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A retrospective study on two cohorts of immunocompetent women treated with nonavalent HPV vaccine *vs.* Ellagic acid complex: outcome of the evolution of persistent cervical HPV infection

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Abstract. – OBJECTIVE: The nonavalent HPV vaccine has demonstrated its efficacy in women and men who already suffer from HPV genital lesions, with little chances to clear the infection. The efficacy of new therapeutic or complementary alternatives as *Ellagic acid* plus *Annona Muricata* (*Ellagic acid complex)* has emerged recently. Our retrospective study compares the evolution of persistent cervical HPV infection in two cohorts of immunocompetent women after the administration of nonavalent vaccine or *Ellagic acid complex*.

PATIENTS AND METHODS: At Tor Vergata University Hospital, Rome, forty women in childbearing age, suffering from persistent cervical HPV infection, were enrolled in two study's groups: nonavalent HPV vaccine (20 women) *vs. Ellagic acid complex* tablets (20 who refused the vaccine). Cytological features, HPV DNA genotypes and mRNA oncogenic genes E6/E7 presence and clearance were analyzed and confronted between the groups.

RESULTS: Demographics and clinical features of the cohorts were comparable. Evaluation of Pap smear, HPV DNA test and mRNA genes E6/E7, were performed at baseline (T0) and after 6 months (T1) and 12 months (T2) from the last dose of vaccine/tablet. At T1 and T2, *Ellagic acid complex* group showed a statistical reduction of abnormalities in Pap smears ($p = 0.018$ and 0.006, respectively), probably due to its direct anti-inflammatory, antioxidative and antiviral activities. At T1, vaccinated group showed a higher rate of HPV clearance ($p = 0.001$), instead *Ellagic acid complex* group didn't report significative differences. At T2, respect to T0, both groups showed an increase in percentage of negative HPV DNA detection, although more marked for vaccinated group respect to Ellagic acid complex group ($p = 0.039$ and 0.062 respectively). Regarding mRNA E6/E7 clearance, at T1 and T2, the group of vaccinated women showed a higher negativization respect to the other group ($p = 0.077$ and 0.042, respectively).

CONCLUSIONS: Despite the limited sample of women enrolled for the present study, the results confirmed the clinical usefulness of HPV vaccination as adjuvant agent for the immune system of women affected by persistent HPV infection. Moreover, in women who refused to be vaccinated, the administration of a biocompound like *Ellagic acid* plus *Annona Muricata,* represented an interesting clinical strategy in terms of increasing chance of HPV viral clearance.

Key Words:
Nonavalent HPV vaccine, Ellagic acid, Annona Muricata, Persistent HPV infection.

Introduction

HPV (Human Papilloma Virus) is considered the leading agent of neoplastic evolution of cervical, vulvar, and anal lesions in women and men, mainly due to the expression of E6 and E7 oncogenic genes[1]. Although HPV infection is transient, several studies have demonstrated that the persistence of high-risk HPV genotypes could lead to the progression of cervical intraepithelial neoplasia[2]. Among the factors that influence the persistence of HPV infection or the development of low grade cervical preinvasive lesions, the most relevant are age, immune system status, smoking, nutritional factors, concomitant sexually transmitted

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diseases and HIV, estroprogestinic (E/P) use, and the viral integration into the host cell genome. Although the "persistence" of HPV infection is characterized by the continuing detection of HPV or by the viral latency, the distinction with the "recurrent" infection is still considered arbitrary because it could depend on the natural history of infection, the timing of samplings and the interval between samplings[3]. In '40s Papanicolau test (Pap smear or Pap test) has modified the approach of public health as screening test for cervical cancer. More recently, HPV testing has allowed both the knowledge of biology of HPV related cancers and the development of HPV prophylaxis. In 2020 the World Health Assembly adopted a global strategy to accelerate the elimination of cervical cancer to speed up the prevention of cervical cancer worldwide, requiring the introduction of HPV vaccines in the national immunization programmes[4]. In Italy, HPV vaccination has been offered to the young girls since 2007 and young boys were included ten years later. Vaccination is still offered out of pocket to MSM (men who had sex with men), immunocompetent women older than 25 years and HIV (Human Immunodeficiency Virus) patients[5]. The introduction of HPV primary prophylaxis for adolescents by bivalent (HPV16/18) and quadrivalent (HPV 16/18/6/11) vaccines has proven to be the best way to reduce the burden of HPV-related cancers, as well to enhance the herd immunity against the oncogenic HPV genotypes[6]. In addition, the most recent introduction of nonavalent (9-valent) HPV (6/11/16/18/31/33/45/52/58) vaccine has demonstrated its safety, tolerability, efficacy even in women and men who already suffer from recurrent or persistent HPV genital lesions with poor chances to clear the infection. Clinical trials and observational studies have showed the reduction of H-SIL risk (high grade squamous intraephitelial lesion) in previously infected patients, but there are few data available regarding the reduction of recurrence of HPV infections along with the viral clearance[7,8]. Although the topical therapies (e.g., imiquimod cream, sinecathechin ointment, epypodophyllotoxin compound) or the ablative therapies (e.g., laser) against HPV lesions haven't always showed benefit for such patients, in recent studies, the efficacy of complementary therapeutic strategies with biocompounds like *Ellagic acid* and *Annona Muricata* emerged as a new therapeutic option[9,10]. *Ellagic acid* is a phenolic compound naturally present in nuts and berries and *Annona Muricata* is a tropical plant. Both show antioxidant, chemopreventive and antiviral activity[11,12]. Nevertheless, in those patients suffering from recurrent or persistent HPV infection it hasn't been compared the efficacy of nonavalent HPV vaccine *vs.* therapeutic alternatives. In this study, we analysed two groups of immunocompetent women suffering from persistent HPV infection for more than one year and resistant to the conventional treatments. One group of twenty women received the nonavalent HPV vaccine and the other group of twenty, who refused the vaccine, received the *Ellagic acid complex*. We evaluated both the effect of nonavalent HPV vaccination and *Ellagic acid complex* on the evolution of cervical lesions, such as ASC-US (atypical cells of undetermined significance) and L-SIL (low grade squamous intraepithelial lesion), and on the HPV genotypes clearance.

Patients and Methods

Study Design

From June 2017 to August 2020, in collaboration with the Infectious Diseases Unit, 40 women affected by HPV infection were evaluated at the Cervical and Vaginal Pathology Outpatient Service at Tor Vergata University Hospital (Rome, Italy).

Inclusion criteria: immunocompetence, age between 18-53 years and persistent HPV infection. Persistent infection was defined by the second detection in a year of the same HPV genotype, both low-risk and high-risk, in absence of any HPV negative result.

Exclusion criteria: Pregnancy, genital/anal neoplasms, H-SIL, treatment with salicylic acid or cortisones or immunosuppressive therapy, treatment with antibiotics, other infections, allergy to yeasts or other vaccines, relevant diseases (such diabetes or hypertension).

All women fulfilling criteria were invited to participate at a counselling session conducted by the Infectious Diseases consultant and the Gynecology consultant. During the session, the patients received detailed information on HPV infections and HPV vaccines and all of them were visited and evaluated for the enrolment in the study groups (HPV nonavalent vaccine *vs. Ellagic acid complex*).

Patients

40 enrolled women were divided into two groups: group A (nonavalent HPV vaccine) represented by twenty women that accepted to receive nonavalent HPV vaccine called Gardasil 9® (Merck), containing L1 Virus Like Particle of 6,11,16,18,31,33,45,52,58 genotypes according to the schedule of the three doses. Before and after the administration of each dose of HPV vaccine, all women were monitored for the vital parameters and observed after the injection. Clinical status and late side effects were also assessed by a phone call seven days after each dose of vaccine.

The other group of women, group B (Ellagic acid complex), was represented by twenty women, who re-

Table I. Demographic data of group of HPV 9-valent vaccinated women (group A) confronted with group of women treated with Ellagic acid Complex (group B).

Demographic data	Group A (*n*=20) HPV 9-valent vaccine	Group B (*n*=20) *Ellagic acid complex*	*p*-value
Age years (mean ± SD)	35.0 ± 8.9	35.5 ± 8.8	0.846*
BMI kg/m^2 (mean ± SD)	24.1 ± 1.3	24.9 ± 1.9	0.760*
Current smokers n (%)	7 (63.6%)	4 (36.4%)	0.288*
Cigarette/day (mean ± SD)	12.3 ± 9.1	8.4 ± 4.7	0.374*
E/P use n (%)	4 (20%)	5 (25%)	0.910*
Previous LEEP n (%)	3 (15%)	4 (20%)	0.890*

(*) Test ANOVA One-way.
Abbreviation: n: number of women; BMI: body mass index; E/P: estroprogestinic; LEEP: Loop Electrosurgical Excision Procedure.

fused the vaccination and accepted to receive 16 mg *Ellagic acid*, 100 mg *Annona Muricata* in a single tablet, called *Ellagic acid complex,* marketing as OasitK® (BioSTILOGIT Pharmaceuticals, Florence, Italy) once a day for 12 months.

Aim of Retrospective Study

Evaluation of cervical cytology, detection of HPV DNA and research of E6/E7 oncogenic transcripts, in both groups at T0 (baseline) and T1 (after 6 months) and T2 (after 12 months) from the last dose of HPV vaccine (Group A) or tablet of *Ellagic acid complex* (Group B) to detect:

1) A change in cytological features after treatment with nonavalent HPV vaccine (group A) or *Ellagic acid complex* (group B).
2) A clearance of HPV genotypes in group A and group B.
3) A clearance of E6/E7 mRNA transcripts in group A and group B.

Evaluation at Baseline (T0) and Follow-Up

At baseline (T0) group A and group B were evaluated for antibodies against sexually transmitted diseases (HIV, Hepatitis B and C, Syphilis) by blood sampling and for other cervico-vaginal infections (*Neisseria gonorrhoeae*, *Mycoplasma*, *Chlamidya trachomatis, Candida species*) by genital swabs. At T0 other concomitant factors potentially involved in the persistence of HPV infection (e.g., smoking, estro-progesterone assumption) were evaluated. Previous surgical procedures, like Loop Elettrosurgical Excision Procedure (LEEP), were considered too.

All those factors were similarly distributed in group A and group B, as well as demographics features like the age and the Body Mass Index (BMI) and are reported in Table I.

Both groups were monitored for HPV infection at baseline (T0) and six months (T1) and twelve months (T2) after the last dose of vaccination or tablet. HPV evaluation included PAP test, HPV DNA genotyping and research of the E6/E7 HPV transcripts. Colposcopy (if necessary) was performed according to the recommendations of the Italian Society of Colposcopy and Cervical Pathology (SICPCV) 2016-2019.

Side Effects

In the group A (nonavalent HPV vaccine) four women (20%) complained mild swollen and pain at injection site after the first dose, lasting no more that 7-10 days. Nobody complained major side effects. In the group B (*Ellagic acid complex*) nobody reported side effects during the period of treatment.

Analysis of Samples

The following examinations were performed by the same specialized health professionals.

Cervical cytology

Ecto and endocervical cells were taken using an Ayre's spatula and cytobrush, respectively. The cells were streaked out on two distinct sections of a glass slide, fixed and sent to the Pathology Department of Tor Vergata University Hospital. Smears were classified according to the 2014 Bethesda system: ASCUS, L-SIL, H-SIL[13].

HPV DNA testing and typing

HPV screening and genotyping were performed at the Laboratory of Molecular Virology, Tor Vergata University Hospital. Cervical cells were collected from the transition zone of the cervical canal, suspended in 20 ml of PreservCyt solution (Hologic, Marlborough, MA, USA) and stored according to the manufacturer's instructions until analysis. An aliquot of 5 ml was transferred into a collection tube and centrifuged at 839 x g for 3 min. The supernatant was then discarded, and the cellular pellet stored at 80°C prior to analysis.

Table II. Baseline T0 features (PAP test, HPV genotype and E6/E7 mRNA) of Group A (9valent HPV vaccine) and Group B (Ellagic acid complex) patients.

Baseline features		Groups			*p*-value
		A (*n*=20) *n* (%)	B (*n*=20) *n* (%)	Total (*n*=40) *n* (%)	
PAP test	Negative	10 (50.0)	14 (70.0)	24 (60.0)	0.197^
	ASC-US/L-SIL	10 (50.0)	6 (30.0)	16 (40.0)	
HPV genotype	Low Risk	4 (20.0)	3 (15.0)	7 (17.5)	0.677^
	High Risk	16 (80.0)	17 (85.0)	33 (82.5)	
E6/E7 mRNA	Negative	16 (80.0)	12 (60.0)	28 (70.0)	0.168^
	Positive	4 (20.0)	8 (40.0)	12 (30.0)	
Colposcopy	Negative	15 (88.2)	12 (60.0)	27 (73.0)	0.054^
	CIN1	2 (11.8)	8 (40.0)	10 (27.0)	

(^) Chi^2 test.
Abbreviation: ASC-US: atypical squamous cells of undetermined significance; L-SIL: low grade squamous intraepithelial lesion; CIN: cervical intraepithelial neoplasia. n: number.

Total DNA/RNA was extracted using a NucliSENS® easyMag® automatic extractor (BioMerieux, Marcy l'Etoile, France). HPVDNA was detected by the Anyplex™ II HPV28 kit HPV according to the manufacturer's instructions[14].

Detection of the E6/E7 transcripts of HPV 16, 18, 31, 33 and 45

The presence of the oncogenic transcripts of HPV 16, 18, 31, 33 and 45 was detected by the Pretec-HPV proofer kit (BioMerieux, Florence, Italy) as previously described[15].

Statistical Analysis

All data were initially entered into an Excel database (Microsoft, Redmond, WA, USA) and the analysis was performed using the IBM SPSS version 25.0 (IBM Corp. Armonk, NY, USA). Descriptive statistics consisted of the mean ± standard deviation (SD) for parameters with gaussian distributions (after confirmation with histograms and the Kolgomorov-Smirnov test). Comparison among groups was performed with the ANOVA one-way or *t*-test Paired Samples for continuous parametric variables or the Chi-Square test or Fisher's exact test (if cells<5) for frequencies variables. A p-value of < 0.05 was considered statistically significant.

Ethics

This retrospective and no profit study was conducted according to the principles of the Declaration of Helsinki and approved by Ethical Committee of Policlinico Tor Vergata (Experimentation register 199/19, approved on 30 October 2019). All patients signed the informed consent.

Results

We evaluated and confronted at T0 (baseline) the status of cervical cytology, colposcopy, HPV genotyping and E6/E7 mRNA in group A (nonavalent HPV vaccine) and group B (Ellagic acid complex) (Table II). All those clinical features were similar between the groups: positive and negative results for cytological screening and colposcopy were proportionally present in both, as well as low and high-risk HPV genotypes and E6/E7 mRNA positivity. In both groups, no sexually transmitted diseases were found neither cervico-vaginal infections. Notably, in group A, cytological abnormalities as ASC-US/L-SIL were found in 50% of Pap smears at T0.

Regarding follow up of cytology (Pap test), at T1 and T2, confronted between group A (nonavalent HPV vaccine) and group B (Ellagic acid complex) all results are presented in Table III. Whilst in group A there was not a significant decrease in cytological abnormalities at T1 and T2 ($p = 0.466$ and 0.121 respectively), in group B a significant decrease in cytological abnormalities was observed at T1 ($p = 0.018$) and T2 ($p = 0.006$). In Figure 1 is represented, as percentage, the marked reduction of cytological alterations at T1 in the group B. Indeed, at T1 the percentage of negative Pap test is 20% if compared with 36% of group A. The activity of vaccine seems to be less rapid to reduce the abnormalities of Pap smears over the time. Such difference between the groups becomes less marked at T2 (9% *vs.*13%). It is important to underline that we reported cases of progression of cytological abnormalities (as L-SIL becoming H-SIL) in both groups, during the study period. Regarding the detection and follow up of HPV genotyping, at T1 and T2, in group A (nonavalent

Table III. Results of cytology (negative *vs.* positive -ASC-US/LSIL – pap test) confronted between group A (nonavalent HPV vaccine) and group B (*Ellagic acid complex*), according to the time (T0: baseline, T1: 6 months, T2: 12 months after the last dose of vaccine/tablet).

	Time		Groups A (*n*=20) *n* (%)	Groups B (*n*=20) *n* (%)	*p*-value A *vs.* B
PAP test	T0	Negative	14 (70.0)	10 (50.0)	0.197^
		Positive	6 (30.0)	10 (50.0)	
	T1	Negative	16 (80.0)	17 (85.0)	0.677^
		Positive	4 (20.0)	3 (15.0)	
	T2	Negative	19 (95.0)	18 (90.0)	0.548^
		Positive	1 (5.0)	2 ((10.0)	
			***p*-value**	***p*-value**	
	T0 *vs.* T1		0.466^	0.018^	
	T0 *vs.* T2		0.121^	0.006^	

(^) Chi^2 test.

HPV vaccine) and group B (Ellagic acid complex), all results are represented in Table IV.

Confronting group A with group B, we appreciated a significant decrease for HPV DNA genotypes detection at T1 (*p*-value = 0.011). Similar observation was observed at T2, when HPV DNA detection decreased more in group A than group B (*p*-value 0.047). Indeed, in group A there was a significant decrease in the detection of HPV at T1 ($p = 0.001$) and T2 ($p = 0.039$) respect to T0, instead in group B there was not a statically significant decrease of positivity of high-risk HPV test at T1 ($p = 0.144$) and T2 ($p = 0.062$) respect to T0. In Figure 2 it is shown the percentage of HPV positive confronted between group A *vs.* B, over the time. Probably in group B the period of treatment with *Ellagic acid complex* was too short to exert its antiviral activities for HPV clearance. On the other hand, HPV vaccine has demonstrated its efficacy earlier, just after the second dose, in line with recent studies[16-18].

Regarding results of follow up and clearance of E6/E7 mRNA in group A and group B, all data are represented in Table V. In group A, four patients were HPV positive with detection of E6/E7 mRNA at T0 (two of them showed HPV genotypes 16, one HPV 31 and one HPV 18). In group B, eight patients were HPV E6/E7 positive at T0 (two showed HPV 31, three HPV 45, two

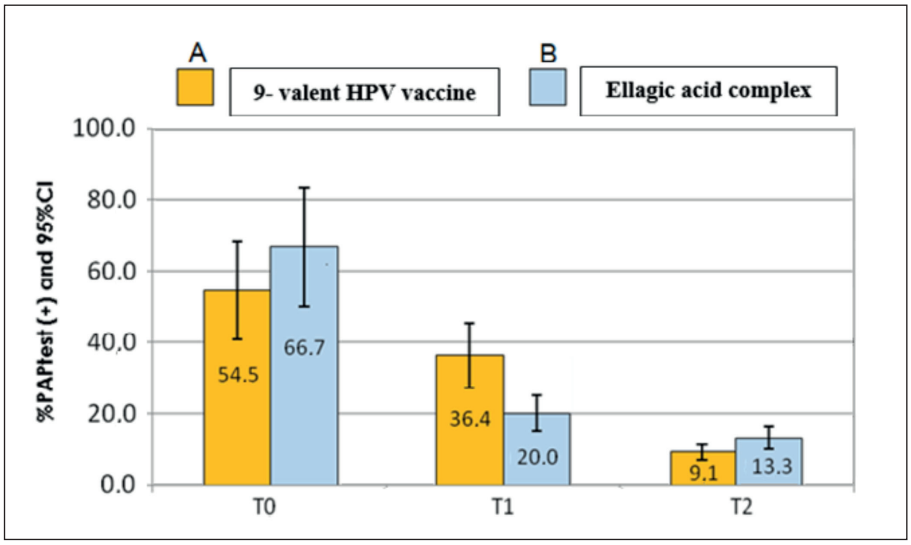

Figure 1. Percentage (%) of cytological abnormalities at baseline (T0), six months (T1) and twelve months (T2) from last dose of vaccine/tablet between group A (20 women treated with 9valent HPV vaccine) and group B (20 women treated with *Ellagic acid complex*).

Table IV. HPV DNA detection test (low and high-risk HPV genotypes) confronted between group B (*Ellagic acid complex*) and group A (nonavalent HPV vaccine) according to the time (T0: baseline, T1: 6 months, T2: 12 months after the last dose of vaccine/tablet).

	Groups				
	Time		A (*n*=20) *n* (%)	B (*n*=20) *n* (%)	*p*-value A *vs.* B
HPV test	T0	LowRisk	4 (20.0)	3 (15.0)	
		HighRisk	16 (80.0)	17 (85.0)	0.677^
	T1	LowRisk	15 (75.0)	7 (35.0)	
		HighRisk	5 (25.0)	13 (65.0)	0.011^
	T2	LowRisk	16 (80.0)	10 (50.0)	
		HighRisk	4 (20.0)	10 (50.0)	0.047^
			***p*-value**	***p*-value**	
	T0 *vs.* T1		0.001^	0.144^	
	T0 *vs.* T2		0.039^	0.062^	

(^) Chi^2 test.

HPV 16 and one HPV 18) and a significant decrease of transcripts was not found at T1 and T2. Therefore, at time T1, mRNA E6/E7 positive test is not significantly different between two groups (*p* value= 0.077). On the contrary, at time T2, the result of mRNA test is significantly different, because group A showed a higher negativization rate than group B, waning to zero (*p*-value 0.042). Indeed, in group A, confronting T0 *vs.* T2, we appreciated a statistically significant reduction of presence of mRNA E6/E7 (*p*= 0.001). Moreover, the percentage of negative HPV E6/E7 mRNA, especially at T2, resulted more marked in the arm of vaccinated women (0% *vs*. 18.8%) (Figure 3).

Discussion

Persistent HPV infection, along with H-SIL, is considered the main risk factor for the development of cervical, vulvar and anal cancers. Women suffering from persistent HPV infection leading to L-SIL and H-SIL, do not often receive benefit from the conventional treatments (for example laser ablation or topical therapy with immunomodulating agents) and facing the recurrence of lesions for months or years and the risk of developing cancer[2,18]. Among the oncogenic HPV genotypes (high-risk types) linked to a worsening of the cervical cytology, HPV 16 is the major responsible of

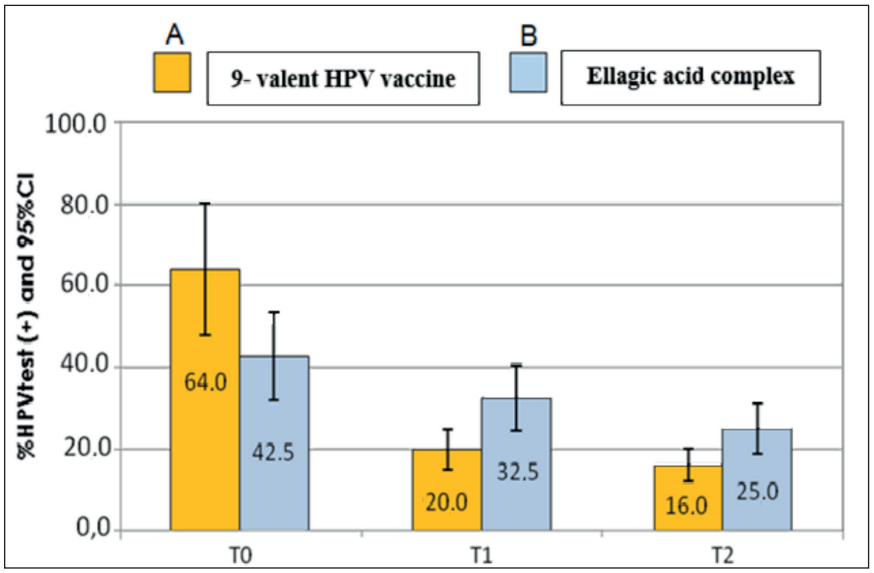

Figure 2. Percentage (%) of HPV DNA test positivity at baseline (T0), six months (T1) and twelve months (T2) from last dose of vaccine/tablet between group A (20 women treated with 9valent HPV vaccine) and group B (20 women treated with *Ellagic acid complex*).

Table V. Results of mRNA E6/E7 detection (negative and positive) confronted between group A (nonavalent HPV vaccine) and group B (*Ellagic acid complex*), according to the time (T0: baseline, T1: 6 months, T2: 12 months after the last dose of vaccine/tablet)

	Groups				
	Time		A (*n*=20) *n* (%)	B (*n*=20) *n* (%)	*p*-value A *vs.* B
mRNA test E6/E7	T0	Negative	16 (80.0)	12 (60.0)	0.168^
		Positive	4 (20.0)	8 (40.0)	
	T1	Negative	19 (95.0)	15 (75.0)	0.077^
		Positive	1 (5.0)	5 (25.0)	
	T2	Negative	20 (100.0)	17 (85.0)	0.042^
		Positive	0 (0.0)	3 (15.0)	
			p-value	*p*-value	
	T0 *vs.* T1		0.151^	0.311^	
	T0 *vs.* T2		0.001^	0.198^	

(^) Chi^2 test.

invasive cervical cancer followed by HPV 18, whilst HPV6 and HPV11 are frequently related to the development of skin and genital warts[19,20]. Indeed, about 3% of women in childbearing age are positive for HPV 16 in absence of abnormalities in their Pap smear[21,22]. Today again the management of persistent or rapidly recurring ano-genital and cervical H-SIL still remains a challenge for physicians. HPV vaccination has demonstrated to protect women without evidence of infection and, partially, women with current infection from further infections or re-infections with the same HPV genotype. As observed by PATRICIA trial, HPV vaccines can keep immunogenicity against cervical intraepithelial neoplasia (CIN) grade 3 associated with HPV16/18 in adulthood[23,24]. VIVIANE study also confirmed that the protection is higher after vaccination in women without HPV infection[25,26]. Observational studies reported that vaccine may reduce the risk of H-SIL recurrence, although it is not known the exact protective mechanism, and that the most of precancerous genital lesions are preventable with HPV vaccines[27-29]. As observed by SPERANZA project, HPV vaccination is also beneficial if added to the surgical treatment for CIN grade 2, showing 80% of clinical effectiveness in prevention of the disease relapse up to 4 years[30]. Because HPV vaccination, in women aged up to 55 years,

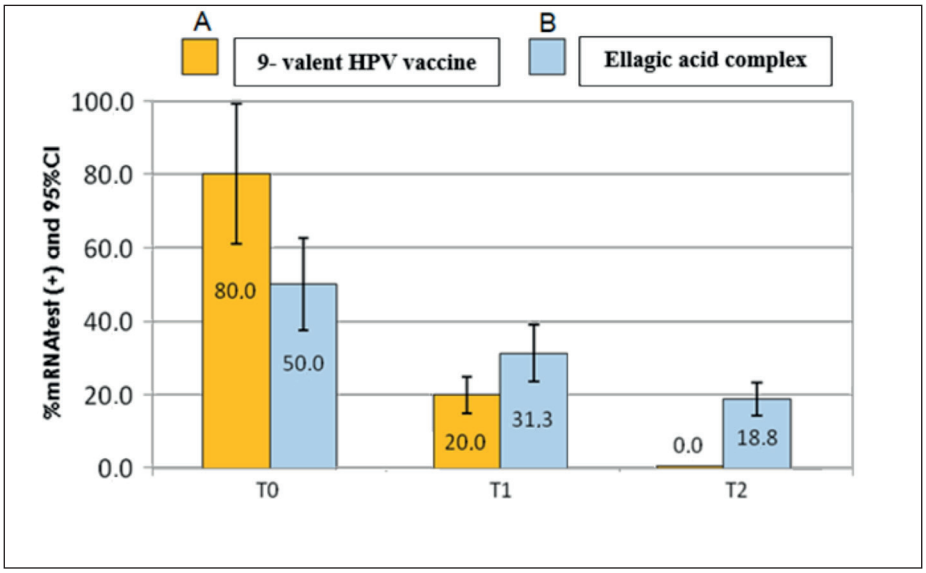

Figure 3. Percentage (%) of E6/E7 mRNA detection at baseline (T0), six months (T1) and twelve months (T2) from last dose of vaccine/tablet between group A (20 women treated with 9valent HPV vaccine) and group B (20 women treated with *Ellagic acid complex*).

have shown almost 90% protection from cervical precancerous lesion caused by HPV16/18 in HPV16/18 negative women, the authors of HPV-FASTER study proposed to expand the indications for HPV vaccination for women up to 30 years old and, in some setting, up to 45-50 years old[23]. In Europe the current HPV vaccines authorized by EMA (European Regulatory Agency) are Cervarix® (HPV 16/18), Gardasil® (HPV 6/11/16/18), Gardasil 9® (HPV 6/11/16/18/31/33/45/52/58). Nonavalent vaccination demonstrated a primary prophylaxis of both cervical cancer and vulvar cancer up to 80% and precancerous lesions up to 70%, with great benefit for health expenses and a cost-effectiveness if including men and boys in immunization campaigns[31]. Indeed, HPV vaccination elicits an immune response that contributes to viral clearance, better than the immune response elicited by the natural infection[32-34]. The Italian HPV Study Group also recommended the HPV vaccination as secondary prophylaxis for males and women already affected by HPV and for those who underwent conservative treatment of genital HPV lesions[35]. Many women with persistence of HPV lesions, who usually receive the topical therapies (as imiquimod cream, sinecathechin ointment, epypodophyllotoxin compound) or the ablative therapies (as laser), not always have received benefit from such treatments[9]. Additionally, several studies have investigated the role of some nutrients suggesting that certain natural compounds provide a protective effect against cervical dysplasia and HPV persistence. Among them, the antioxidant and chemopreventive activity of *Ellagic acid* and *Annona Muricata* have been demonstrated. *In vitro*, both compounds induce cell cycle arrest in the G1 phase, DNA repair and apoptosis[10-12]. A recent study investigated *Ellagic acid* and *Curcumin* at various concentrations showing better anticancer properties than either of the drug when used alone. Besides this, *Curcumin* and *Ellagic acid* also restore p53, induce ROS (radical oxygen species) formation and DNA damage in HeLa cells with decrease of E6 protein[36]. Such result confirms that antioxidants as *Ellagic acid* plus *Annona Muricata* (so called *Ellagic acid complex*) may counterbalance the damaging effects of oxidative stress by arresting the cell cycle, promoting DNA repair, and inducing apoptosis, thus hampering HPV disease progression[37]. In the light of these studies, we decided to compare the efficacy of nonavalent HPV vaccine with the efficacy of *Ellagic acid complex,* in the evolution of persistent HPV infection in immunocompetent women. A retrospective study was designed to analyse the outcomes of administration of both nonavalent HPV vaccine and *Ellagic acid complex,* in two cohorts of women in childbearing age with similar demographics and clinical features (Table I). Twenty of them accepted the course of vaccination (three doses) while the other twenty refused it and received *Ellagic acid complex* (one tablet a day). Regarding the results of cervical cytology, an impressive reduction of abnormalities at T1 were observed in group B (women not vaccinated), in which a lower number of ASC-US lesions were detected at T1 and T2, respect to T0 (Table III). In our opinion, such results could be ascribed to the direct anti-inflammatory, anti-oxidative and antiviral activities of *Ellagic acid complex* maybe more marked than the activity exerted by vaccine in group A. It is significant to point out that during the period of observation, in both groups we didn't appreciate a worsening in Pap test results (L-SIL switching into H-SIL). Regarding the HPV-DNA genotypes assessment (Table IV), we assume that in group B (women not vaccinated) the time of treatment with *Ellagic acid complex* was too short to allow a viral clearance, if compared with group A, for which HPV vaccine is efficacious early, just after the second dose, as reported by previous studies[16-18]. Regarding the detection of E6/E7 mRNA, at T1 (six months from the last dose of vaccine/tablet), both groups didn't show statistical differences respect to T0, instead at T2 (twelve months from the last dose of vaccine/tablet) women who decided to be vaccinated reported a significant decrease of HPV oncoprotein expression, than women treated with *Ellagic acid complex* (Table V). Our results confirm that nonavalent HPV vaccine plays a role in shortening the time of clearance for high-risk HPV types, among sexually active adult women, as already reported by other studies[38,39]. Additionally, women who refused the vaccine but assumed *Ellagic acid complex,* reported a reduction of mRNA E6/E7 detection at T2, although at lower rate than vaccinated women. Indeed, in our study, the variable of time has showed to have the main statistical significance. We argue that because E6/E7 genes are responsible of p53 and Rb inactivation as well as of ROS generation in tissues, HPV vaccine can act stimulating the immune response rapidly, whereas the biocompounds act modulating the signal transduction pathways and showing an intrinsic chemo -preventive action in long term. We are aware that the main limitations of our study are represented by the retrospective analysis, the small cohort of enrolled women, the lack of an adequate randomization and of a control group of women not vaccinated/treated with therapy. The lack of this control group doesn't permit to completely answer the questions of spontaneous clearance *vs.* intervention. In fact, most HPV infections are transient and around 80% are cleared by the immune system after two years since the evidence of the presence of HPV genotypes, before developing a pathogenic and cytopathic effect, especially in women < 30 years old[40]. Our study's population, in

both groups, is instead around 35-year-old, and this data may impact negatively on HPV virus natural clearance with low chances to spontaneous negativization[41,42]. To date, immune mechanisms involved in HPV clearance are not completely clear, and many immune factors, further than cell-mediated immune response, could contribute differently in HPV clearance, as aging progresses[43,44]. Anyway, our results obtained in groups of women older than 30 years old, demonstrate an evident clinical benefit both on cervical cytology and on HPV genotypes presence, in both groups, after 6 months and 1 year from the two different treatments. Nevertheless, in literature we did not find similar analysis that compares the outcomes of administration of both HPV vaccine and biocompounds like *Ellagic acid* and *Annona Muricata*, among women in childbearing age. It will be interesting to conduct further studies that could explore the molecular mechanisms underlying the activity of *Ellagic acid complex* against the persistent HPV infection, and that could follow up different cohorts of vaccinated women and those treated with *Ellagic acid complex*, for more years after the end of treatment.

Conclusions

Our study confirms the clinical usefulness of nonavalent HPV vaccination, not only as preventing agent against HPV in primary prophylaxis, but also as an adjuvant agent of HPV viral clearance, in secondary prophylaxis, in women in childbearing age affected by persistent HPV infection. Notably, among the new therapeutic strategies currently used against HPV infection, the biocompounds like *Ellagic acid* plus *Annona Muricata* (*Ellagic acid complex*) demonstrated efficacy in terms of viral clearance, in women affected by persistent HPV infection, after one year of follow up. Further studies, including larger randomized samples, will surely contribute to improve the knowledge of both the evolution of persistent HPV infection and the better treatment for women or men with persistent HPV infection and HPV related diseases.

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Authors' Contributions

GMFM wrote the manuscript, searched literature, and conducted the patient counselling. FG improved the discussion and conducted the patient counselling. AAC performed both colposcopy and LEEP and conducted the patient counselling. MC providing the characterization of HPV DNA and improved the discussion. RS reviewed the database. SR reviewed the database and provided the statistical analysis. MA and FS read and approved the final manuscript.

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Ethics Approval

This retrospective and no profit study was conducted according to the principles of the Declaration of Helsinki and approved by Ethical Committee of Policlinico Tor Vergata (Experimentation register 199/19, approved on 30 October 2019).

Informed Consent

Informed consent was obtained from all patients involved in this study. Written informed consent has been obtained from each patient to publish this paper.

Conflicts of Interest

All the authors declare no conflict of interest.

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