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Original Article

Evaluation of the management of Hr-HPV+/PapTest- women: results at 1-year recall

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Abstract: With cervical cancer screening the choice of 1-year as a period of follow-up in positive high-risk HPV women without cytological lesions is still under discussion. We evaluated the management of these women and the role of HPV genotyping test. We did a cervical cancer screening study of women aged 35-64 with primary high-risk HPV test. Women positive for high-risk HPV with negative cytology were followed-up after 1 year. In this study we selected women with high-risk HPV+/PapTest- resulted high-risk HPV+ at recall and performed the PapTest and HPV genotyping test. The detection rate of squamous high grade (CIN2+) relative to the total screened cohort was 2.1‰, and it was 0.2‰ at the 1-year recall. The colposcopy performed in women referred at the 1-year recall accounted for 48.8% of the total (baseline + 1-year recall), and 84.3% of these women had no cytological lesions. The most frequent hr-HPV genotype detected was HPV16 and 66.7% of co-infections were due to HPV16 and HPV18. 54.5% of women presented a persistent infection at 1-year recall with the same HPV subtype, 50% of persistent infections was due to HPV16 and 16.7% of these were determined to be CIN2+ histological lesions. Our data show that it may be useful to extend the period of follow-up for women hr-HPV+/PapTest- so as to reduce the number of unnecessary colposcopies due to the transitory infections and that the genotyping test could help to identify the persistent infections in which HPV16 is involved.

Keywords: Cervical cancer, screening, HPV, PapTest, follow-up, genotyping

Introduction

Cervical cancer represents the fourth most common cancer in women worldwide [1]. The strength of cervical screening is the possibility to get an early diagnosis of high grade cervical intraepithelial neoplasia (CIN2+), the precursor lesion of invasive cervical cancer, before the appearance of symptoms [2, 3]. It is well established that Human Papillomavirus (HPV) infection is a necessary condition for cervical cancer development [4] and recently, the use of molecular methods for the detection of HPV DNA in cervical cell samples was proposed [5]. The International Agency for Research on cancer has identified 12 high-risk HPV (hr-HPV), classifying them as carcinogens [6]. The use of automatized molecular test to detect hr-HPV in cervical screening increases the diagnostic capability of CIN2+compared to PapTest [7, 8], especially in women 35 years of age and over [9, 10]. Given this evidence, the Italian screening guidelines of GISCi (Italian Association of Cervical Screening Programs) actually recommend the use of Hybrid Capture 2 hr-HPV DNA test (HC2; Qiagen, Gaithersburg, MD) as primary screening test in women aged 35-64 [3, 11]. Nevertheless, HPV infection is very common and usually clears spontaneously within 1-2 years [12, 13] and an appropriate cytological triage is considered a necessary strategy to maintain an adequate level of specificity of the test and to avoid unnecessary colposcopies and possible over-diagnosis [3, 14]. The GISCi guidelines in case of normal cytology after a positive molecular test (hr-HPV+/PapTest-) recommend that women be recalled to perform HC2 test after 1-year and, if they result positive again, they are to be directly referred forcolposcopy [3].

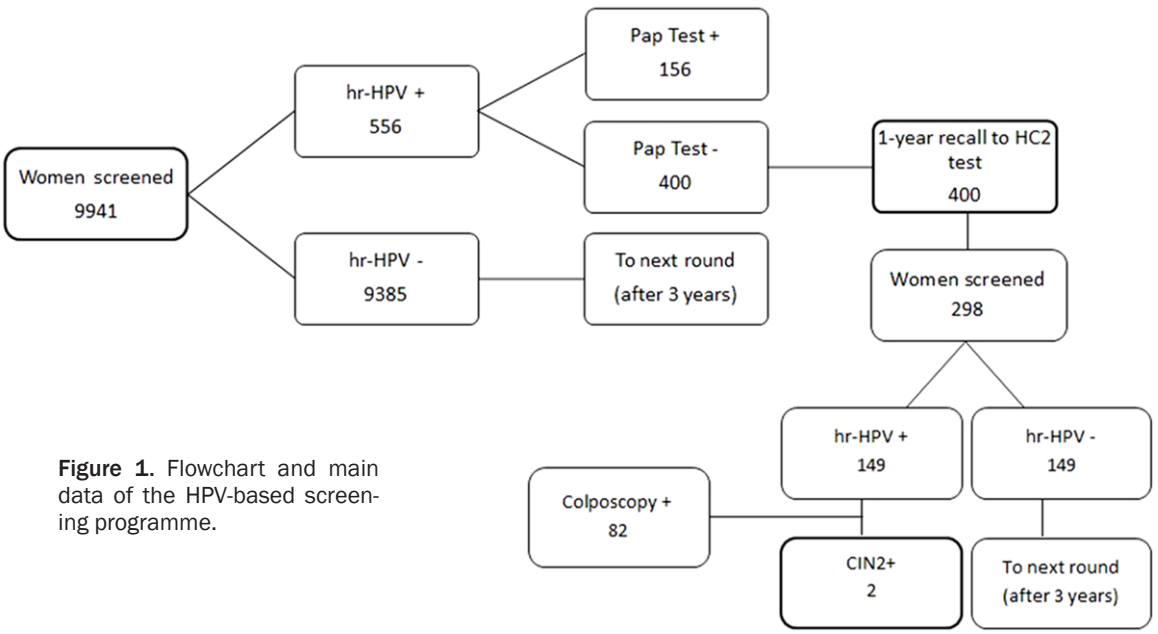


Figure 1. Flowchart and main data of the HPV-based screening programme.

A critical issue of HPV screening program that can affect the screening efficacy, is the compliance to 1-year recall of women hr-HPV+/PapTest- at baseline [14], indeed the choice of 1-year as a period of follow-up is still subject matter for discussion [15, 16]. Currently, it has been hypothesized that the use of HPV genotyping tests that identified specific HPV subtypes could improve the screening program [17]. However, literature data is not yet sufficient to confirm that view.

The aim of the study was to assess the follow-up period of hr-HPV+/PapTest- women in order to understand if 1 year is the correct period to be able to identify CIN2+ lesions without increasing unnecessary colposcopies. Moreover, we will try to understand if the genotyping test could improve the effectiveness of the screening program.

Materials and methods

Study population

The Pathology Unit of ICOT Hospital, Department of Medical-Surgical Sciences and Bio-Technologies, Sapienza University of Rome and Screening Unit of Local Health Unit of Latina, have been running a new organized cervical-screening in the Latina district since 2012. The study population include women aged 35-64, which were invited by mail to perform HC2 test from April 2012 to June 2013 that had resulted hr-HPV+/PapTest- at baseline and that were recalled to perform HC2 test after 1 year. hr-HPV+ women at the 1-year recall were referred to colposcopy in accordance with GISCI guidelines. Therefore, we invited the hr-HPV+ women at the 1-year recall to perform PapTest (BD SurePath™, Franklin Lakes, NJ, USA) and we performed the HPV sign® Genotyping Test (Diatech Pharmacogenetics, Jesi, IT) in all women hr-HPV+/PapTest+ at the 1-year recall, in selected women hr-HPV+/PapTest- at the 1-year recall and on samples of the same women at baseline. We used the women hr-HPV- at the 1-year recall as control.

Hybrid capture 2 high-risk HPV DNA test (HC2)

Exfoliated cervical cells were collected using a cytobrush and eluted in the Sample Transport Medium (STM, Qiagen, Hilden, DE). Cervical specimens were denatured to disrupt the virus and release the target DNA. The RNA probes were diluted in a probe diluent and once loaded all the samples, calibrators, controls and reagents, the hybridization phase began according to supplier's instructions. The chemiluminescent reaction was measured by luminometer (DML instrument, Qiagen, Hilden, DE) and the emitted light was measured as RLU. For each reaction were used three negative controls, three positive controls, one quality control for low-risk HPV (lr-HPV) and one quality control for hr-HPV. The HPV subtypes detected

Table 1. Results of HPV screening program in women aged 35-64

	Baseline			HPV+/PapTest- at the 1-year recall		
	N	%	‰	N	%	‰
Women invited	32,988			400		
Women screened	9,941	30		298	74.5	
Compliance to HC2 test	9,941/32,988	30		298/400	74.5	
Proportion of HPV positive tests	556/9,941	5.6		149/298	50	
Proportion of HPV+/Pap test +	156/556	28		10/64*	15.6	
Proportion of HPV+/Pap test -	400/556	72		54/64*	84.3	
Referral rate to colposcopy	156/9,941	1.5		149/9.941	1.5	
Compliance to colposcopy	150/156	96		140/149	94	
PPV for CIN2+	21/150	14		2/140	1.4	
DR CIN2+ relative to the total	21/9,941		2.1	2/9,941		0.2
DR CIN2+ at recall				2/298		6.7
Overall DR CIN2+	23/10239		2.25			

*234 women who didn't want to perform PapTest.

by the assay are 12 hr-HPV (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) and 1 probable carcinogen HPV subtype (HPV 68), without determining the specific HPV subtype present in the sample. Samples that showed a RLU ≥ 1 pg/ml were considered positive. After HC2 test, the specimens were stored at -20°C.

Cytology: Pap test

The cervical cell samples were obtained by using a cytobrush and were put in PreservCyt solution; liquid-based cytology was performed by using the Sure Path system (BD SurePath™, Franklin Lakes, NJ, USA). One slide per woman was prepared according to the supplier's instructions. Cytological and histological diagnosis was reported according to 2001 Bethesda System [2].

HPV sign® genotyping test

Total DNA was extracted from the same STM sample used previously for HC2 test by QIAamp DNA Mini Kit (Qiagen, Hilden, DE) following the manufacturer's instructions. The concentration and quality of extracted DNA was assessed by amplification of housekeeping gene β-actin on Agilent Bioanalyzer (Agilent Technologies, Waldbronn, DE). For each sample, according to the supplier's instructions, a hypervariable region of a highly conserved HPV L1 gene was amplified by Real-Time PCR (Rotor-Gene Q, Qiagen, Hilden, DE) based on EvaGreen™ dye chemistry, using human β-globin gene as internal control. A negative control and four positive controls provided by the manufacturer were included in the process. Samples that showed amplification of HPV L1 gene were considered suitable for pyrosequencing. 20 µL of biotinylated amplification products were immobilized in the sepharose beads coated with streptavidin (Streptavidin Sepharose™ High Performance, GE Healthcare Bio-Sciences AB, Upsala, SE). Immobilized amplification products were denatured and washed using PyroMark Q24 Vacuum Workstation (Qiagen, Hilden, DE) and subsequently they were annealing with four sequencing primers recognizing a broad spectrum of HPV subtypes, including hr-HPV (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), intermediate-risk (ir-HPV: HPV26, 53, 66, 67, 68, 73, 82, 84, 90, 91) and lr-HPV (HPV6, 11, 34, 40, 42, 43, 44, 54, 70, 72, 81, 89). At the end of this step, the clinical samples and the positive controls were mixed with enzymes, substrate, dNTP (Qiagen, Hilden, DE) and they were processed for sequencing reaction. Pyrosequencing was performed on PyroMark Q24 instrument (Qiagen, Hilden, DE) and results were analyzed using IdentiFire version 1.0.5.0 software package (Biotage AB, Uppsala, SE). The identification of HPV genotype was considered correct if the score of the correlation between the reference sequence and the sequence of the clinical sample was higher than or equal to 85%.

Results

32,988 women aged 35-64, were invited to perform HC2 test in health district of Latina from April 2012 to June 2013 and 9,941 (30%)

Table 2. Results of the genotyping test at the baseline and at the 1-year recall

	Baseline		1-year recall			
ID	Genotype		Genotype		Cytology	Histology
1	33		16		LSIL	CIN1
2	58		58		LSIL	
3	16		66*		ASCUS	CIN1
4	59		16		LSIL	
5	35		35		LSIL	CIN2
6	16		56		LSIL	
7	16	70*	16	70*	HSIL	CIN1
8	16	18	16		ASCH	CIN2
9	33		33		ASCH	CIN1
10	31		18		NILM	
11	43**		33		NILM	
12	16		16		NILM	
13	45		18		NILM	
14	16	31	16		NILM	
15	66*		18		NILM	
16	16		16		NILM	CIN1
17	18		54**		NILM	
18	18		81**		NILM	
19	81**		81**		NILM	
20	45		45		NILM	
21	16	18	16		NILM	
22	18		18		NILM	
23	16		negative			
24	16		negative			
25	70*		negative			
26	67*		negative			
27	16		negative			
28	16		negative			
29	16		negative			
30	18	16	negative			
31	18		negative			
32	18	16	negative			
33	16		negative			

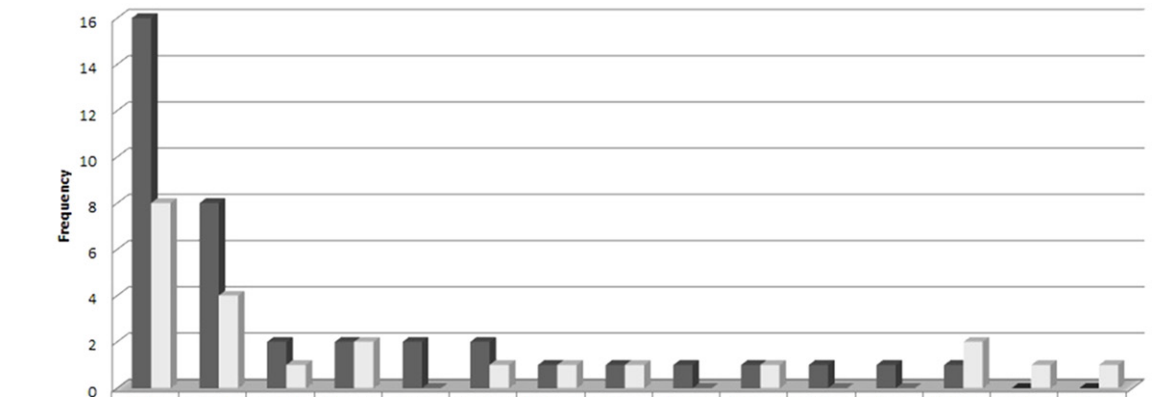
*Intermediate-risk-HPV/**low-risk-HPV. NILM: Negative for Intraepithelial Lesions or Malignancy.

were screened, as detailed in **Figure 1**. Overall, 556 (5.6%) women resulted hr-HPV+ at baseline and 156 (28%) of them resulted positive to PapTest. Women hr-HPV+/Pap test- (400, 72%) were invited to repeat hr-HPV test after 12 months, with referral rate (RR) for HC2 test to 1-year of 4%. Among these, 298 (74.5%) attended to the hr-HPV test: 149 (50%) resulted still positive to hr-HPV test and were directly referred to colposcopy, with RR for colposcopy to 1-year of 1.5%. Overall, 140 (94%) women were examined and 82 (58.6%) presented a positive colposcopy result, moreover, CIN1 and CIN2+ were detected in 77 (94%) and 2 (2.4%) cases, respectively (**Figure 1**). The detection rate (DR) for CIN2+ at the 1-year recall was 6.7‰ (2/298), while the positive predictive value (PPV) for CIN2+ was 1.4%. The DR for CIN2+ at the 1-year recall relative to the total screened cohort was 0.2‰ (2/9941) while the overall DR for CIN2+ of the HPV program (baseline and 1-year recall) was 2.2‰ (23/10239). The proportion of CIN2+ detected at the 1-year recall was 0.67% (2/298) (**Table 1**).

For the aim of our study, 64 (43%) women hr-HPV+ at the 1-year recall agreed to be subjected to the Pap test and 10 (15.6%) of them presented a positive cytology result. Atypical squamous cells of undetermined significance (ASC-US) was detected in 1 (10%) case, low-grade squamous intraepithelial lesion (L-SIL) was detected in 6 (60%) cases, atypical squamous cells-cannot exclude high grade SIL (ASC-H) in 2 (20%) cases and high-grade squamous intraepithelial lesion (H-SIL) was detected in 1 (10%) case. Between them, the 60% (6/10) showed a histological lesion (4 CIN1 and 2 CIN2+) (**Table 2**).

HPV genotyping test was performed on 33 women with baseline hr-HPV+/PapTest- which, 9 (27.3%) resulted hr-HPV+/PapTest+ (one woman was excluded due to insufficient HC2 residual material to perform the molecular analysis), 13 (39.4%) resulted hr-HPV+/PapTest- and 11 (33%) resulted hr-HPV- at the 1-year recall.

Most frequent genotype detected was HPV 16, identified in 16/33 (48.5%) baseline and 8/22 (36.4%) 1-year recall hr-HPV positive women (**Figure 2**); between the 8 women positive to HPV16 at the 1-year recall, the 50% (4/8) showed a histological lesion (3 CIN1 and 1 CIN2+). The HPV18 was observed in 8/33 (24%) and 4/22 (18%) women baseline and 1-year recall hr-HPV+ respectively; in any case we didn't observe a histological lesion. Moreover, we identified at base line 6/33 (18%) co-infection of HPV 16 with other HPV type (HPV 18, 31, 70); the 66.7% (4/6) of these co-infection was due to HPV16 with HPV18 (**Table 2**). At the 1-year recall only 1 co-infection (HPV16/70) was persistent without shows histological



	HPV 16	HPV 18	HPV 45	HPV 33	HPV 31	HPV 70	HPV 35	HPV 58	HPV 59	HPV 66	HPV 67	HPV 43	HPV 81	HPV 54	HPV 56
Baseline	16	8	2	2	2	2	1	1	1	1	1	1	1	0	0
1-year recall	8	4	1	2	0	1	1	1	0	1	0	0	2	1	1

Figure 2. Distribution of HPV type detected.

lesions while, a woman with an HPV16/18 co-infection at the baseline and only HPV 16 infection at the 1-year recall developed CIN2+ lesion.

We observed that between women resulted hr-HPV+/PapTest- at the baseline, the 15% (5/33) women resulted positive to the hr-HPV test due to cross-reaction because they were infected with an intermediate- (43, 66, 67) or low-risk HPV subtypes (70, 81) (**Table 2**); therefore, none of these developed an histological lesion.

54.5% (12/22) of women hr-HPV+ at the 1-year recall presented a persistent infection with the same HPV subtypes and 50% was due to HPV16. Moreover, 16.7% (2/12) showed a high grade histological lesion (CIN2+) and one of these was due to HPV16. The 45.5% (10/22) hr-HPV+ women at the 1-year recall, developed new HPV subtype infection but none of these developed a high grade histological lesion.

Discussion

This study describes the follow-up of women baseline hr-HPV+/PapTest- who attended the new HPV-based screening program organized by the Pathology Unit of ICOT Hospital, Department of Medical-Surgical Sciences and Bio-Technologies of Sapienza University of Rome and Screening Unit of Local Health Unit of Latina, since 2012. Women hr-HPV+/PapTest- have been tested from April 2012 to June 2013 and they have been followed for 1 year.

We observed that 30% of invited women attended the hr-HPV test and this value is consistent with the regional average (29.3%) reported in the previous three years [18] as we already described in our previous work [19]. The baseline hr-HPV positive rate (5.6%) was in line with the value observed in NTCC study where the hr-HPV test was performed on women aged 35-60 and the proportion of women with baseline hr-HPV+/PapTest- was comparable to data reported in the literature (4% vs. 3.6%) [20].

We observed that compliance to 1-year recall of women hr-HPV+/PapTest- at baseline was 74.5% and this value was similar to that observed in other Italian national studies [21], it was probably due to the success of the information campaign carried out in the Italian and Latina district. Compared to the population screening at baseline, in the group of women hr-HPV+/PapTest- at the 1-year-recall we found a higher percentage of cases hr-HPV+ (50% vs. 5.6%); moreover, we detected only a small number of high-grade lesions (2/298). Indeed, the contribution rate for the detection of CIN2+ in the group hr-HPV+/PapTest- at the 1-year recall was much less than expected (0.67%). Even if the data of HPV clearance (50%) was consistent with the literature [15], the 1-year recall resulted in an increase of unnecessary colposcopies and negatively affected the PPV for CIN2+ (1.4%); also, 84.4% (54/64) of women at the 1-year recall had no cytological lesions. Therefore, to improve the effectiveness of the screening program it may be useful to lengthen the follow-up period to repeat the hr-HPV test (e.g. 18-24 months), in order to increase the clearance rate, although a too long follow-up

period could affect the compliance of colposcopy.

Nearly 3% (305/9941) of women were referred to colposcopy at baseline and at the 1-year recall and the colposcopy performed in women referred at 1-year recall accounted for 48.8% (149/305) of the total. Indeed, the detection rate of CIN2+ it was only 0.2‰ at the 1-year recall compared to 2.1‰ of the total screened cohort; so, an additional triage at this stage could decrease the amount of unnecessary colposcopies.

The genotyping test allowed us to confirm that HPV16 is the most common HPV subtype as well as reported in literature and also in our previous study [22, 23] both at baseline and at the 1-year recall (48.5% and 36.4%) and that 50% of histological lesions at the recall were observed in women infected with this HPV subtype. The persistence of infection with an hr-HPV is most important in the development of cervical cancer [24]; in this study we observed that 54.5% of women had a persistent infection with the same HPV subtype and that 16.7% of them developed a CIN2+ histological lesion. We noted that this persistence was linked prevalently to the presence of HPV16 (50%) effectively, infection with HPV16 tend to persist longer than with other HPV subtypes regulating the proliferation and promoting the neoplastic transformation of epithelial cells [25].

HPV18 represents the second HPV subtype responsible of the development of cervical cancer [22]; we observed that it was present in 24% and 18% of women hr-HPV+ at baseline and 1-year recall respectively but in our study we observed that HPV18 did not cause cervical lesions probably due to the age of the examined women (35-64). This data is in agreement with the hypothesis already considered by us that the proportion of high grade lesions caused by HPV18 is prevalent among young women [19, 26].

It is known that co-infection with HPV subtypes could lead to cervical lesions [27] and its role is studied for the possible implication in vaccine efficiency [28]. In our study 18% of women hr-HPV+ at baseline presented a co-infection and 66.7% was due to HPV16 with HPV18. We observed that a woman with an HPV 16 persistent infection and with an HPV16-18 co-infection at baseline showed a high grade cervical lesion at the recall.

The use of cytological triage in combination with the hr-HPV test was recommended by the Italian national guidelines to improve the detection rate of high grade CIN and to reduce colposcopy referral rate, especially in women aged 35-64 [3]. Indeed, the hr-HPV test alone determined a high rate of positive tests and this is sometimes due to a cross-reaction with intermediate or low risk HPV subtype that does not determine a cervical lesion [29]. In this study, we observed that at baseline in 5/33 women (15%) the positivity to the hr-HPV test was the consequence of a cross-reactivity with non-oncogenic HPV types and this data was comparable to the literature [29]; indeed, they did not show a cytological alteration at the 1-year recall but, even if only little, the cross-reactions increased the number of colposcopies. Our data, even if it needs to be confirmed on a larger population, could lead us to speculate that it may be more appropriate to lengthen the follow-up period of baseline hr-HPV+/PapTest- women in order to reduce unnecessary colposcopies without increasing the risk of CIN2+.

Moreover, as already described in our previous study the genotyping of HPV16-18 at baseline may be a useful tool but not as alternative method to cytological triage [22]. However, it could be also useful to identify HPV16 persistent infections and HPV16 and 18 co-infections in order to discover the CIN2+ lesions in hr-HPV+/PapTest- women independently of the follow-up period.

Disclosure of conflict of interest

None.

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