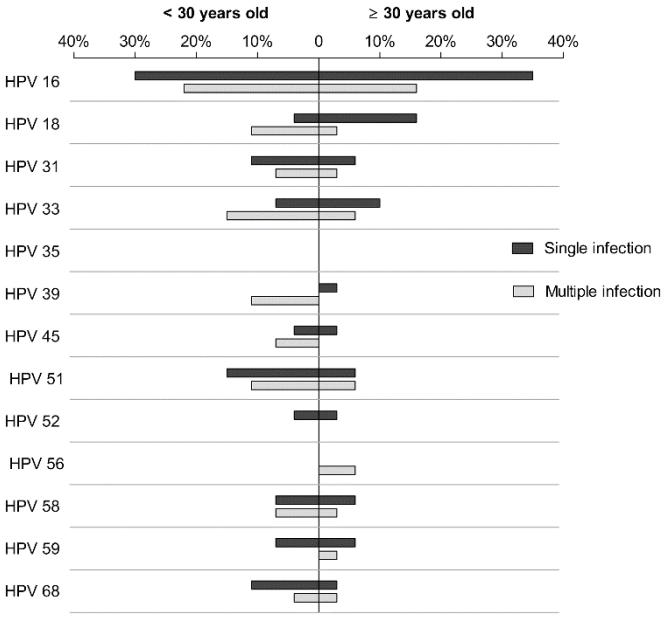
Out of 1726 investigated women, 611 subjects aged between 17-58 years old (mean age ± standard deviation: 31.56 ± 7.38) met the inclusion criteria for the study. From these, 121 women (19.8%) were found HPV positive (comprising 26 of 37 tested genotypes). Among the positive cases, 68.6% (n = 83) presented single infection and 31.4% (n = 38) multiple infections. Low, medium or undetermined HPV genotypes were found in 31 cases: HPV 11 (n = 10; 8.26%), HPV 66 (n = 9; 7.38%), HPV 6 (n = 9; 7.38%) and HPV 63 (n = 5; 4.13%). On the other hand, hrHPVs were identified in 90 women (14.73% of all cases, 74.38% of HPV positive ones). HPV 16 was the most common oncogenic type (n = 30; 24.79% of all HPV-positive samples) with a frequency of 17.54%. Other hrHPV were HPV 33, HPV 51 (n = 8; 6.61% each), HPV 18 (n = 7; 5.78%) and HPV 31 (n = 6; 4.95%). Multiple infections harboring two hrHPV were found in 10 of 121 positive cases (8.26%), and three oncogenic types in one case.

Differences between women under 30 years and above were observed both in overall HPV and hrHPV prevalence either as single or as multiple infections. Age stratification of subjects older than 30 years showed the lowest HPV prevalence in the 30-34 years group and the highest in the 35-39 years group. On the other hand, comparable percentages of hrHPV were found in groups 30-34 (n = 17; 11.64%) and 35-39 (n = 12; 11.88%) and higher in women over 40 years (n = 14; 14.43%) (Table 1).

The distribution of the hrHPV in women under and over 30 years old is shown in Figure 1.

HPV 16 as a single infection was equally distributed between these groups. HPV 18 was prevalently encountered as multiple infections in women under 30 and as single infections over this age. HPV 51 was found (as single and multiple infections) in a higher number in younger women (< 30 years old). HPV 33 has a higher share of multiple infections in the group under 30 and in single infections in subjects over 30 years old, while HPV 52 was found only as single infections in both groups.

Figure 1. Distribution of hrHPV genotypes by age groups (under and over 30 years old).



Based on the questionnaires filled by the enrolled women, the correlations between hrHPV infections and socio-behavioral factors were further investigated.

In this context, statistical calculations were performed versus the acquisition of oncogenic genotypes, but also *vs* genotype 16 whose prevalence was significantly higher in the study group.

Table 1. The prevalence of HPV infection according to age.

Age (years)	Cases (n)	HPV positive n (%)	High-risk HPV genotypes (single/multiple infections)	Age groups (years)	Cases (n)	HPV positive n (%)	High-risk HPV genotypes (single/multiple infections)	Multiple infections with 2 hrHPV (n)	Multiple infections with 3 hrHPV (n)	Other HPV genotypes (single/ multiple infections)
< 30	267	61 (22.85)	47 (27/20)	17-24	113	28 (24.78)	19 (10/9)	3	1	9 (6/3)
				25-29	154	33 (21.43)	28 (17/11)	2	0	5 (5/0)
≥ 30	344	60 (17.44)	43 (31/12)	30-34	146	23 (15.75)	17 (12/5)	3	0	6 (4/2)
				35-39	101	20 (19.80)	12 (9/3)	1	0	8 (8/0)
				≥40	97	17 (17.53)	14 (10/4)	1	0	3 (2/1)

Table 2. Correlation between the acquisition of hrHPV infections and socio-behavioral factors.

		Marital status		**Number of partners**			**Age at first sexual intercourse**			**Contraceptives measures**				**Age of primiparity**			**Number of births**		
		M*	**UM**	**<3***	**3-5**	**>5**	**< 17**	**17-20**	**≥ 21**	**C***	**ACO**	**IUD**	**other**/ none**	**≤ 25***	**26-30**	**≥ 31**	**0***	**1**	**2**	**≥ 3**
Total		401	210	460	126	25	28	348	235	62	222	31	296	285	120	22	184	247	155	25
HPV +	(n)	63	58	85	24	12	5	76	40	14	43	7	57	56	15	0	50	45	24	2
	OR	*	**2.047**	*	1.038	**4.072**	1.060	1.362	*	*	0.8236	1.000	0.8177	*	0.5842	**0.1291**	*	**0.5970**	**0.4910**	**0.2330**
	CI 95%		*1.37-3.07*		*0.628-1.72*	*1.80-9.24*	*0.380-2.96*	*0.891-2.08*			*0.416-1.63*	*0.357-2.81*	*0.422-1.59*		*0.316 -1.08*	*0.008-2.17*		*0.378-0.944*	*0.285-0.845*	*0.053-1.03*
	RR	*	1.325	*	1.008	1.103	1.006	1.211	*	*	0.8609	1.000	0.8482	*	0.8679	0.9124	*	0.7577	0.7484	0.8876
	CI95%		*1.11-1.56*		*0.903-1.13*	*1.02-1.19*	*0.899-1.13*	*0.922-1.59*			*0.512-1.45*	*0.710-1.41*	*0.496-1.45*		*0.755-1.00*	*0.878-0.958*		*0.601-0.955*	*0.614-0.912*	*0.816-0.966*
	p	-	***0.0006***		*ns*	***0.0011***	*ns*	*ns*	-	-	*ns*	*ns*	*ns*	-	*ns*	***0.0536***	-	***0.0342***	***0.0119***	***0.0467***
Hr HPV	(n)	48	42	57	23	10	3	60	27	11	40	4	35	39	14	0	37	34	17	2
	OR	*	**1.946**	*	1.483	**5.061**	0.9420	1.593	*	*	0.9751	0.7273	0.6390	*	0.7829	**0.0903**	*	0.6096	**0.4700**	**0.3149**
	CI 95%		1.23-3.07		0.872-2.52	*2.12-12.1*	*0.265-3.35*	*0.976-2.60*			*0.465-2.04*	*0.210-2.53*	*0.303-1.35*		*0.408-1.50*	*0.005-1.51*		*0.364-1.02*	*0.252-0.876*	*0.071-1.40*
	RR	*	1.293	*	1.103	1.136 *1.03-*	0.9939	1.345	*	*	0.9804	0.9091	0.6994	*	0.9318	0.9124	*	0.7653	0.7380	0.8996
	CI 95%		*1.06-1.58*		*0.953-1.28*	*1.26*	*0.875-1.13*	*0.966-1.87*			*0.549-1.75*	*0.643-1.29*	*0.393-1.25*		*0.781-1.11*	*0.878 -0.948*		*0.591-0.991*	*0.594-0.916*	*0.816-0.992*
	p	-	***0.0050***	-	*ns*	***0.0006***	*ns*	*ns*	-	-	*ns*	*ns*	*ns*	-	*ns*	***0.0185***	-	*0.0645*	***0.0169***	*ns*
HPV 16 s	(n)	11	8	12	5	2	1	12	6	2	12	0	5	10	2	0	7	9	2	1
	OR	*	1.617	*	1.532	**4.808**	1.413	1.434	*	*	1.609	**0.3959**	**0.5021**	*	0.8228	**0.4857**	*	0.8529	**0.2923**	0.8323
	CI 95%		*0.638-4.10*		*0.525-4.45*	*0.974-23.7*	*0.163-12.3*	*0.529-3.89*			*0.348 -7.44*	*0.018-8.58*	*0.095-2.67*		*0.632-1.07*	*0.028-8.57*		0.310-2.35	*0.060-1.43*	*0.098-7.09*
	RR	*	1.191	*	1.114	1.128	1.044	1.253	*	*	1.480	0.6667	0.5854	*	0.4362	0.9124	*	0.9116	**0.6501**	0.9754
	CI 95%		*0.808-1.76*		*0.817-1.52*	*0.910-1.40*	*0.769-1.42*	*0.646-2.43*			*0.400 -5.47*	*0.566-0.785*	*0.176-1.94*		*0.094-2.03*	*0.878-0.948*		*0.515-1.61*	*0.450-0.940*	*0.745 -1.28*
	p	-	*ns*	-	*ns*	*ns*	*ns*	*ns*	-	-	*ns*	*ns*	*ns*	-	*ns*	*ns*	-	*ns*	*ns*	*ns*

*reference, ** Rhythm method, *coitus interruptus*, tubal ligation; ns = non-significant; C = condom; ACO = oral contraceptives; IUD = Intrauterine devices.

The data obtained indicates a strong and statistically significant association of the number of sexual partners (more than 5) with HPV infection OR = 4.072 (1.80-9.24, p = 0.0011) and with hrHPV positivity OR = 5.061 (2.12-12.1, p = 0.0006). Even if a high prevalence of HPV 16 was noticed, no statistical significance for the association between acquiring this genotype and behavioral factors was observed, maybe due to the small number of cases.

The unmarried status favours the acquisition of both HPV infections OR = 2.047 (1.11-1.56, p = 0.0006) and hrHPV genotypes OR = 1.946 (1.23-3.07, p = 0.005) although the data might suggest a difference between a stable partner or not. Number of births seems to play a protective role rather than a risk for HPV infection as seen in Table 2. Older age at primiparity and births appear to be protective factors against HPV 16 acquiring, as RR and OR were found to be sub-unitary in these cases. No association was found between HPV infection and contraceptive measures.

Among the women enrolled in the study, 63 subjects were cytological and viral retested after 12 months since recruitment. From 34 negative cases, 10 became positive, 5 of them being infected with high-risk genotypes. The other 24 cases retested negative for HPV DNA but two of them developed abnormal cytology (LGSIL - *Low Grade Squamous Intraepithelial Lesion*). Of the 29 positive women at base-line, at follow-up 12 subjects (mean age 31.45 years) retested negative for viral infection. The other 17 women were still HPV DNA positive, 8 harbored the same genotype of which one developed HGSIL (*High Grade Squamous Intraepithelial Lesion)* cytology. The results of the cytological and viral investigations of the re-tested patients as well as their socio-behavioral characteristics are presented in Table 3. The data revealed a link between the number of partners and HPV DNA positivity (acquisition and/or carring).

Discussion

Women with cytology within normal limits who tested negative for hrHPV have a very low risk of cervical pre-cancer for the next 5-10 years. On the other hand, HPV infections are less frequent in adult women compared with younger ones, but the risk of persistence increases with age. Therefore, screening strategies recommend viral testing for high-risk genotypes in women over 30 and retesting in an interval between 3 to 5 years or more [15,16]. In this study, a method that allowed the detection of individual genotypes was used in order to better illustrate the distribution of each hrHPV genotype. Out of 611 enrolled subjects with normal cytology, 19.8% tested HPV positive. The prevalence of the viral infection decreased with age, from 24.78% in women under 30 years old to 17.44% in those of 30 and over. Burchell *et al.* found the highest HPV prevalence in younger women (< 20), a decrease in the middle age groups, and an increase again at age 65 and older [17]. In a meta-analysis of women with normal cytology, de Sanjosé *et al.* reported the highest HPV prevalence in women younger than 35 years old with a second peak in women aged 45 years or older [18]. Our results showed that HPV prevalence was highest in young (< 25 years old) and middle-aged women (35-39 years old, 19.80%).

We detected hrHPV in 90 of the 611 enrolled cases (14.72%), the lowest prevalence being found in groups of 30-34 and 35-39 years (17 of 23 and 12 of 20

Table 3. Results of follow-up investigations.

Enrolment (n)		Follow-up			Socio-behavioral characteristics		
		HPV test (n)	Cytology (n)	Age (mean age)	Marital status (married/ unmarried)	Number pregnancies/ births (average)	Number partners (average)
NILM cytology n = 63	HPV negative n = 34	negative n = 22	22 NILM	31.29	13/9	3/1.11	1.32
		negative n = 2	2 LGSIL	42	1/1	2/2	2
		positive n = 10*	10 NILM	36.14	4/6	1.4/1.2	4.6
	HPV positive n = 29	negative n = 12	12 NILM	31.45	4/8	2.5/1	1.8
		positive n = 13**	13 NILM	32.33	5/8	0.83/0.4	2.14
		positive n = 4	2 ASCUS 1 LGSIL 1 HGSIL	32.75	2/2	2.25/1	2.25

*5 cases with hrHPV; ** 8 cases harboring the same hrHPV genotype as enrolling.

respectively). In our country, Voidazan *et al.* reported that in women having NILM cytology 20.8% were HPV positive and hrHPV was found in 12 out of 15 HPV positive cases [19].

We found that the most commonly detected hrHPV types are similar to those described in pre-neoplastic and cancer cases, although the frequency of HPV 16 is significantly higher than other genotypes. Also, Ursu *et al.* confirms the high-risk genotypes identified in our study, along with HPV 16, genotype 51 being common, while the prevalence of HPV 18 decreased compared to previous studies [20].

The data we obtained on the prevalence of oncogenic genotypes support screening based on viral testing. HPV 45 is less common in our country, but other hrHPV types should be tested (HPV 31, 33, 51) [4]. Some of HPV types, such as HPV 53 and HPV 66 were classified for cautionary reasons as possibly carcinogenic to humans even in the absence of adequate epidemiological data on their carcinogenic potential. They are relatively common in women with normal cytology and in women with low-grade cervical lesions [21]. In our study, three HPV positive patients (carrying genotypes 53, 66, 70) turned negative after one year. Multiple infections were considered to be a risk if any HPV detected was a high-risk one [22]. While some authors correlate the presence of multiple HPV types with the severity of cervical intraepithelial neoplasia [23], other authors have reported that infection with multiple types does not increase the risk of developing or progression to cervical cancer [24,25]. In this study 30.58% of the infected women carried more than one viral type. The peak of all single infections was noticed in 35-39 age group (16.83%) while two peaks were observed for single hrHPV: in group of 25-29 years old (11.04%) and women over 40 (10.31%). The frequency of high-risk multiple infections decreased with age, the highest values being noted for women under 30 (8.58% for all HPV types and 7.46% for high risk types), while the lowest were found in 35-39 age group (2.97% for both oncogenic and all HPV types).

Analyzing the questionnaires filled by the enrolled women, we found that the risk factor associated with HPV infection is the number of partners (>5), while protective role seems to be linked to the marital status (married), contraceptive measures and the number of pregnancies (the explanation for this fact could be that these women are generally married and are more likely to address the gynecologist for investigations). In fact, other authors underlined that among risk factor associated with genital warts are the number of sexual partners and other sexually transmitted diseases in the previous 24 months [26].

The most carcinogenic HPV types concentrated in species alpha-9 and alpha-7 clades were known for an elevated risk of progression given persistence rather than persistence alone. Although several reports [8] have suggested that one HPV infection reduces the risk of contracting another infection from the same species by sharing group-specific immune protection or general protection, we found one multiple infection case (HPV 16 and 31) belonging to alpha-7 whose persistence in follow-up may be the cause of progression to HGSIL cytology. These results may suggest that co-testing is a suitable approach for identifying women at risk. Bulkman*s et al.* reported that HPV 16 and HPV 31 have a great risk of progression to CIN and the lowest rates of clearance [27]. In our study, 4 cases of multiple infections at baseline (genotypes belonging to two different alpha families), tested positive only for one HPV type at folow-up (HPV 18 and 53 at enrollment retested HPV 18; HPV 51 and 40 retested HPV 51; HPV 31 and 66 retested HPV 66; HPV 33 and 54 retested HPV 54). Our results showed that 12 of 29 HPV positive women retested negative after one year. The POBASCAM study that addressed HPV testing in the Netherlands drew attention to the risk of developing CIN 3+ lesions in women who tested intermittently, compared to women who consistently had HPV-negative results. These cases, which often carry the same genotype, suggest reactivation rather than new infection. The authors recommended that viral screening intervals should be determined separately for HPV DNA positive and negative women [28].

Primary HPV screening is very effective in reducing the risk of cervical cancer and screening intervals should be extended also to negative HPV and NILM cytology. In this study out of the 36 subjects who retested HPV negative at follow-up, 2 developed LGSIL cytology. We supposed that HPV DNA was not detectable by the test we used and these observations were also reported by Wright *et al.* [29]. In fact, Schiffman *et al*. emphasized that co-testing brings benefits to women who test negative for HPV but have altered cytology [30].

The acquisition of hrHPV during the follow-up period significantly increased the risk of cytological abnormalities. Thus, after follow-up Pap test revealed that 2 patients developed ASCUS (*Atypical Squamous Cells of Undetermined Significance*), 3 LGSIL and one HGSIL. We found a low rate of new HPV infections among women who tested negative at enrollment. This makes repeated HPV-screening with intervals of every

5-6 years an even more attractive option to prevent cervical cancer, the test being as safe and effective as three-year cytology screening [31]. On the other hand, Eurogin Roadmap 2017 suggested that the results from epidemiological studies could improve the accuracy of retesting intervals and methods for hrHPV positive women with negative cytology [32].

Although our follow-up group study was carried out on a limited number of individuals, it generated results that may permit the policy makers to initiate cervical cancer screening using co-testing strategy, based on hrHPV detection and cytology, mainly when the prevalence of the oncogenic types varies among countries and according to age and behavioral factors.

Conclusions

HPV infection is common in women with negative cytology in our country (19.8%), 74.38% of positive cases presenting high-risk genotypes, emphasizing the importance of HPV DNA testing in Romania. Viral genotyping has a significant role both in identifying women at risk of developing pre-neoplastic lesions and in their monitoring, particularly in women who have negative Pap smears allowing for discrimination between viral persistence and the acquisition of new infections. Genotyping has also an epidemiological importance for Romania as the distribution of hrHPV types can establish the impact of prophylactic vaccines and the utility of the HPV DNA screening tests.

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Authors' contributions

All authors contributed equally to this work.

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