Italian Journal of
Gynæcology & Obstetrics
September 2021 - Vol. 33 - N. 3 - Quarterly - ISSN 2385 - 0868

Multiple human papillomavirus infection and ASCUS-LSIL progression: a review

B. Gardella, M. Dominoni, S. Morsia, A. Gritti, C. Sosso, A. Arrigo, A. Spinillo

Department of Obstetrics and Gynaecology, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy

ABSTRACT

Recent literature highlighted the role of Multiple Human Papillomavirus (HPV) infections in the development of CIN2+. A better understanding of single-genotype and combined multiple-genotype oncogenic potential has become essential to plan future screening and to evaluate the prospective susceptibility to high risk cervical lesions progression. Analysing the literature data, we evaluated the prevalence and the prognostic significance of multiple HPV infections and their type-specific interactions in women with ASCUS and L-SIL cytology. Multiple HPV infections are detected in about 16.7% of women with ASCUS cytology and in 28.7% of LSIL Pap smear results. In ASCUS patients the rates of severe biopsy proven lesions are 8.6% and 31.6% in women with single and multiple HPV infections respectively. Similarly, in women with LSIL cytology, the likelihood of CIN2+ is 12.5% in patients with single and 28.4% in those with multiple HPV infection. Despite the well-known oncogenic risk of HPV16 and HPV18, recent studies provide a new insight in the high prevalence of other HR-HPVs and their significant contribution to a large proportion of high-grade cervical lesions in women with ASCUS/LSIL. This review provides further evidence that multiple HR-HPV infection is a significant risk factor for severe cervical lesions in women with ASCUS and LSIL cytology, and highlights that increased oncogenic risk might be strictly associated with peculiar type-specific profiles.

SOMMARIO

La letteratura recente ha evidenziato il ruolo delle infezioni multiple da papillomavirus umano (HPV) nello sviluppo di CIN2+. Una migliore comprensione del potenziale oncogeno del genotipo singolo e dei genotipi multipli è diventata essenziale per pianificare lo screening futuro e per valutare la potenziale suscettibilità della progressione delle lesioni cervicali ad alto rischio. Analizzando i dati della letteratura, abbiamo valutato la prevalenza e il significato prognostico di più infezioni da HPV e le loro interazioni tipo-specifiche nelle donne con citologia ASCUS e L-SIL. Infezioni multiple da HPV vengono rilevate in circa il 16.7% delle donne con citologia ASCUS e nel 28.7% dei risultati del Pap-test LSIL. Nei pazienti ASCUS le percentuali di lesioni gravi comprovate da biopsia sono dell'8.6% e del 31.6% nelle donne con infezioni da HPV singole e multiple, rispettivamente. Allo stesso modo, nelle donne con citologia LSIL, la probabilità di CIN2+ è del 12.5% nelle pazienti con infezione da HPV singola e del 28.4% in quelle con infezione multipla da HPV. Nonostante il ben noto rischio oncogeno di HPV16 e HPV18, studi recenti forniscono una nuova visione dell'elevata prevalenza di altri HPV HR e del loro contributo significativo a un'ampia percentuale di lesioni cervicali di alto grado nelle donne con ASCUS/LSIL. Questa revisione supporta il razionale che l'infezione multipla da HPV HR è un fattore di rischio significativo per lesioni cervicali gravi nelle donne con citologia ASCUS e LSIL e sottolinea che un aumento del rischio oncogeno potrebbe essere strettamente associato a profili specifici per tipo particolare.

Corresponding Author: Barbara Gardella
E-mail: barbara.gardella@gmail.com

Copyright 2021

DOI: 10.36129/jog.33.03.02

Key words
ASCUS; high-grade CIN; human papillomavirus; LSIL; multiple infection.

INTRODUCTION

Multiple Human Papillomavirus (HPV) infections in Cervical Intraepithelial Neoplasia (CIN) have gained increasing attention in recent literature. To date, both the biology of inter-genotypic molecular interactions and their clinical implications are yet unclear, even though greater awareness would be necessary for enhanced primary and secondary prevention programs. A large number of surveys (1), point out that immunity towards HPV is predominantly type-specific, with limited cross-action. Hence, a better understanding of single-genotype and combined multiple-genotype oncogenic potential has become essential to plan future vaccination schedules and to evaluate the prospective susceptibility to high risk cervical lesions caused by no-targeted high risk (HR)-HPV genotypes in vaccinated cohorts (2).

Most of the published population data addressing the role of multiple HR-HPV infections on the severity of cervical dysplasia in women with abnormal cytology consider co-infections as a whole rather than highlighting the interactions of single HPV genotypes. Analysing the literature data, we evaluated the prevalence and the prognostic significance of multiple HR-HPV infections and their type-specific interactions in women with Atypical squamous cells of undetermined significance (ASCUS) and Low-grade squamous intraepithelial lesion (LSIL) cytology, providing useful information for their future clinical management.

MATERIALS AND METHODS

We consulted international databases such as PubMed, Cochrane Database of Systematic Reviews, EMBASE, Web of Science, with the following terms and their combinations "multiple Papillomavirus infection AND ASCUS", and "multiple Papillomavirus infection AND LSIL". We included all available article published until 2020 in English, inclusive retrospective or prospective trials, and reviews. We found 459 records from the preliminary bibliographic search. After the elimination of duplicates and after the excluding the works that were manifestly irrelevant, we carefully examined the 96 most recent or significant papers as it appears below. In particular, we excluded abstracts, letters, and oldest article about multiple HPV infection. For the specific purposes of the present work, we performed a further selection of the preliminary set of articles, with a more restrictive criterion, *i.e.*, the role of multiple infection in low grade citology and its clinical application. Finally we consider a total of 15 articles which, in our opinion, represent better the aim of this review (**figure 1**).

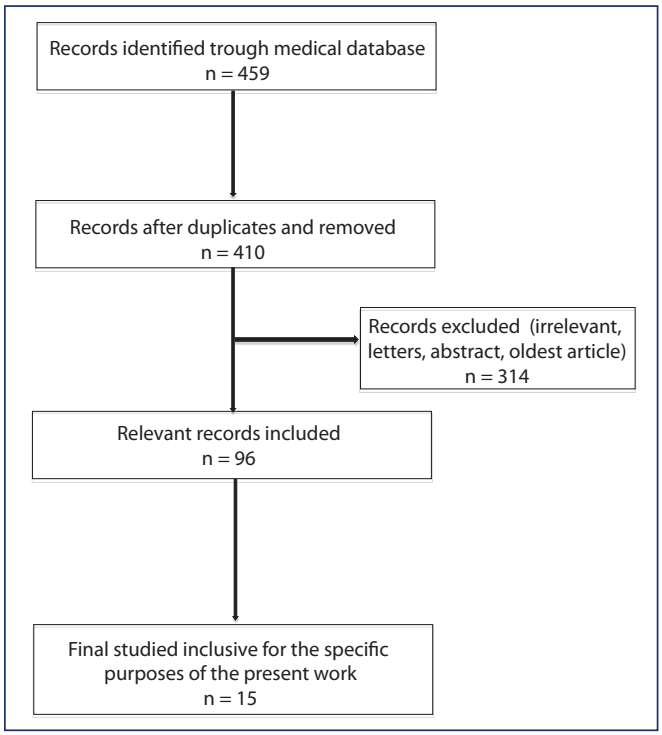

Figure 1. *PRISMA flow chart of research strategy.*

RESULTS

ASCUS and LSIL cytological results are reported in approximately 5% and 2.5% of all cervical Pap smears respectively (3). Recent studies have demonstrated an average HR-HPV positivity rate ranging from 43% to 48.7% in women with ASCUS cytology (4) and from 68% to 82% in women with LSIL cytology (5). The likelihood of CIN2$^+$ is considered to be 7.4 to 12.6% after ASCUS cytology and 17.4 to 18.6% after LSIL results (6, 7). Nonetheless, previous papers have shown that while CIN2$^+$ lesions can be identified in 10.7% of women with ASCUS and HR-HPV positivity, they are found in only 1.5% of women with ASCUS and HR-HPV negativity (4). With regards to LSIL cytology, there is evidence that biopsy-diagnosed High-grade Squamous Intraepithelial Lesion (HSIL) is seen in 16% to 26% of HR-HPV positive but in only 4.3% to 13% of HR-HPV negative LSIL pap tests (5). Therefore, these data provide evidence that

HR-HPV positivity is significantly associated with an increase in risk of high-grade cervical lesions in women with ASCUS/LSIL cytology.

Despite the well-known oncogenic risk of HPV16 and HPV18, recent studies provide a new insight in the high prevalence of other HR-HPVs and their significant contribution to a large proportion of high-grade cervical lesions in women with ASCUS/LSIL (8, 9). Wang *et al*., report that, while based on HPV16/18 model test the rate is only 55.2%, the combination of HPV16/18/31/33/58/52 is able to identify the 93.1% of women with ASCUS cytology but histologically proven CIN2$^+$ (9). These findings are consistent with Demarco *et al*., analyses, which confirm that risk of progression differs substantially by HPV type and can be meaningfully categorized into four groups: (1) HPV16, whose cumulative risk of CIN2$^+$ is 21.5% at 7 years of follow-up; (2) HPV18 and HPV45, whose cumulative risk of CIN2$^+$ is over 10% at 7 years of follow-up; (3) HPV31;33;52;58;35, whose risk of CIN2$^+$ is over 5% at 7 years of follow-up; (4) HPV39;51;56;59;68, whose cumulative risk is below 5% at 7 years of follow-up (10).

According to these results, Bonde *et al*., recommend that ASCUS/LSIL cytology combined with any of HPV16,31,18,33,45 would merit direct colposcopy referral, whereas ASCUS/LSIL cytology combined with any of HPV35,39,51,56,59,66,68 could be at low risk enough to be followed by a 12-month follow-up retesting regimen (11). In accordance with this, some of our previous studies have demonstrated that in women with low-grade cytological abnormalities HPV16,18,31,51,52 genotypes contribute to over 90% of histologically-proven high-grade cervical disease (6, 12). Moreover, we found that in women with ASCUS/LSIL cytology and CIN2$^+$ histology, the rates of single and multiple infections stratified by each of the most prevalent HPV types were respectively 4.1% and 50.8% for HPV16, 1.6% and 24.9% for HPV18, 4.7% and 29% for HPV31, 2.1% and 16.6% for HPV51, 2.1% and 22.8% for HPV52 (6).

In addition, we observed that HPV infection caused by unknown or untypable genotypes can be detected in 11.7% of cervical cytological samples, and that their prevalence is much significantly higher in women with mild than in those with severe lesions in their Pap smears. Although the risk of high-grade CIN associated with untypable HPV infection is higher than that associated with uninfected patients, it is significantly lower than that associated with known low-risk HPV types. Finally, the low rate of progression to CIN2/3 of subjects with untypable HPV infection is reassuring and suggests that these women could be followed-up with the same protocol used for HPV negative patients (12).

Multiple HPV infections are detected in about 16.7% of women with ASCUS cytology and in 28.7% of LSIL Pap smear results (7). According to literature, the most frequent HR-HPV types found in multiple infections are HPV16/18, HPV18/51, HPV18/52, HPV39/51 and HPV39/52 (13). To date, several studies have reported that 79.5 to 80.8% of CIN2$^+$ cervical lesions in women with mild cytological abnormalities may harbor multiple HPV infections (6, 14). While only 18.2% of severe cervical lesions are sustained by single HR-HPV infections, 32.7% and 47.2% of them present multiple low/high-risk HPV types and multiple HR-HPV types respectively (6).

Besides, recent studies report that the Odds Ratio (OR) of high-grade CIN among women with multiple compared to single HPV infection is 2.54 (95% CI = 1.70-3.79, $p < 0.001$) (14). These findings confirm the high prevalence of multiple HPV infections in patients undergoing colposcopy because of ASCUS or LSIL, and suggest their potentially significant association with increased risk of CIN2$^+$ compared to single infections, especially in patients with previous history of SIL/CIN (14). With regards to each cytological outcome, in ASCUS patients the rates of severe biopsy proven lesions are 8.6% and 31.6% in women with single and multiple HPV infections respectively. Similarly, in women with LSIL cytology, the likelihood of CIN2$^+$ is 12.5% in patients with single and 28.4% in those with multiple HPV infection (6). Hence, these data confirm that compared to subjects with single infection, women with multiple HPV infections have up to four-fold increased risk of CIN2$^+$.

Previous research reports that 52% of HPV persistent infections are caused by multiple genotypes. Conversely, 54.8% of samples with multiple HPV infection at baseline show persistent infection during follow-up (13). These results provide further evidence that multiple HPV infections are significantly associated with persistence and, thus, increased oncogenic risk. In addition, our previous studies show that the relative risk of CIN2 and CIN3$^+$ in women with multiple compared to those with single HPV infection are 2.14 (95% CI = 1.44-3.18) and 2.31 (95% CI = 1.54-3.47) respectively among HPV16-positive patients, and 2.19 (95% CI = 1.3-3.68) and 3.25 (95% CI = 2.29-4.61) in HPV16-negative women (7). It follows that multiple HR-HPV infections are associated

with an increased risk of CIN2 and CIN3+ compared with single HR-HPV infections among women with abnormal cytology, in both HPV16-positive and HPV16-negative subjects, suggesting that multiple genotypes might interact synergistically, increasing the risk of CIN, regardless of HPV16-positivity. According to recent literature, among women with ASCUS/LSIL the rates of histologically-diagnosed CIN3+ are 9% in patients with single HR-HPV infection, 15.9% in subjects with multiple high and low-risk HPV infection, and 20.1% in those with multiple high-risk HPV infection (12). Vice-versa, among women with multiple high and low-risk HPV infection, the prevalence of LSIL and ASCUS cytology is 57% and 16.5% respectively and the rate of histopathological diagnosed CIN2+ is 39% (14). Similarly, among women with multiple high-risk HPV infections, LSIL and ASCUS cytology are reported in 57% and 12% of cases respectively and the rate of CIN2+ at histology is 46% of cases (14). An incremental risk of severe cervical lesions can be observed not only for infections sustained by high-risk strains but also for the association between low and high-risk types. Previous research has shown that in women with abnormal cytological results, the rate of CIN2+ histological lesions is 19.5% in patients with single infection, 31.5% in those coinfected with 2 genotypes, 57% in those coinfected with 3 genotypes and 90% in subjects with more than 3 types (14). Hence, these findings suggest a significant linear trend for increasing severity of cervical lesions and number of co-infecting types.

With respect to clinical practice, several surveys have demonstrated that in severe CIN lesions diagnosed in Loop Electrosurgircal Excision Procedure (LEEP) or cold-knife conization, multiple HR-HPV infections are associated with larger cervical lesions as detected by colposcopy (15, 16). Of note, we reported that multiple HPV infections can be detected in 41.2% of subjects with no colposcopic lesions, and 49.8%, 53.4%, 58.1% and 54.7% among women with extension < 25%, 25-50%, 50-75%, > 75% respectively (16). Among women with ASCUS/L-SIL, the Positive Predictive Value (PPV) for CIN2/3 lesions associated with any colposcopic abnormality is higher among patients with multiple than those with single HR-HPV infection, with a linear trend between the extension of colposcopic lesions and the number of high-risk HPVs detected (16). In addition, multiple HPV status and/or HPV16 positivity do not seem to influence the accuracy of colposcopy in the detection of CIN3+ lesions (16). Finally, as we reported in previous papers, among women undergoing conization because of persistent low-grade CIN, multiple HR-HPV infections during follow-up correlate with increased rates of high-grade CIN recurrence (17). **Table I** summarizes the main results.

Table I. *Main features of frequency and prevalence or relative risk measures of CIN2+ lesions in ASCUS/LSIL patients with different type of multiple HPV infections.*

Authors, title, publication year	Design	N° examined patients	Main results
(3) Wright TC *et al*. Risk detection for high-grade cervical disease using Onclarity HPV extended genotyping in women, ≥ 21 years of age, with ASC-US or LSIL cytology. Gynecol Oncol 2019.	Multicentric clinical trial	33.858 patients	1. 5% and 2.5% of PT cytological results are ASCUS and L-SIL respectively. 2. In women with ASCUS/L-SIL, the highest risk of CIN2+ is associated with HPV16, followed by HPV31, 18, 33, 58, 52. 3. Overall risk for CIN2+ in patients with ASCUS/HR-HPV+ and LSIL/HR-HPV+ is 14,2% and 10,9% respectively. 4. If women with ASCUS/LSIL and HPV16, 31, 18, 33/58 are referred to colposcopy, risk for CIN2+ in the remaining HR-HPV positive women drops to only 4,5%.
(4) Tao X, *et al*. Atypical squamous cells of undetermined significance cervical cytology in the Chinese population: Age-stratified reporting rates, high-risk HPV testing, and immediate histologic correlation results. Cancer Cytopathol 2020.	Retrospective study	1.597.136 patients	1. HR-HPV positivity rate ranges from 43% to 48,7% in women with ASCUS cytology. 2. While CIN2+ lesions can be identified in 10.7% of women with ASCUS and HR-HPV positivity, they are found in only 1.5% of women with ASCUS and HR-HPV negativity.
(5) Rufail M, *et al*. Low-grade squamous intraepithelial lesion on Papanicolaou test: follow-up rates and stratification of risk for high-grade squamous intraepithelial lesion. J Am Society Cytopathol 2020.	Retrospective review	526 patients	1. HR-HPV positivity rate ranges from 68% to 82% in women with L-SIL cytology. 2. While CIN2+ lesions can be identified in 16-26% of women with L-SIL and HR-HPV positivity, they are found in only 4.3-13% of women with L-SIL and HR-HPV negativity. 3. Higher rates of CIN2+ are found in ASCUS/HR-HPV+ women who have already had previous abnormal PT results (23%).

Authors, title, publication year	Design	N° examined patients	Main results
(6) Spinillo A, *et al*. Multiple human papillomavirus infection and high grade cervical intraepithelial neoplasia among women with cytological diagnosis of atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions. Gynecol Oncol 2009.	Single-centered clinical trial	1.218 patients	1. The prevalence of high-grade CIN is 12,6% in patients with ASCUS and HR-HPV positivity and 17,4% in patients with L-SIL and HR-HPV positivity. 2. In women with ASCUS/LSIL cytology and CIN2+ histology, the rates of single and multiple infections stratified by each of the most prevalent HPV types are respectively 4.1% and 50.8% for HPV16, 1.6% and 24.9% for HPV18, 4.7% and 29% for HPV31, 2.1% and 16.6% for HPV51, 2.1% and 22.8% for HPV52. 3. Overall, the rates of multiple infections are 22.5% among women with negative histology, 63.6% and 79.5% in patients with CIN1 and CIN2+ respectively. 4. Among women with ASCUS/L-SIL, the risk of CIN2+ is of 18.7% in patients with single HR-HPV positivity, 32.7% in patients with multiple L/HR-HPV infections, 47.2% in patients with multiple HR-HPV infections. 5. After excluding infections by HPV16/18, the risk of CIN2+ in patients with ASCUS and single or multiple HPV infection is 8.6% and 31.6% respectively; the risk of CIN2+ in patients with L-SIL cytology and single or multiple infection is 12.5% and 28.4% respectively. 6. Increasing number of HPV types is linearly associated with increasing severity of colposcopic/pathologic outcome.
(7) Spinillo A, *et al*. Multiple human papillomavirus infection with or without type 16 and risk of cervical intraepithelial neoplasia among women with cervical cytological abnormalities. Cancer Causes and Control 2014.	Cross-sectional study	3.842 patients	1. Multiple HPV infections are detected in 16.7% of women with ASCUS cytology and 28.7% of women with L-SIL cytology. 2. Infections by multiple HR-HPVs increase the risk of CIN3+ in both HPV16-positive and HPV16-negative subjects. 3. Coinfections with low-risk HPV types does not modify the risk of progression to precancerous cervical lesions associated with high-risk types.
(8) Song F, *et al*. Type-specific distribution of cervical hrHPV infection and the association with cytological and histological results in a large population-based cervical cancer screening program: Baseline and 3-year longitudinal data. J Cancer 2020.	Prospective observational study	10.186 patients	1. High-risk HPV positivity among women with abnormal cytology is 59.3%, which is 7.4 times greater than that among women with normal cytology. 2. The base-line risk for CIN2+ is 25% and 27,3% in women with ASCUS/HPV16 and L-SIL/HPV16 respectively, 7.1% in women with ASCUS/HPV18 (NA in women LSIL/HPV18), 8.4% and 15.5% in women with ASCUS/Other HRHPVs and L-SIL/Other HRHPVs respectively.
(9) Wang Y, *et al*. The efficiency of type-specific high-risk human papillomavirus models in the triage of women with atypical squamous cells of undetermined significance. Cancer Manag Res 2020.	Single-centered clinical trial	34.532 patients	1. In women with ASCUS identified as having CIN2+, the HR-HPV infection rate is 97.7%, and the prevalence rates of HPV-16, – 18, – 31, – 33, – 35, – 39, – 45, – 51, – 52, – 56, – 58, – 59, – 66 and – 68 are 48.3%, 8.0%, 6.9%, 4.6%, 1.1%, 2.3%, 3.4%, 3.4%, 26.4%, 1.1%, 17.2%, 2.3%, 0.0% and 0.0%, respectively. 2. The HPV16/18/31/33/52/58 model shows a higher sensitivity [93.1 (87.8-98.4)], specificity [73.0 (70.7-75.4)], PPV [18.0 (14.5-21.5)], NPV [99.4 (98.9-99.9)], PLR [3.7 (3.1-3.8)] and NLR [0.06 (0.03-0.18)] for the triage of ASCUS patients, as well as colposcopy referral rate (30.9%) is significantly lower than that of the recommended HR-HPV model (44.0%).
(10) Demarco M, *et al*. A study of type-specific HPV natural history and implications for contemporary cervical cancer screening programs. EClinicalMedicine 2020.	Longitudinal observational study	11.573 patients	Risk of progression can be stratified into 4 groups basing on HPV type: 1. HPV16, whose cumulative risk of CIN2+ is 21.5% at 7 years of follow-up; 2. HPV18 and HPV45, whose cumulative risk of CIN2+ is over 10% at 7 years of follow-up; 3. HPV31;33;52;58;35, whose risk of CIN2+ is over 5% at 7 years of follow-up; 4. HPV39;51;56;59;68, whose cumulative risk is below 5% at 7 years of follow-up.
(11) Bonde JH, *et al*. Clinical Utility of Human Papillomavirus Genotyping in Cervical Cancer Screening: A Systematic Review. J Lower Gen Tract Dis 2020.	Systematic review	240.674 patients	Applying US threshold for colposcopy of 5.2%: 1. ASCUS/LSIL cytology combined with HPV16,31,18,33,45 would merit direct colposcopy referral. 2. ASCUS/LSIL cytology combined with HPV35,39,51,56,59,66,68 could be followed by a 12-month follow-up retesting regimen.

Authors, title, publication year	Design	N° examined patients	Main results
(12) Spinillo A, *et al*. Untypable human papillomavirus infection and risk of cervical intraepithelial neoplasia among women with abnormal cervical cytology. J Med Virol 2014.	Prospective observational study	4.258 patients	1. Among women with ASCUS/LSIL cytology and CIN2-3 histology, HPV16,52,32,18,51 are found in the 45.9%, 26.2%, 25%, 14.7%, 14.3% of cases respectively. 2. Untypable HPVs can be detected in 11.7% of PT smears, with higher prevalence in women with low-grade cytologic lesions. 3. Rates of CIN associated with untypable HPVs are higher than those associated with uninfected samples, but lower that those associated with low-risk HPV types. 4. Infection by untypable HPVs shows low rates of progression to high grade CIN. 5. Rates of CIN3+ in patients with ASCUS/L-SIL are of 0.4% in HPV negative women and 1.7%, 2.5%, 15.9%, 9%, 20.1% in women infected by untypable HPVs, single/multiple L-R types, multiple L/H-R types, single H-R types and multiple H-R types respectively.
(13) Oyervides-Muñoz MA, *et al*. Multiple HPV Infections and Viral Load Association in Persistent Cervical Lesions in Mexican Women. Viruses 2020.	Prospective hospital case study	294 patients	1. 52% of HPV persistent infections are caused by multiple genotypes. 2. 54.8% of samples with multiple HPV infection at baseline show persistent infection during follow-up.
(14) Bello BD, *et al*. Cervical infections by multiple human papillomavirus (HPV) genotypes: Prevalence and impact on the risk of precancerous epithelial lesions. J Med Virol 2009.	Prospective observational study	1.323 patients	1. Multiple high-risk HPV infection is associated with the same incremental risk of severe cervical lesions as multiple high/low-risk HPV infection. 2. The rate of CIN2+ histological lesions is 19.5% in patients with single infection, 31.5% in those coinfected with 2 genotypes, 57% in those coinfected with 3 genotypes and 90% in subjects with more than 3 types.
(15) Spinillo A, *et al*. Multiple Papillomavirus Infection and Size of Colposcopic Lesions Among Women With Cervical Intraepithelial Neoplasia. J Low Genit Tract Dis 2016.	Case series	898 patients	In CIN lesions diagnosed by LEEP or cold-knife conization, multiple HR-HPV infections correlate with larger cervical lesions as detected by colposcopy.
(16) Spinillo A, *et al*. Diagnostic accuracy of colposcopy in relation to human papillomavirus genotypes and multiple infection. Gynecol Oncol 2014.	Cohort study	2526 patients	1. In patients with ASCUS/L-SIL, multiple HPV infections are detected in 41.2% of subjects without colposcopic lesions, and 49.8%, 53.4%, 58.1%, 54.7% among subjects with extension < 25%, 25-50%, 51-75%, > 75% respectively. 2. Linear trend between the extension of colposcopic lesions and the number of HR-HPVs detected. 3. Among women with ASCUS/L-SIL, the PPV for CIN3+ lesions associated with any colposcopic abnormality is 23.5% and 15.5% in subjects with multiple and single infections respectively. 4. The accuracy of colposcopy to detect CIN2-3+ is not influenced by neither multiple HPV or HPV16 infections.
(17) Spinillo A, *et al*. Outcome of Persistent Low-Grade Cervical Intraepithelial Neoplasia Treated With Loop Electrosurgical Excision Procedure. J Low Genit Tract Dis 2016.	Prospective observational study	252 patients	Among women undergoing conization because of persistent low-grade CIN, multiple HR-HPV infections during follow-up correlate with increased rates of high-grade CIN recurrence.

DISCUSSION

Co-infection with multiple genotypes of HPV is commonly observed among women with abnormal cervical cytology, as it is reported in 20-50% of cases according to literature (18, 19). Guidelines on the management of low-grade cytological lesions in Western countries are mainly based on the use of HPV DNA test as intermediate triage and direct colposcopy referral for HR-HPV positive ASCUS/LSIL cohorts. A large number of surveys have claimed that HR-HPV positivity is significantly associated with an increase in risk of high-grade cervical lesions in women with ASCUS/LSIL cytology (4, 5). Recent research shows that, although HPV testing increases the sensitivity and negative predictive value of screening programs, the specificity declines compared with cytology, due to the high prevalence of HPV infection in sexually active women. As a consequence, most women with HR-HPV positive ASCUS/LSIL who undergo colposcopy do not have high-grade cervical lesion. There

is a clinical need to develop risk-stratification approaches that would further reduce the number of unnecessary colposcopy exams for women with ASCUS/LSIL while ensuring that most women with CIN2$^+$ receive appropriate treatment.

Several studies claim that significant clustering patterns of HPV types and species may occur in multiple infections. Indeed, noteworthy interactions were found both at inter-species level, with higher rates of co-infection by α7-α9-α10, α6-α9, α7-α10, and at inter-genotype level, with higher risk of co-infection by types HPV31-35-56, HPV16-18, HPV51-52. Besides, α9 species and HPV16 genotype are most frequently represented (1). There is evidence from literature that HPV16 genotype tends to cluster with viral types from different species, as proved by the high rates of co-infection between HPV16 and HPV45 (from α9 and α7 species respectively), while co-infection with other α9- genotypes is quite uncommon, suggesting possible competitive interactions among genotypes from the same species at a cellular level (20). Nonetheless, other studies on multiple HPV infection have reported that individual types and species associate at random based on their relative frequency (21, 22).

The above-mentioned heterogeneity of results also depends on socio-demographic and behavioural reasons, such as the origin and the characteristics of the studied population, their socio-economic status (20), young age, HIV seropositivity and recent sexual intercourse (6, 23). As far as racial influences on HPV genotype distributions are concerned, recent literature (24) reports that, despite the higher proportion of HPV$^+$ ASCUS citology in black than in white women, the prevalence of HPV16 type is higher in white (12.7%) than in black (7.8%) patients. Conversely, black women show a higher proportion of other high-risk HPVs (HPV 31,33,35,39,45,51,52,56,58,59,66,68) than white ones (47% *vs* 38.1%).

Several cross-sectional studies suggest that multiple HR-HPV infections are associated with an increased risk of severe CIN (14). Namely, it has been observed a linear trend between the number of viral types involved and the severity of lesion (1, 20, 14). Multiple HR-HPVs increases the risk of CIN2$^+$ in both HPV16-positive and HPV16-negative subjects, suggesting a potential synergistic interaction between HR-HPVs, favoring the progression of CIN lesions (25). Co-infection with low-risk HPV types, although quite common, does not seem to modify the risk of precancerous cervical lesions associated with the high-risk viral strains involved (25). Nonetheless, other reports disprove multiple HPV infection as a significant risk factor for severe cervical lesions, claiming that any single area of CIN is due to the action of a single carcinogenic type, while other high-risk HPVs detected in cervical smears are related to independent transient infections (26, 27).

Van der Marel J. *et al*., claim that women with High-Grade CIN and concomitant multiple HPV infection, often present with various heterogeneous cervical lesions, each one caused by a single carcinogenic type tough, as proved by Laser Capture Microdissection – Polymerase Chain Reaction (LCM-PCR) genotyping system (26).

In vitro studies have recently proved that coinfection of a single cell with more than one HPV type is possible, and that this could result in significant inter-genotype molecular interactions, affecting the life cycle of the single viral types involved, as well as their own ability to persist and to drive cell transformation (28, 29). In this particular case, Biryukov *et al*., have observed that coinfection with HPV16 and HPV18 decreases HPV18 E1 and E4 transcription, that is a proxy for a significant decrease in HPV18 infectivity compared to a single infection (28). Indeed, these results indicate that there is some degree of inter-genotypic competition or superinfection exclusion, for which HPV16 is able to partially block or interfere with HPV18 life cycle. Besides, it is important to consider that HPV16 and HPV18 show different binding localization patterns and internalization times. In fact, while HPV16 is able to bind directly to the cell surface and be internalized, HPV18 needs to bind to the Extracellular Matrix (ECM) prior to conformational changes and transfer to the cell surface, where L2 is then cleaved by furin in order to enter the cell. Because of these different attachment requirements, HPV16 shows higher cell entry rates than HPV18. Hence, they hypothesized that superinfection exclusion might occur early during the attachment/entry phase of the viral life cycle, suggesting that HPV16 minor capsid L2 protein may play a crucial role in blocking furin-dependent HPV18 cleavage and, thus, decreasing HPV18 infectivity (28). On the other hand, Mori S. *et al*. suggest that the inter-genotypic competition observed in the coinfection of a single cell with HPV16 and HPV18 may be due to a mechanism of genome replication interference as a result of the transcription of heterooligomers composed of HPV16/18 helicase E1 Oligomerization-Domain (OD), which are responsible for a decrease in viral replication rates. This could possibly be explained

by the lower efficiency of chimeric proteins in binding to the DNA replication origin, recruiting cellular factors required for HPV replication and transcription and unwinding double-stranded DNA (29).
Previous analyses have shown that multiple HR-HPV infection correlates significantly with greater period of infection, which in turn stands for enhanced risk of progression (13).
Hence, it is important to note that the reported molecular mechanisms of inter-genotypic competition mostly occur in the early phases of acute viral infection. On the contrary, it has been observed that in cells that harbour persistent HR-HPV infections, there is no block in the ability of a second viral type to superinfect cells (28).
These findings in biology are consistent with the clinical counterpart for which it has been observed that the detection of a new additional viral type in the setting of a previously diagnosed HR-HPV persistent infection increases the risk of $CIN2^{+}$ if compared to simultaneous coinfection (14). Therefore, it is conceivable that significant genotype-specific clustering patterns might mostly be represented in CIN lesions of women infected with multiple HR-HPV types, based on their own ability to give rise to persistent infections.
Since persistent high-risk HPV infection is a prerequisite of cervical dysplasia and cancer, recent primary prevention programs, based on type-specific high-risk HPV vaccination, have led to a significant decrease in CIN1-CIN3 cervical lesions in the vaccinated cohorts (30). In fact, while Gardasil (4vHPV, Gardasil, Merck, 2006) and Cervarix (2vHPV, Cervarix, GlaxoSmithKline, 2009), raising immunity against HPV6-11-16-18 and HPV16-18 respectively, are protective against high-risk HPV type 16 and 18 which are responsible for 70% of all cervical dysplasia, Gardasil 9 (9vHPV, Gardasil 9, Merck, 2015) extends protection also from HPV 31-33-45-52-58, which are responsible for another 18.3-20% of cervical neoplasia (30). Nonetheless, the above mentioned preventive vaccines, which trigger the production of L1-specific neutralizing antibodies, are strictly type-specific. Moreover, it has been shown that the preventive efficacy of HPV vaccination programs seems to decrease in women over 25 who have been previously infected by targeted high-risk HPVs (31), altohough they would still benefit to a certain extent (32). Interestingly enough, the recent prospective case-control study Speranza (32) has reported a decrease of 81.2% in the risk of H-SIL recurrence during four-year follow up time after LEEP procedure in patients undergoing tetravalent HPV vaccination 30 days after the intervention. These results are in accordance with previous studies, such as Future I and II and Patricia, which claim the clinical efficacy of HPV vaccination after LEEP in decreasing the risk of disease recurrence (33). As well as protecting patients from *de novo* infections by targeted HR-HPVs, HPV vaccination after LEEP is supposed to prevent the reduction of immune response against Human Papillomavirus that seems to follow escissional procedures (32). Indeed, recent research (34, 35) suggests that patients with persistent HR-HPV infection show higher levels of TNFalfa in their cervical fluids. However, it has been observed a decline in cervical TNFalfa levels after escissional procedures, leading to a cervical microenvironment similar to that of HPV-naïve patients (32). Hence, it has been hypothesized that HR-HPV vaccination boosts neutralizing antibody production by memory B-cells from the basement membrane, enhancing their titres up to 100-1000 times and, therefore, preventing any virus in the surgical site from entering regenerating epithelial cells (32).
Hence, the introduction of type-specific HR-HPV vaccination brings along with it significant clinical implications in primary and secondary prevention programs, as well as redrawing prevalence and distribution of the main oncogenic viral types, based on which new screening risk-stratification tools should be considered.

CONCLUSIONS

In conclusion, this review provides further evidence that multiple HR-HPV infection is a significant risk factor for severe cervical lesions in women with ASCUS and LSIL cytology, and highlights that increased oncogenic risk might be strictly associated with peculiar type-specific profiles, independent of HPV-16. Besides, it offers interesting insights in the epidemiology of HPV genotype distribution, especially in the post-vaccination era, suggesting new risk-stratification models to be taken into account. These observations could offer useful guidance on proper clinical management of women with mild cytological abnormalities.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

REFERENCES

1. Spinillo A, Dal Bello B, Alberizzi P, *et al.* Clustering patterns of human papillomavirus genotypes in multiple infections. Virus Res 2009;142(1-2):154-9.
2. Venetianer R, Clarke MA, van der Marel J, *et al*. Identification of HPV genotypes causing cervical precancer using tissue-based genotyping. In J Cancer 2020;146(10) 836-44.
3. Wright TC, Stoler MH, Parvu V, Yanson K, Cooper C, Andrews J. Risk detection for high-grade cervical disease using Onclarity HPV extended genotyping in women, ≥ 21 years of age, with ASC-US or LSIL cytology. Gynecol Oncol 2019;154(2):360-7.
4. Tao X, Zhang H, Wang L, *et al.* Atypical squamous cells of undetermined significance cervical cytology in the Chinese population: Age-stratified reporting rates, high-risk HPV testing, and immediate histologic correlation results. Cancer Cytopathol 2021;129(1):24-32.
5. Rufail M, Lew M, Pang J, Jing X, Heider A, Cantley RL. Low-grade squamous intraepithelial lesion on Papanicolaou test: follow-up rates and stratification of risk for high-grade squamous intraepithelial lesion. Journal of the Am Soc Cytopathol 2020;9(4):258-65.
6. Spinillo A, Dal Bello B, Gardella B, Roccio M, Dacco' MD, Silini EM. Multiple human papillomavirus infection and high grade cervical intraepithelial neoplasia among women with cytological diagnosis of atypical squamous cells of undetermined significance or low grade squamous intraepithelial lesions. Gynecol Oncol 2009;113(1):115-9.
7. Spinillo A, Gardella B, Roccio M, *et al.* Multiple human papillomavirus infection with or without type 16 and risk of cervical intraepithelial neoplasia among women with cervical cytological abnormalities. Cancer Causes and Control 2014;25(12):1669-76.
8. Song F, Du H, Xiao A, Wang C, *et al*. Type-specific distribution of cervical hrHPV infection and the association with cytological and histological results in a large population-based cervical cancer screening program: Baseline and 3-year longitudinal data. J Cancer 2020;11(20):6157-67.
9. Wang Y, Gao S, Wang Y, Chen F, Deng H, Lu Y. The efficiency of type-specific high-risk human papillomavirus models in the triage of women with atypical squamous cells of undetermined significance. Cancer Man Res 2020;12:5265-75.
10. Demarco M, Hyun N, Carter-Pokras O, *et al*. A study of type-specific HPV natural history and implications for contemporary cervical cancer screening programs. EClinicalMedicine 2020;22:1-9.
11. Bonde JH, Sandri MT, Gary DS, Andrews JC. Clinical Utility of Human Papillomavirus Genotyping in Cervical Cancer Screening: A Systematic Review. J Low Genit Tract Dis 2020;24(1):1-13.
12. Spinillo A, Gardella B, Roccio M, Alberizzi P, Silini EM, Dal Bello B. Untypable human papillomavirus infection and risk of cervical intraepithelial neoplasia among women with abnormal cervical cytology. J Med Virol 2014;86(7):1145-52.
13. Oyervides-Muñoz MA, Pérez-Maya AA, Sánchez-Domínguez CN, *et al*. Multiple HPV Infections and Viral Load Association in Persistent Cervical Lesions in Mexican Women. Viruses 2020;12(4):380.
14. Bello BD, Spinillo A, Alberizzi P, *et al*. Cervical infections by multiple human papillomavirus (HPV) genotypes: Prevalence and impact on the risk of precancerous epithelial lesions. J Med Virol 2009;81(4):703-12.
15. Spinillo A, Gardella B, Iacobone AD, Cesari S, Alberizzi P, Silini E M. Multiple Papillomavirus Infection and Size of Colposcopic Lesions Among Women With Cervical Intraepithelial Neoplasia. J Low Genit Tract Dis 2016;20(1):22-5.
16. Spinillo A, Gardella B, Chiesa A, Cesari S, Alberizzi P, Silini EM. Diagnostic accuracy of colposcopy in relation to human papillomavirus genotypes and multiple infection. Gynecol Oncol 2014;134(3):527-33.
17. Spinillo A, Gardella B, Iacobone AD, Dominioni M, Cesari S, Alberizzi P. Outcome of Persistent Low-Grade Cervical Intraepithelial Neoplasia Treated With Loop Electrosurgical Excision Procedure. J Low Genit Tract Dis 2016;20(4):307-11.
18. Mollers M, Vriend HJ, van der Sande MA, *et al*. Population- and type-specific clustering of multiple HPV types across diverse risk populations in the Netherlands. Am J Epidemiol 2014;179:1236-46.
19. Dickson EL, Vogel RI, Bliss RL, Downs LS Jr. Multiple-type human papillomavirus (HPV) infections: a cross-sectional analysis of the prevalence of specific types in 309,000 women referred for HPV testing at the time of cervical cytology. Int J Gynecol Cancer 2013;23:1295-302.
20. Del Río-Ospina L, Soto-DE León SC, Camargo M, *et al*. Multiple high-risk HPV genotypes

are grouped by type and are associated with viral load and risk factors. Epidemiol Infect 2017;145:1479-90.
21. Wheeler CM, Hunt WC, Schiffman M, Castle PE. Human Papillomavirus Genotypes and the Cumulative 2 Year Risk of Cervical Precancer. J Infect Dis 2006;194(9):1291-9.
22. Chaturvedi AK, Myers L, Hammons AF, *et al*. Prevalence and clustering patterns of human papillomavirus genotypes in multiple infections. Cancer Epidemiol Biomarkers Prev 2005;14:2439-45.
23. Yang Z, Cuzick J, Hunt WC, Wheeler CM. Concurrence of multiple human papillomavirus infections in a large US population-based cohort. Am J Epidemiol 2014;180:1066-75.
24. Risley C, Clarke MA, Geisinger KR, *et al*. Racial differences in HPV type 16 prevalence in women with ASCUS of the uterine cervix. Cancer Cytopathol 2020;128:528-34.
25. Spinillo A, Gardella B, Roccio M, *et al*. Multiple human papillomavirus infection with or without type 16 and risk of cervical intraepithelial neoplasia among women with cervical cytological abnormalities. Cancer Causes Control 2014;25:1669-76.
26. van der Marel J, Quint WG, Schiffman M, *et al*. Molecular mapping of high-grade cervical intraepithelial neoplasia shows etiological dominance of HPV16. Int J Cancer 2012;131(6):E946-53.
27. Vaccarella S, Franceschi S, Snijders PJ, Herrero R, Meijer CJ, Plummer M. IARC HPV Prevalence Surveys Study Group. Concurrent infection with multiple human papillomavirus types: pooled analysis of the IARC HPV Prevalence Surveys. Cancer Epidemiol Biomarkers Prev 2010;19:503-10.
28. Biryukov J, Meyers C. Superinfection Exclusion between Two High-Risk Human Papillomavirus Types during a Coinfection. J Virol 2018;92:e01993-17.
29. Mori S, Kusumoto-Matsuo R, Ishii Y, Takeuchi T, Kukimoto I. Replication interference between human papillomavirus types 16 and 18 mediated by heterologous E1 helicases. Virol J 2014;11:11.
30. Yusupov A, Popovsky D, Mahmood L, Kim AS, Akman AE, Yuan, H. The nonavalent vaccine: a review of high-risk HPVs and a plea to the CDC. Am J Stem Cells 2019;8:52-64.
31. Fogleman C, Leaman L. Prophylactic vaccination against human papillomavirus to prevent cervical cancer and its precursors. Am Fam Phys 2019;99:15-16.
32. Ghelardi A, Parazzini F, Martella F, *et al*. SPERANZA project: HPV vaccination after treatment for CIN2+. Gynecol Oncol 2018;151:229-34.
33. Clark KT, Trimble CL. Current status of therapeutic HPV vaccines. Gynecol Oncol 2020;156:503-10.
34. Scott ME, Shvetsov YB, Thompson PJ, *et al.* Cervical cytokines and clearance of incident human papillomavirus infection: Ha- waii HPV cohort study, Int J Cancer 2013;133:1187-96.
35. Saftlas AF, Spracklen CN, RyckmanKK, Stockdale CK, Penrose K, Ault K, Rubenstein LM, Pinto LA. Influence of a loop electrosurgical excision procedure (LEEP) on levels of cytokines in cervical secretions. J Reprod Immunol 2015;109:74-83.