Original Article
Correlation of Human Papillomavirus multiple infections with cytological findings in a cohort of Greek women

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Abstract
Infection with the human papillomavirus (HPV) causes precursor intraepithelial lesions in women which may lead to the development of cervical cancer. There are several HPV types which show a variable distribution pattern worldwide. Therefore, identifying the prevalence of cancer-associated HPV types is important for the prevention of cervical cancer. In the current study, 627 cervical samples from individual women were collected by primary care clinics in Greece. The majority of women (70.17%) presented abnormal cytology as indicated by their Pap Test smear. Samples were sent to our laboratories for the detection of HPV by DNA analysis, PCR and reverse hybridization using HPV genotype-specific probes. Then, the prevalence of 28 different genotypes was estimated. Approximately 70% of all samples were infected with at least one HPV type. The most prevalent high risk (HR) HPV types associated with the development of cervical cancer were HPV types 16, 31 and 51. Prevalence of HPV types, as well as appearance of them as a single or multiple infection were also studied in samples with normal and abnormal cytology. Our analysis showed that infected women with normal cytology in our study population tend to have single HPV infections. In contrast, those with abnormal cytological phenotype tend to have multiple infections of HR HPV types.

Keywords: HPV, cytology, multiple infections

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Abbreviations:

HPV: Human Papillomavirus

HR: High Risk

MR: Moderate Risk

LR: Low Risk

SILs: Squamous Intraepithelial Lesions

ASCUS: Atypical Squamous Cells of Undetermined Significance

LGSILs: Low-grade Squamous Intraepithelial Lesions

HGSILs: High-grade Squamous Intraepithelial

Introduction

The human papillomavirus (HPV) infection is one of the most common sexually transmissible infections in the world. More than 120 different HPV types have been catalogued and more than 40 are known to infect the epithelium of the anogenital tract and other mucosal areas of the body (de Villiers et al., 2004). Based on their association with cervical cancer and precursor lesions, HPVs can be classified as high (HR), moderate (MR) or low (LR) oncogenic risk according to their involvement in the genesis of benign or malignant conditions (Munoz et al., 2003).

Among them, at least 16 HPV types are considered as high risk HPV and are strongly associated with the progression of cervical lesions and invasive cancer. HR HPV include 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 types. MR HPV types 26, 53 and 66 are less frequently found in cancers but are often detected in squamous intraepithelial lesions (SILs). Finally, LR HPV types (6, 11, 40, 42, 43, 44, 54, 55, 69, 70, 71 and 74) are occasionally found in cervical carcinomas (Bernard et al., 2010). However, two of them, HPV type 6 and HPV type 11 are associated with low-grade cervical cell abnormalities and laryngeal papillomas (Quiney et al., 1989). Recent studies demonstrate that more than 90% of genital warts are caused by these low-risk HPV types (Lacey, 2005).

There are several studies indicating the prevalence of HPV types in different regions around the world (Bosch et al., 2008; Clifford et al., 2005b). However, the frequency of HPV types may vary according to age, geographic, demographic and clinical-pathological factors (Clifford et al., 2005a; Li et al., 2011). Focusing on cytological phenotype, Centers for Disease Control and Prevention (CDC) indicates that HPV infects the cutaneous epithelium which may lead to cervical cell abnormalities. HPV infection may be the result of a single type (single infection) or more than one type of the virus (multiple infections). Multiple infections seem to play an important role in the progression of cervical cancer and are detected in high frequency in cervical carcinomas (Huang et al., 2004).

Considering the need to prevent HPV infection, it is essential to identify the prevalence of cancer-associated genotypes. Among Greek population, the prevalence of HPV in women is high, with HPV infection being quite common especially in younger ages (Papachristou et al., 2009). HR HPV type 16 seems to be the most prevalent in Southern Greece, detected either as a single or a multiple infection (Stamataki et al., 2010). Here, we studied the prevalence of HPV types in a cohort of Greek women and attempted to correlate single or multiple HPV infections with their cytological phenotype.

Materials and Methods
Samples

Cervical samples (n=627) from individual women ages 16-66 having their annual check-up, cervical screening or gynaecological exam were routinely collected by medical doctors-gynaecologists in primary care clinics. Cervical samples were collected using ThinPrep Pap Test (Hologic), either with a plastic spatula for adequate sampling from the ectocervix or an endocervical brush device. Samples were rinsed into sterile ThinPrep bottles containing PreservCyt solution and sent to the laboratories of Biohellenika for HPV DNA testing. Each cervical sample obtained a code upon arrival in our labs. Samples included in the study were collected from 2009 to 2015. All

women were informed by their gynaecologists that the results of the DNA test may be used for a retrograde statistical analysis of HPV prevalence in Greece. Experimental data were handled according to the regulations of the Hellenic Data Protection Authority. All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

In clinical practice, women are mainly subjected to a DNA test for HPV when there are indications of cytological abnormalities detected by either a Pap test or a histological analysis. To this end, Pap results were classified according to the most severe diagnosis as negative for intraepithelial lesion or malignancy, Atypical Squamous Cells of Undetermined Significance (ASCUS), Low-Grade Squamous Intraepithelial Lesion (LGSIL) and High-Grade Squamous Intraepithelial Lesion (HGSIL) (Solomon et al., 2002). For study purposes, ASCUS, LGSILs and HGSILs were considered as abnormal cytology. Thus, cervical samples were categorized into two groups, according to their cytological phenotype. Group 1 comprises of 440 samples from women having abnormal cytology and Group 2 consists of 187 samples from women that presented normal cytology according to their Pap Test. Furthermore, infected samples were divided into groups harbouring either single or multiple HPV infections. In single infected samples, only one type of the virus was detected. On the other hand, up to 7 HPV types were detected simultaneously in samples with multiple infections.

Sample handling and DNA isolation

All samples were handled under sterile conditions. Experimental procedures were performed in a cleanroom. DNA was isolated from each sample using the Nucleospin Tissue Kit (Macherey-Nagel), according to the manufacturer's instructions. DNA concentration was measured using the Nanodrop Spectophotometer.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed using the INNO-Lipa HPV Genotyping Extra Amp Kit (Innogenetics). With this PCR-based amplification kit, 28 different genotypes of the human papillomavirus are identified. Detection is performed using the SPF10 primers which amplify a 65bp region in the conserved L1 region of the HPV genome. An additional set of primers for the amplification of the human HLA-DPB1 reference gene is contained in the reaction mix to monitor sample quality and extraction.

Reverse Hybridization

PCR products were reverse-hybridized using the INNO-Lipa HPV Genotyping Extra Kit (Innogenetics) which detects HR, MR and LR HPV genotypes. The reverse-hybridization assay offers a platform for highly specific probe hybridization. All DNA probes are immobilized as parallel lines on membrane strips, on which 28 HPV sequence-specific DNA probe lines and 4 control lines are fixed. HPV types 44/51 and 69/71 are detected by a single DNA probe, respectively. Control lines are: a) the Conjugate control which controls the addition of reactive Conjugate and Substrate solution during the detection procedure, b) the human DNA control line, which is the fragment of the human HLA-DPB1 reference gene, c) two HPV control lines. PCR products (10ul) were mixed with equal volume of Denaturation solution for 5min at room temperature. Then, Hybridization solution (2ml) was added and samples

were hybridized to the immobilized probes at 49°C for 1h. Probes were washed and incubated with streptavidin-conjugated alkaline phosphatase for 30min at room temperature. Finally, HPV-specific hybrids were detected using NBT/BCIP chromogen. According to the manufacturer, a sample was considered HPV positive when the human DNA control was detected and at least one of the type-specific lines and one of the HPV control lines were positive. Moreover, all HPV types were scored as negative in the sample if the type-specific line pattern (columns of the Interpretation chart) was a subset of the full line pattern observed on the strip. When the human DNA control line was detected but no HPV specific line was positive, the sample was considered as negative.

Validation of the results
The results of the INNO-Lipa HPV Genotyping Extra Amp Kit were validated using the PapilloCheck kit (GreinerBioone) for HPV DNA typing. Twenty samples of our group population were randomly selected and used to perform the microarray method according to the manufacturer's instructions.

Statistical Analysis
Statistical analysis was performed using the SPSS software. The type-specific HPV prevalence and 95% Confidence Interval (CI) were estimated for all HPV types detected within our study population (Supplementary Table 1). A two tailed *z*-test of a single proportion was used to discover whether statistical difference exists between the proportion detected from multiple infections rather than that from single infections. The analysis was performed either for infected samples with either abnormal/normal cytology (Table 2) or

any HR HPV type detected in our study population (Supplementary Table 2). The null hypothesis was that the occurrence of any of the two type of infections is random (50%-50%, Ho: p=0.5, H$_1$≠0.5). If the *z-value* of the test was found to be either less than -1.96 or greater than 1.96 (z<-1.96 or z>1.96) the statistical difference (alpha = 0.05) between the two proportions is significant and the null hypothesis is rejected. In the opposite case, the null hypothesis stands and no significant difference is reported.

Results
Detection of HPV infections
Consecutive cervical samples (n=627) were collected. Pap smear reported that 440 samples had abnormal cytological findings (70.17% of total population) while 187 samples (29.82% of total population) displayed normal cytology. Among those with abnormal cytology, 290 cervical samples (46.25% of total population) presented ASCUS, 122 samples (19.46% of total population) had LGSIL and 28 samples (4.46% of total population) presented HGSIL in the Pap smear. Samples were then routinely tested for HPV infection. Samples from 439 women (~70% of total population) were found to be positive for infection with various HPV types, while 188 women were negative for any HPV infection (~30% of total population). Further analysis indicated that 325 samples (ASCUS: n=208, LGSIL: n=98, HGSIL: n=19) from those with abnormal cytology (n=440) harboured HPV infections, indicating the high correlation between epithelial lesions and HPV infection (Table 1).

Prevalence of HPV types
The prevalence of HR, MR and LR HPV types in infected women of our study population (n=439) was then analyzed. The prevalence of any HPV

Table 1: Cytology and HPV infection of total samples. Table shows the number of total cervical samples classified according to their cytology and HPV infection.

HPV infection	Abnormal Cytology						Normal Cytology		Total samples	
	ASCUS		LGSIL		HGSIL					
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
n	208	82	98	24	19	9	114	73	439	188
Total samples per category	290		122		28		187		627	
	440									

Table 2: Single/Multiple Infections of HPV infected samples. Table shows the number of infected cervical samples detected with either single or multiple infections, according to their cytology. It also shows the statistical score (p-value); this indicates whether a statistical difference exists between the proportion of single vs multiple infection in Groups 1 and 2 (ns: non significant, **: p-value <0.01).

| Infection	Group 1 (Abnormal Cytology)			Group 2 (Normal Cytology)		
	Single	Multiple	p-value	Single	Multiple	p-value
n	170	155	ns	74	40	**
Total	325			114		

type is shown in Figure 1. HPV types 16, 51 and 31 were the most common HR types detected in these samples. Type 16 appeared in 22.1% (95%CI: 21.5-22.7), type 31 in 18% (95%CI:17.4-18.6) and type 51 in 17.5% (95%CI: 17-18.1) of infected samples. Moreover, HPV types 53 and 66 were the most prevalent MR types, being detected in 13.9% (95%CI: 13.4-14.4) and 12.5% (95%CI: 12-13) of all infected women, respectively. Finally, types 44/55, 74 and 6 were the most common LR HPV types appearing in 6.4% (95%CI: 6-6.8), 5% (95%CI: 4.7-5.3) and 5% (95%CI: 4.7-5.3) of infected samples (Figure 1, Supplementary Table 1). Validation of the results obtained with the INNO-Lipa HPV Genotyping Extra Kit was performed in twenty randomly selected samples using a secondary screening method (PapilloCheck kit).

Prevalence of HPV types according to their cytology
Infected samples were also analyzed according to their cytology. They were categorized in Group 1, samples from women with abnormal cytology (n=325) and Group 2, samples from women with normal cytology (n=114). 170 samples (52.3%) belonging to Group 1, harboured a single HPV infection and 155 samples (47.7%) multiple HPV infections. On the other hand, 74 samples (64.9%) belonging to Group 2 were detected with a single HPV infection and only 40 samples (35.1%) with multiple HPV infections (Table 2). The results of a single proportion two tailed *z*-test showed that statistical difference (p<0.01) exists only between the proportions of Group 2 (women with normal cytology), indicating that women with normal cytology in our study population tend to harbour single HPV infections.

We also analyzed the prevalence of any HPV type in infected samples (n=439) according to their cytology. The most prevalent HR HPV types in samples from Group 1 (abnormal cytology) were 16, 51 and 31; in samples belonging to Group 2 (normal cytology) HPV types 16, 31 and 56 were mainly detected (Supplementary Figures 1 and 2). Interestingly, the most prevalent HR HPV types in Group 1 tend to appear within the context of multiple infections. Single
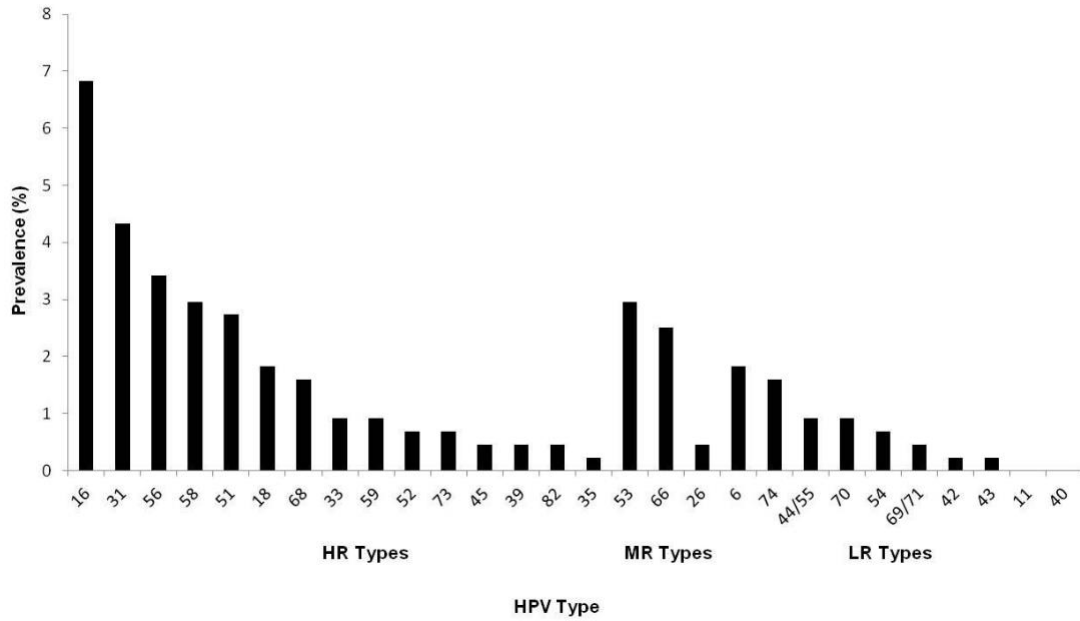
Figure 1: Prevalence of HPV genotypes in a population of Greece. Figure shows the prevalence (%) of High risk (HR), moderate risk (MR) and low risk (LR) HPV types in infected women.

and multiple infections of the most prevalent HR HPV types were then studied for infected samples of Group 1 (Supplementary Figure 1). Two tailed *z*-test of a single proportion showed that for 5 HPV types (HPV31, 18, 39, 73, 33), statistical difference between the two proportions is significant ($p < 0.05$); in all cases multiple infections are detected more often than single ones (Supplementary Table 2).

Discussion

HPV infections are fairly common nowadays. Genetic studies have shown the prevalence of various HPV types worldwide, indicating that HPV infection correlates with the age and the sexual behavior of the individual across different geographical regions (Smith et al., 2008). In the present study, HPV types in consecutive samples tested in our laboratories (n=627) were retrospectively statistically analyzed. As expected, a high percentage of these samples were positive for HPV infection. Several

women are preventively tested for HPV infection. However, clinical doctors tend to propose a DNA test for HPV mainly when cytological abnormalities are detected. In our case, 70.17% of all samples presented cytological abnormalities. This may explain the high ratio of HPV infected samples in our study population. Our results are in agreement with other population studies which also indicate a high prevalence of HPV infection in women with abnormal cytological findings in Greece (Argyri et al., 2013; Stamataki et al., 2010).

We also observed that several samples with abnormal cytology, were negative for HPV infection. This suggests that an abnormal cytological phenotype may not be solely associated with an HPV infection. However, we cannot exclude the possibility that this represents a limitation of our method which may fail to detect viral loads in some samples. Additionally, several samples with normal cytology were also scored as positive for HPV infection. In these samples, it is

possible that low concentration of the virus can be detected by DNA analysis earlier than the appearance of epithelial lesions (Liaw et al., 1999).

We also studied the prevalence of the different HPV types and found that the high risk types 16, 51 and 31 were more often detected in this study population. The results are in agreement with similar studies, indicating the high prevalence of these types in Southern European countries (Castellsague et al., 2012; Giorgi Rossi et al., 2010; Pista et al., 2011a). HPV type 16 is the most prevalent type worldwide, an observation which was also reported in a recent cross-sectional study carried out in Greece (Agorastos et al., 2014). It should be noted that over 70% of all cervical cancer cases are related to the presence of HPV types 16 and 18. Based on these observations highly effective vaccines against this type have been designed (Joura et al., 2007; Paavonen et al., 2007) and a large vaccination program has been launched in order to prevent cervical cancer.

Furthermore, we studied the prevalence of single and multiple HPV infections in infected women with abnormal or normal cytology. In agreement with previous observations (Dickson et al., 2014; Pista et al., 2011b), we found a tendency for multiple HR HPV infections in samples with abnormal cytological findings. Specifically, our results indicate a statistically significant detection of HR HPV 31 within multiple infections. Similar tendencies were found for other HR HPV types (Supplementary Table 2). However, more samples should be tested before drawing a concrete conclusion for the participation of these genotypes in multiple infections. Concerning infected women with normal cytology, we found a statistically significant difference towards single HPV infections (p<0.05). Therefore, a normal phenotype may gradually transit to a more severe cytological status when there are multiple HPV infections.

In conclusion, here we show that single HPV infections are often detected in Greek women with normal cytology. Also, HR HPV genotypes are mainly detected within multiple infections in women with abnormal cytology. This suggests that women with single HPV infections are more likely to present epithelial lesions associated with cervical cancer, if they are infected with more than one type of the virus. Thus, we propose that regular HPV DNA testing in all ages, regardless the result of the Pap test, public information about the consequences of HPV infection and promotion of the prophylactic methods may help to reduce HPV prevalence within the Greek population.

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Supplementary Materials
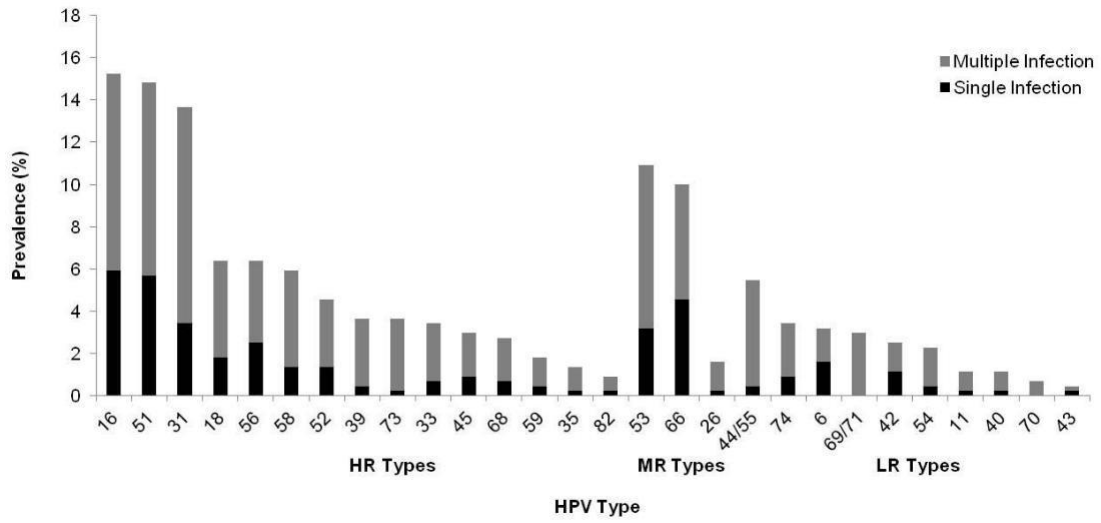
Supplementary Figure 1: Prevalence of HPV genotypes in samples with abnormal cytology. Figure shows the prevalence (%) of High risk (HR), moderate risk (MR) and low risk (LR) HPV types in infected women with abnormal cytology. Columns also indicate the participation of each genotype in single or multiple
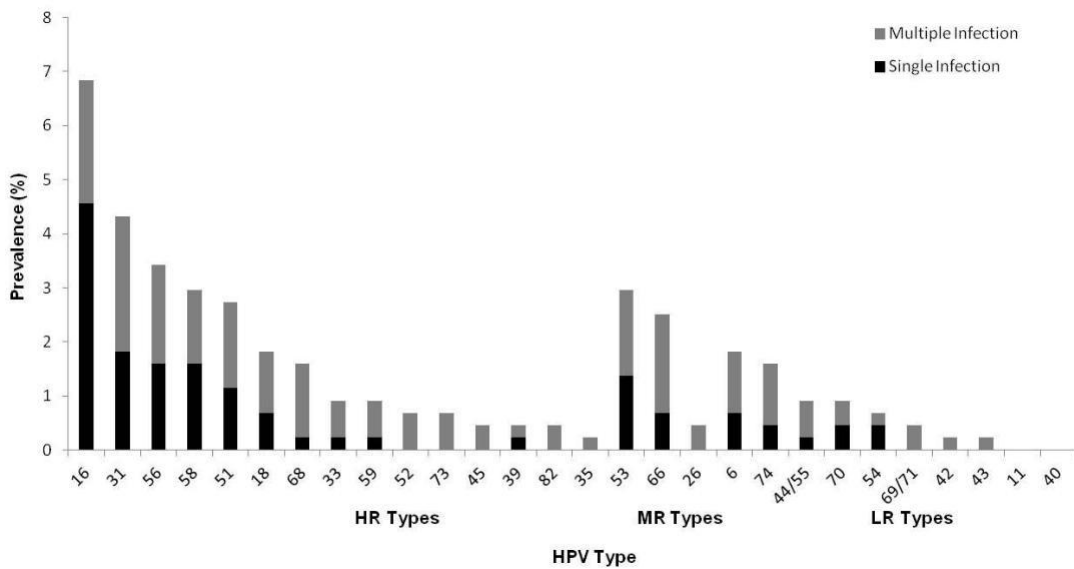
Supplementary Figure 2: Prevalence of HPV genotypes in samples with normal cytology. Figure shows the prevalence (%) of High risk (HR), moderate risk (MR) and low risk (LR) HPV types in infected women with normal cytology. Columns also indicate the participation of each genotype in single or multiple infections.

Supplementary Table 1

HPV Type		Infected Samples		CI	
		n	**%**	**low**	**high**
HR	**16**	97	22,096	21,462	22,729
	31	79	17,995	17,409	18,582
	51	77	17,540	16,959	18,121
	56	43	9,795	9,341	10,249
	58	39	8,884	8,449	9,318
	18	36	8,200	7,781	8,619
	52	23	5,239	4,899	5,579
	33	19	4,328	4,017	4,639
	68	19	4,328	4,017	4,639
	39	18	4,100	3,797	4,403
	45	15	3,417	3,139	3,694
	73	13	2,961	2,702	3,220
	59	12	2,733	2,484	2,983
	35	7	1,595	1,403	1,786
	82	7	1,595	1,403	1,786
MR	**53**	61	13,895	13,367	14,423
	66	55	12,528	12,023	13,034
	26	9	2,050	1,834	2,267
LR	**44/55**	28	6,378	6,005	6,751
	6	22	5,011	4,678	5,345
	74	22	5,011	4,678	5,345
	69/71	15	3,417	3,139	3,694
	54	13	2,961	2,702	3,220
	42	12	2,733	2,484	2,983
	70	7	1,595	1,403	1,786
	40	6	1,367	1,189	1,544
	11	5	1,139	0,977	1,301
	43	3	0,683	0,558	0,809

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Supplementary Table 1: Prevalence of HPV genotypes in Greek women. Table shows the number (n) and prevalence (%) of HPV types detected in infected samples (n=439). It also shows the range of values for a 95% confidence interval (CI) of our analysis.

Supplementary Table 2

HPV type		n	Single Infection	Multiple Infection	*z*-value	p-value
HR	**16**	67	26	41	-1,7883	ns
	51	65	25	40	-1,8226	ns
	31	60	15	45	-3,4641	***
	18	28	8	20	-2,0815	*
	56	28	11	17	-1,1384	ns
	58	26	6	20	-1,9085	ns
	52	20	6	14	-1,6609	ns
	39	16	2	14	-2,4	*
	73	16	1	15	-2,634	**
	33	15	3	12	-1,9926	*
	45	13	4	9	-1,2943	ns
	68	12	3	9	-1,549	ns
	59	8	2	6	-1,2649	ns
	35	6	1	5	-1,3587	ns
	82	4	1	3	-0,8944	ns

Supplementary Table 2: Detection of HR HVP types in samples with abnormal cytology. Table shows the number (n) of HR HPV infected samples harbouring single or multiple infections. It also shows the z-value and statistical score (p-value); this indicates whether a statistical difference exists between the proportion of single vs multiple infection of a HR HPV type (ns: non significant, *: p <0.05, **: p<0.01, ***: p<0.001).