

***Chlamydia trachomatis* prevalence and chlamydial/HPV co-infection among HPV-unvaccinated young Italian females with normal cytology**

Donatella Panatto¹, Daniela Amicizia^{1,*}, Silvia Bianchi², Elena Rosanna Frati², Carla Maria Zotti³, Piero Luigi Lai¹, Alexander Domnich¹, Daniela Colzani², Roberto Gasparini¹, and Elisabetta Tanzi²

¹Department of Health Sciences; University of Genoa; Genoa, Italy; ²Department of Biomedical Sciences for Health; University of Milan; Italy; ³Department of Public Health and Paediatrics Sciences; University of Turin; Italy

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Abbreviations: CC, cervical cancer; CI, confidence interval; CIN, cervical intraepithelial neoplasia; Ct, *Chlamydia trachomatis*; DNA, deoxyribonucleic acid; HR, high-risk; HPV, human papillomavirus; IARC, International Agency for Research on Cancer; LHU, local health unit; LR, low-risk; NT, untypeable; OR, odds ratio; ORF, open reading frame; PCR, polymerase chain reaction; PR, possible/probable-risk; RFLP, restriction fragment length polymorphism; SD, standard deviation; STI, sexually transmitted infection

Infections caused by *Chlamydia trachomatis* (Ct) and human papillomavirus (HPV) are the two main sexually transmitted infections; however, epidemiological data on Ct prevalence and Ct/HPV co-infection in Italy are scant. This study aimed at estimating the prevalence of Ct infection and Ct/HPV co-infection in young HPV-unvaccinated females with normal cytology, and placed particular attention on the possible association between Ct-DNA positivity and different HPV infecting genotypes. Five hundred 66 healthy females aged 16–26 years without cervical lesions, previously assessed for HPV infection (HPV-DNA prevalence: 18.2%), were tested for Ct-DNA. The overall prevalence of Ct was 5.8% (95% CI: 4.2–8.1), while Ct/HPV co-infection was recorded in 2.7% (95% CI: 1.6–4.3) of subjects. Compared with HPV-DNA-negative females, HPV-DNA positive subjects had significantly ($P < 0.001$) higher odds of being infected with Ct (odds ratio of 4.20, 95% CI: 2.01–8.71). Both Ct and Ct/HPV infections were much more prevalent in under 18-year-olds than in older women. Subjects positive for single high-risk HPV genotypes and various multiple HPV infections had higher odds of being Ct-DNA positive. Our findings confirm that HPV and Ct infections are very common among asymptomatic young Italian females. This underlines the urgent need for nationwide Ct screening programs and reinforcement of sexual health education, which would be the most important public health strategies, since no Ct vaccines are currently available.

Introduction

Sexually transmitted infections (STIs) are a worldwide public health challenge, with more than a million new cases occurring every day; these are caused by more than 30 different bacteria, viruses and parasites.¹ Among these, human papillomavirus (HPV) and *Chlamydia trachomatis* (Ct) are the two main sexually transmitted pathogens.^{2,3}

It is now universally recognized that several oncogenic HPV genotypes are the necessary, but not sufficient, cause of cervical cancer (CC); most women infected with HPV do not develop CC.^{4,5} Various endogenous and exogenous co-factors, including other STIs, that influence the risk of progression from HPV infection to invasive CC exist and have been extensively studied.⁵ Among these potential co-factors, urogenital Ct infection is of

particular concern owing to its wide diffusion and heavy public health burden. Indeed, genital Ct infection is the most prevalent bacterial STI, especially among adolescents and young women.^{6,7} Although often asymptomatic, genital Ct infection in women may lead to numerous serious and costly complications, such as pelvic inflammatory disease, tubal factor infertility, ectopic pregnancy and chronic pelvic pain.^{8,9} In Europe, the prevalence of Ct in unscreened asymptomatic women varies significantly among countries, ranging from 1.7 to 17%.⁶ In Italy, no nationwide population-based studies on the prevalence of Ct infection have yet been performed;¹⁰ Ct case notification is not mandatory and no screening policy exists.¹¹ A few ad hoc studies conducted in Italy^{11–17} have found Ct prevalence rates ranging from 1.8–10.4% among sexually active women, depending on the study population and geographic area.

*Correspondence to: Daniela Amicizia; Email: daniela.amicizia@unige.it

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A specific role of Ct infection in the pathogenesis of cervical intraepithelial neoplasia (CIN) and subsequent CC is biologically plausible, although as yet there is no univocal and well-established evidence on this issue.^{18,19} Earlier studies have found a significantly higher prevalence of Ct-DNA among HPV-positive women than HPV-negative ones^{2,20-22} as well as evidence that Ct is an HPV co-factor in the etiology of displastic and neoplastic cervical abnormalities and CC.²³⁻²⁵ Cervical Ct infection has been clinically documented to be associated with cytological atypia and cervical metaplasia, thus damaging epithelial integrity, allowing the HPV access to the basal epithelium and increasing viral load.^{19,24} Ct may be responsible for increasing the persistence of HPV infection by affecting viral clearance. Furthermore, the natural history of HPV infection may be influenced by Ct-induced chronic cervical inflammation, a reduction in antigen-presenting cells and inhibition of cell-mediated immunity. A local deficit of immunity during the reparative phase of the epithelial surface under inflammatory conditions may make cervical epithelial cells more susceptible to other pathogens.^{18,19}

On the other hand, it has been also suggested that the association between Ct and HPV lies only in their shared route of transmission, as both pathogens are markers of sexual risk behaviors.²⁵

Our previous study²⁶ revealed an overall HPV prevalence of 18.2% among unvaccinated young females with normal cytological findings and a particularly high prevalence (10.1%) of high-risk HPV genotypes, underlining the need to optimize preventive measures against STIs among healthy young females. Owing to the absence of a national surveillance system and few epidemiological data available, we conducted a study to estimate the prevalence of Ct infection and Ct/HPV co-infection in young asymptomatic women without cervical lesions. Our second goal was to assess the possible association between Ct-DNA positivity and various HPV infecting genotypes.

Results

In our previous study²⁶ 566 cervical samples were studied to determine the prevalence of different genotypes of HPV in 16–26-y-old Italian females with normal cytology; the results are reported elsewhere.²⁶ Briefly, 103 HPV-DNA-positive and 463 HPV-DNA-negative samples were identified; in the present study, these samples were analyzed for Ct. HPV-DNA-positive and -negative groups were comparable in terms of age [19.8 (SD 2.5) and 19.7 (SD 2.4) years, respectively; $t_{(564)} = 0.19, P = 0.85$].

Prevalence of *Chlamydia trachomatis* infection

Overall, 33 out of 566 young women were positive for Ct: a prevalence of 5.8% (95% CI: 4.2–8.1%). A higher prevalence of Ct infection was found in Genoa [7.7% (95% CI: 4.5–13.0%)] than in Milan [6.4% (95% CI: 4.1–9.9%)] and Turin [2.3% (95% CI: 0.8–6.6%)]. This difference was not, however, statistically significant ($\chi^2_{(2)} = 4.04, P = 0.13$).

Ct positivity among 16–17-y-olds (n = 27) was at least 3.5-fold higher [18.5% (95% CI: 8.2–36.7%)] than in older age-classes [18–20 y (n = 376): 5.3% (95% CI: 3.5–8.1%); 21–23 y (n = 105): 4.8% (95% CI: 2.1–10.7%); 24–26 y (n = 58): 5.2% (95% CI: 1.8–14.1%)] with a borderline significance level (Fisher exact test: $P = 0.075$).

Chlamydia trachomatis/HPV co-infection

Ct/HPV co-infection was found in 15 subjects [2.7% (95% CI: 1.6–4.3%)]. The prevalence of Ct-DNA positivity was about 3.7-fold higher among HPV-DNA-positive subjects than among HPV-DNA-negative subjects [14.6% (95% CI: 9.0–22.6%) vs. 3.9% (95% CI: 2.5–6.1%); $\chi^2_{(1)} = 17.49, P < 0.001$] with an odds ratio (OR) of 4.20 (95% CI: 2.01–8.71). The Ct-DNA prevalence was highest among adolescents 16–17 y old and tended to be higher among HPV-DNA-positive subjects than HPV-DNA-negative ones across almost all age-classes, although a statistically significant difference was observed only in the 18–20 age-class (Fig. 1). Indeed, within the HPV-DNA-positive group, the mean

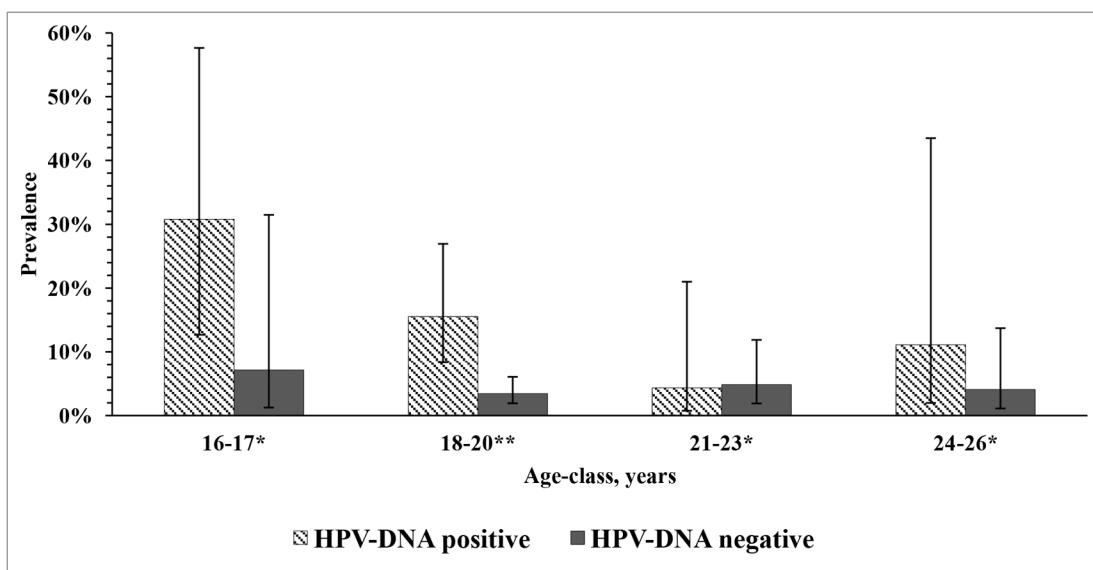


Figure 1. Prevalence of *Chlamydia trachomatis* DNA positivity by age-class and HPV positivity status. *Not significant; **Fisher exact test $P = 0.002$.

age of females with Ct/HPV co-infection was significantly lower ($t_{(101)} = 2.05, P = 0.043$) than that of Ct-DNA-negative females [18.6 (SD 2.1) vs. 20.0 (SD 2.5) years]. The effect size was medium (Cohen's $d = 0.61$). By contrast, no difference emerged between the mean ages of Ct-DNA-positive and Ct-DNA-negative subjects within the HPV-DNA-negative group [19.8 (SD 2.7) and 19.7 (SD 2.3) years, respectively; $t_{(461)} = 0.18, P = 0.86$].

Among Ct/HPV co-infected females, 12 out of 15 [80.0% (95% CI: 54.8–92.3%)] were positive to at least one high-risk (HR) or possible/probable-risk (PR) HPV. The most frequent HPV genotype was HPV-16, which was detected in 4 subjects. By contrast, low-risk (LR) HPV genotypes were much less frequent [13.3% (95% CI: 3.7–37.9%)] (Table 1).

Logistic regression revealed that females infected with single PR [OR = 6.51 (95% CI: 1.60–26.47) $P = 0.009$], double LR+PR [OR = 27.35 (95% CI: 1.47–508.16) $P = 0.026$] and triple HR+HR+LR [OR = 22.90 (95% CI: 1.36–385.80) $P = 0.030$] HPV infections had significantly higher odds of being Ct positive. Although females infected with HR or HR+HR HPV genotypes did not reach statistical significance at the $P < 0.05$ level, the effect sizes were large [OR = 2.98 (95% CI: 0.94–9.44) $P = 0.064$ and OR = 9.84 (95% CI: 0.98–98.84) $P = 0.052$, respectively]. Other types of single or multiple HPV infections (LR, LR+LR, LR+PR, PR+PR, LR+HR, HR+PR and HR+HR+PR) were not statistically significant.

Discussion

This study is among the first to explore some epidemiological features of Ct/HPV co-infection among Italian female adolescents and young women with normal cytology. These results potentially have several implications for future health policy planning. Indeed, knowledge of the prevalence of and risk factors for these two most common STIs is a prerequisite for undertaking effective preventive and control measures.

Most of the few studies on Ct prevalence conducted in Italy have involved women with abnormal cytological findings or symptoms of STIs.^{14,15,17} This makes direct comparison difficult, since our study population was made up of asymptomatic female adolescents and young women with normal cytology. Our Ct prevalence estimate is in line with those of several European population-based studies among sexually active female adolescents and young women, which have reported prevalence rates ranging from 3.6 to 8.0%.^{27–30} Notably, the majority of the women in our sample accessed gynecology centers

seeking the prescription of oral contraceptives (results not shown), and the mode of Ct prevalence among European women seeking contraception is estimated, as in our study, to be about 6%.⁶ In a recently performed meta-analysis, Ct prevalence in high-income countries was estimated to be 4.32% among sexually active females aged ≤ 26 y.¹⁰ By contrast, in a study conducted in Brescia (Italy),¹² an unexpectedly low Ct prevalence of 1.9% among sexually experienced healthy female students was documented. This inconsistency with our results could be explained in several ways. First, it could be due to the difference in study locations, as Ct prevalence varies geographically.⁶ Indeed, we observed a 3.3-fold difference in Ct prevalence between Genoa and Turin; thus, the estimate from Brescia is almost consistent with the figure we recorded in Turin. Second, our study population also included female adolescents aged 16–17 y—the age-class which displayed the highest Ct prevalence—while the study by Matteelli et al.¹² enrolled only women aged ≥ 18 y. Finally, the difference could be ascribed to the recruitment process; the participants in our study were enrolled at the gynecology centers of local health units (LHUs), while those in Brescia were recruited at high schools.

Our results confirm previous findings of significantly higher Ct prevalence among HPV-positive than HPV-negative women.^{2,20–22} A systematic review by Silva et al.¹⁹ reported Ct-DNA positivity prevalence rates of 4–59% among HPV-positive women living in different continents; however, when only estimates from Europe were considered, this range narrowed to 10–24%. By contrast, Ct-DNA positivity among HPV-negative controls reported in their paper was substantially lower, rarely exceeding 10%.¹⁹ Research conducted in Italy by Verteramo et al.¹⁷ found a Ct frequency of 13.9% among HPV-positive women, as against 5.4% among HPV-negative women; these figures are similarly to our estimates. However, in a recent paper by Sammarco et al.,¹³ Ct prevalence in HPV-positive women aged 18–63 y without cervical lesions was estimated to be as low as 6.3%, which is about half

Table 1. HPV genotypes found among *Chlamydia trachomatis* DNA-positive subjects, by urban area and age

Residence	Age, years	HR HPV genotypes	PR HPV genotypes	LR HPV genotypes	Untypeable HPV (NT)
Genoa	16	–	–	11	–
Genoa	17	–	67	–	–
Genoa	17	–	–	87	–
Genoa	17	–	53	–	–
Milan	18	52, 58	66	–	–
Milan	18	52	–	–	–
Milan	18	16	–	–	–
Milan	18	16, 31	–	40	–
Genoa	18	52	–	61	–
Genoa	18	16	–	–	–
Milan	19	–	73	–	–
Turin	19	16, 58	–	61	–
Turin	20	–	–	–	+
Genoa	22	56	–	–	–
Milan	24	–	66	54	–

our figure. Again, the difference observed is very probably due to the different age profiles of the study populations.

In our study, both Ct-DNA positivity and HPV/Ct co-infection displayed age disparities, with a much higher prevalence among female adolescents under 18 y old; this is in line with the well-established evidence that sexually active adolescent females are at higher risk of genital Ct infection than older females.³¹⁻³⁴ Indeed, a meta-analysis by Adams et al.³⁵ revealed that the OR of being infected with Ct diminished as age increased. Similarly, in a large Italian study,¹⁶ female adolescents between 15 and 19 y had the highest Ct prevalence. Another interesting finding concerns the association between the lower age of Ct-infected subjects than of Ct-DNA-negative ones within the HPV-DNA-positive group. That patients with concurrent STIs tend to be younger has already been suggested by Griffiths et al.³⁶ However, Tamim et al.² did not find any association between age and Ct positivity within the HPV-positive group; this was probably the result of the lower sample size in their study. By contrast, our finding not only reached statistical significance at α level <0.05 , this between-group age difference of about 1.5 y is also of practical significance, as demonstrated by the effect size. This suggests the need to strengthen health education, counseling and other preventive measures before sexual debut, which has been estimated to be around 15 y old.³⁷

An association between Ct and HR HPV genotypes has been described previously.¹⁹ As expected, the most frequently detected HPV genotype among our subjects co-infected with Ct was HPV-16, and most of co-infections involved at least one HPV genotype belonging to groups 1, 2A or 2B of the International Agency for Research on Cancer (IARC).³⁸ This finding is not surprising, since HPV-16 is the most prevalent genotype in Italy^{39,40} and in many other geographical areas.⁴¹ Analogous results have been reported by Tamim et al.² who found HPV-16 to be the most frequent genotype in HPV/Ct co-infections. The results of our regression analysis showed that women infected with single HR/PR and different combinations of multiple HPV genotypes with at least one HR/PR HPV type had significantly higher odds of being co-infected with Ct. This finding is of particular importance for the primary prevention of CC, since concurrent Ct infection has been found to be associated with the persistence of HR and multiple HPV genotypes in female adolescents.⁴² In turn, the persistence of HR HPV genotypes among cytologically normal women leads to a greatly increased risk of CC.⁴³

It is noteworthy that the findings from our cross-sectional study did not allow us to determine which infection, HPV or Ct, occurred first. However, our main aim was to determine Ct and Ct/HPV infection prevalence among HPV-unvaccinated young women, and not to establish any causality. Another limitation of this explorative study is its relatively small sample size; this explains the large confidence intervals of the coefficients obtained from the logistic regression model. At the same time, the sample size was sufficient to identify large effect sizes associated with some specific HPV genotypes and their combinations.

In conclusion, the results of the present study highlight the fact that genital Ct infection is common among young HPV-unvaccinated females with normal cytology in three large

metropolitan areas in northern Italy. Approximately one fifth of asymptomatic females in our sample tested positive for HPV and/or Ct, suggesting that the epidemiological features of STIs in Italy may fit into the “silent epidemic” context.⁴⁴ Indeed, the study population referred no symptoms of the STIs confirmed by subsequent gynecological examination. This observation stresses the need for nationwide Ct screening programs, which would be the most important viable and potentially cost-effective public health strategy in the absence of a Ct vaccine.⁴⁵ With regard to Ct vaccines, several candidates are under preclinical development, and a safe and effective vaccine is seen as the ultimate goal in the control of Ct;⁴⁵ indeed, mathematical modeling has shown that fully protective vaccination would eliminate Ct epidemics in about two decades.⁴⁶

Finally, in Italy public health policies towards these two most common STIs display several critical features, including far from optimal HPV vaccination⁴⁷ and cervical screening coverage,⁴⁸ and inadequate Ct case notification and Ct screening;¹¹ these issues must be addressed as public health priorities.

Materials and Methods

The study was approved by the Ethics Committee of the LHU in Genoa, Italy.

Study population

In this study, cervical samples which had already been tested for HPV-DNA were examined to detect Ct-DNA.¹⁶ Briefly, subjects aged 16–26 y were recruited in three cities in northern Italy (Turin, Milan and Genoa). All consecutive eligible young women who spontaneously accessed the gynecology centers of LHUs for medical consultations from 1st January to 30th June 2010 were enrolled.

All participants gave written informed consent. The inclusion criteria were: being sexually active, not being pregnant, not being symptomatic for any STI, not having been vaccinated against HPV, and no previous history of cervical abnormalities. All participants underwent a Pap smear and only those with normal cytology were studied.

Sample collection

Cervical smear samples were collected by means of a spatula, immersed and rinsed in a vial containing 20 ml of PreservCyt® solution (ThinPrep Pap Test, Hologic; cod.70136-001) and stored until processing at room temperature. The samples were analyzed in the molecular laboratory of the Department of Biomedical Sciences for Health of Milan University.

PreservCyt® solution (10 ml for each sample) containing cervical cells was centrifuged at 3800 g for 10 min at room temperature. After centrifugation, pellets were resuspended in 1 ml of phosphate buffered saline, transferred into a new 1.5 ml collection test-tube and stored at -20 °C until nucleic acid extraction.

Nucleic acid extraction

The NucliSENS® EasyMAG® (bioMérieux; cod. 200111) commercial kit was used for DNA extraction following the manufacturer's instructions. A Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc) was used to

ascertain the concentration and purity of the DNA extracted, while DNA integrity was evaluated by amplification of a 268 base pair (bp) fragment in the ubiquitous β -globin gene by using the primer pair PCO₄ and GH₂₀.⁴⁹

HPV DNA detection and Restriction Fragment Length Polymorphism genotype analysis

HPV DNA was detected by PCR amplification of a 450-bp segment of ORF L1, as previously described.^{50,51} Briefly, 50–100 ng of DNA was added to the PCR master mix containing an equimolar mixture of each primer ELSI-f and ELSI-r (30 pmol/ μ l), and the GoTaq[®] DNA Polymerase (Promega; cod. M3175). Amplification reaction conditions were 5 min denaturation at 94 °C, followed by 40 cycles of amplification consisting of a denaturation step at 94 °C for 30 s, an annealing step at 55 °C for 30 s and an elongation step at 72 °C for 30 s. The last cycle was followed by a 7 min elongation step at 72 °C.

Positive (DNA extracted from HPV 16-positive cells, Caski) and negative (water) control samples were inserted in each PCR run. The amplified products were revealed by electrophoresis analysis of 2% agarose gels containing ethidium bromide. The amplification product bands were then compared with molecular weight standards (DNA Molecular Weight, Marker 100, Sigma-Aldrich; cod. G8291).

All amplified fragments were genotyped by means of Restriction Fragment Length Polymorphism (RFLP) capable of identifying all genotypes, including HR, PR and LR genotypes, according to the latest IARC classification.³⁸

One μ g of each amplified product was added to three different digestion solutions, each containing 1U of either *Rsa*I, *Dde*I or *Hae*III (New England BioLabs; cod. R0167L; cod. R0175L; cod. R0108L) restriction enzymes diluted in their respective buffers for 1 h at 37 °C. The digestion products were determined following separation in 3% agarose gels, and restriction patterns were compared with appropriate standards (DNA Molecular Weight, Marker 100+20, Sigma-Aldrich; cod. D7808). The pattern of fragments generated by the three restriction enzymes allowed genotypes to be identified.^{52,53}

Each sample that displayed a complex/undetermined pattern on RFLP was retested. Samples that could not be assigned to an HPV genotype after 2 consecutive analyses were defined as “untypeable” (NT).

Chlamydia trachomatis

DNA detection

A nested PCR assay targeting a 150 bp segment of the cryptic plasmid of Ct was performed. The two primer pairs of primers used in the two PCR runs have previously been described by Jalal et al.⁵⁴ Briefly, 50–100 ng of DNA was added to the PCR master mix containing an equimolar mixture of each primer (25 pmol) and the GoTaq[®] DNA Polymerase (Promega; cod. M3175). Each run included positive controls (DNA extracted from human Ct-positive cervical cells) and negative (water) controls. Amplification reaction conditions were 5 min of denaturation

at 94 °C, followed by 30 cycles (first run) and 25 cycles (second run). Each cycle consisted of a denaturation step at 94 °C for 30 s, an annealing step at 50 °C for 30 s, and an elongation step at 72 °C for 30 s. The last cycle was followed by a 7 min elongation step at 72 °C. The amplification products were revealed by means of electrophoresis analysis of 2% agarose gels containing ethidium bromide. Amplified product bands were compared with molecular weight standards (DNA Molecular Weight, Marker 100, Sigma-Aldrich; cod. G8291).

Statistical analysis

The normally distributed continuous variable “age” was expressed as mean and standard deviation (SD) and the two-sample Student’s *t* test was used to evaluate between-group difference in age. As the measure of effect size for the *t* test, Cohen’s *d* was used. Cohen’s *d* was interpreted as follows: small (*d* = 0.2), medium (*d* = 0.5) and large (*d* = 0.8).⁵⁵ The prevalence of Ct-DNA positivity with 95% confidence intervals (CIs), estimated by using Wilson’s score, was calculated both for the overall study population and by age-class (broken down into 4 age-classes: 16–17, 18–20, 21–23, and 24–26 y old on the basis of previous research²⁶), province of residence and HPV-DNA positivity status. To compare frequency distributions, Fisher’s exact test was performed if 320% of expected frequencies were <5; otherwise, the chi-square test was applied. To detect a possible association between Ct-DNA positivity (dependent variable) and specific HPV genotypes (dummy-coded independent variable with the reference category of HPV-DNA-negative observations), a multi-variable logistic regression analysis was performed; all ORs were adjusted for age and province of residence. Categories of single HPV infections were dummied in accordance with the latest IARC classification³⁸ and presented as HR, PR and LR genotypes. Double and triple infections were dummied on the basis of our previously reported²⁶ different HPV combinations: LR+LR, LR+PR, PR+PR, LR+HR, HR+PR, HR+HR, HR+HR+LR and HR+HR+PR. A two-sided p-value < 0.05 was considered statistically significant. All analyses were performed by means of the R stats package, version 3.0.1.⁵⁶

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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