Received: 13 January 2024 | Accepted: 5 February 2024

DOI: 10.1002/jmv.29474

RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

Human papillomavirus genotyping in high-grade vaginal intraepithelial neoplasia: A multicentric Italian study

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Abstract

This study aimed to analyze the human papillomavirus (HPV) genotype distribution in a large cohort of high-grade vaginal intraepithelial neoplasia (VaIN) (vaginal HSIL, VaIN2/3) patients from two Italian referral centers. We included all patients with histologically confirmed VaIN2/3 from the Department of Surgical Sciences, Sant'Anna Hospital, University of Torino, Torino, Italy, and Ospedale Maggiore della Carità, Novara, Italy, between 2003 and 2022. After the histological evaluation of formalin-fixed paraffin-embedded samples, we performed HPV genotyping with VisionArray HPV Chip 1.0. We detected HPV DNA in 94.4% of VaIN2/3 (168/178), with HPV 16 as the most prevalent genotype, accounting for 51.8% of all infections, 41.2% of VaIN2 and 77.6% of VaIN3 cases. Other frequent genotypes were HPV 58 (8.3%, 10.9% of VaIN2 and 2.0% of VaIN3), HPV 73 (5.4%, 5.0% of VaIN2 and 6.1% of VaIN3), and HPV 31 (5.4%, 6.7% of VaIN2 and 2.0% of VaIN3). 73.2% of VaIN2/3 had a single HPV genotype infection and 26.8% a multiple infection (20.8% a double infection, 4.8% a triple infection, and 1.2% a quadruple infection). Single infection was more frequently present in VaIN3 than VaIN2 (81.6% vs. 69.8%). 69.1% of single infections and 73.3% of multiple infections had one or more genotypes covered by nine-valent HPV vaccine. HPV vaccination is expected to have a large impact on reducing the incidence of vaginal intraepithelial neoplasia.

KEYWORDS

HPV, HPV genotyping, HPV vaccine, Vagina, vaginal intraepithelial neoplasia, VaIN, VaIN-HSIL

1 | INTRODUCTION

Vaginal cancer is a rare malignancy accounting for only 3% of all gynecologic tumors.[1] The most common histological type (>90%) is vaginal squamous cell carcinoma (VaSCC) followed by adenocarcinoma, carcinosarcoma, and melanoma.[2] VaSCC has an incidence rate of 0.4 cases per 100 000 women per year,[1] and, due to its rarity, there are no established guidelines for routine screening of the general population.

VaSCC is associated with human papillomavirus (HPV) infection in more than 70% of cases.[3] Persistent HPV infection may lead to the development of vaginal intraepithelial neoplasia (VaIN)[4]: VaIN 1 represents a benign lesion that may regress spontaneously in most cases, while VaIN2 and VaIN3 are considered preinvasive lesions. The prevalence and progression rate of VaIN are not well understood, mainly because VaIN is often asymptomatic and underdiagnosed. HPV genotype, immunodepression, age, and other HPV-related lesions of the cervix and vulva[5] are risk factors for developing VaIN and vaginal cancer.

The diagnosis of VaIN has increased in the last 10 years[6] due to increased awareness, use of cytology and HPV testing, and colposcopy performed by expert gynecologists. Indeed, when abnormalities are

detected in Pap test results or persistent positivity in HPV testing is present, colposcopists face the suspicion of vaginal lesions, especially when the cervical colposcopic examination is negative or in hysterectomized patients.

As well as VaIN diagnosis, its treatment may be difficult, requiring specific expertise and depending on VaIN grade, presence of multifocal and multicentric lesions, age and immunodepression of the patients.[7,8] In particular, special attention should be given to HPV 16 positive women, as it is the most frequent genotype involved in multicentric and recurrent disease.

Excisional methods are preferred when occult invasion cannot be ruled out, as ablative methods and Imiquimod do not allow histological examination, and should be preceded by multiple mapping biopsies.[9] Patients with cervical cancer submitted to combined radiotherapy and surgery represents a high risk group and HSIL VaIN should always be excised to rule out invasive foci or lesion persistence after treatment.

Knowing the oncogenic role of HPV 16 in multicentric lower genital tract intraepithelial neoplasia, a better information about genotype prevalence distribution in VaIN could have a positive impact on early disease detection, treatment and follow up of this rare disease.

Due to VaIN rarity, limited HPV prevalence and genotype distribution studies are available, and 92.3% HPV prevalence in VaIN is reported in the largest Chinese study so far,[10] with worldwide geographical variations due to sexual behavior in different populations and different susceptibility. HPV 16 is the leading genotype, as in other sites, while the secondary genotypes have wide regional variance. The present study aims to analyze HPV DNA prevalence and genotype distribution in a large multicentric cohort of VaIN2/3 in Italy, as so far no studies are available in our country.

2 | METHODS

A retrospective cross-sectional study was performed, including all women treated for VaIN2/3 at the Department of Surgical Sciences, Sant'Anna Hospital, University of Torino, Torino, Italy, and at the Ospedale Maggiore della Carità, Novara, Italy, from 2003 to 2022. All cases were retrospectively retrieved from a dedicated institutional database. The following clinical and pathological data were recalled from medical charts: age at diagnosis, presence of concomitant or previous histologically proven cervical or vulvar lesions, and clinical reason for hysterectomy if performed, HPV vaccination status.

VaIN were diagnosed histologically according to World Health Organization (WHO) and Lower Anogenital Squamous Tract (LAST) terminology and further divided into grades 2 and 3 (VaIN2 and VaIN3).[2,4]

2.1 | Specimens, DNA extraction, and quantification

Formalin-fixed paraffin-embedded (FFPE) blocks were retrieved from histological archives and processed under strict protocol to avoid cross contaminations. Tissues were fixed in 10% neutral buffered formalin for 24–72 h, processed, and embedded in paraffin. Before DNA extraction, hematoxylin–eosin-stained slides were revised by an experienced pathologist to assess the adequacy of the material and to confirm the previous diagnosis. Later, DNA extraction was performed, starting with a manual macro-dissection of the tumor from the FFPE. Five micrometers thick sections were cut and collected in a 1.5 mL tube. Xylene was used to clean the microtome blade after sectioning each block to avoid sample cross-contamination. DNA was extracted using the Maxwell® RSC DNA FFPE Kit method loaded on the Maxwell® RSC Instrument (Promega Corporation). All DNA samples were quantified using a Qubit 4 Fluorometer (ThermoFisher Scientific).

2.2 | HPV testing

At least 15 ng of DNA was necessary to proceed with the PCR reaction, using the VisionArray HPV PreCise Master Mix (ZytoVision GmbH). The processed DNA specimens were run together with positive and negative controls. Specific regions of the L1 gene (amplicon length: 139–148 bp) of the HPV genomes were amplified and biotinylated together with the human HLA-DQA1 gene (amplicon length: 227 bp) which was used as internal PCR-positive control. Later, the VisionArray HPV Chip 1.0 (ZytoVision GmbH) was used for the qualitative detection and genotyping of 41 HPV genotypes. The potentially detected HPV genotypes were classified according to the current scientific literature as Low-Risk HPV (6, 11, 40, 42,43, 44, 54, 55, 57, 61, 62, 72, 81CP8304, 83MM7, 84MM8, 90, and 91), probably High-Risk (HR) HPV (26,34, 53, 66, 67, 68a, 68b, 69, 70, 73, 82IS39, and 82MM4), and HR HPV (16, 18, 31, 33, 35,39, 45, 51, 52, 56, 58, and 59). The reagents provided with the VisionArray Detection Kit (ZytoVision GmbH) were used to allow the hybridization between the L1 gene biotinylated amplicons and the specific complementary DNA probes captured on the glass chip. Later, a streptavidin–peroxidase conjugate was applied followed by tetramethylbenzidine staining. As a final step, the VisionArray Analyzer Software (ZytoVision GmbH) was used to digitally scan and analyze the glass chips. The results obtained were interpreted following the instructions provided by the manufacturer.

2.3 | Statistical analysis

Data were analyzed using IBM SPSS statistical software (version 28.0). Descriptive statistics were reported as mean, median, standard deviation (SD), range, and interquartile range (IQR) for continuous variables and as absolute frequency and percentage for categorical variables. To test the difference between the two groups, Wilcoxon–Mann–Whitney's test and Fisher's exact test were applied to continuous and categorical variables, respectively. A p-value of <0.05 was considered statistically significant.

2.4 | Ethical approval

The study was approved by the Research Ethics Committee for Human Biospecimen Utilization (Department of Medical Sciences—ChBU) of the

University of Turin (n°2/2022, date of approval February 22, 2022). All patients included in our retrospective study were treated according to the ethical standards of our local committee on human experimentation and with the Helsinki Declaration and signed informed consent for the anonymous use of clinical and instrumental data for research purposes at the time of diagnosis.

3 | RESULTS

A total of 227 patients with VaIN2/3 were included, 159 (70.0%) from Torino and 68 (30.0%) from Novara. Among the 227 patients, 164 (72.2%) were diagnosed with VaIN2 and 63 (27.8%) with VaIN3. The mean age at the time of diagnosis was 55.7 years (range: 26–87, SD 13.8 years). The mean age for VaIN3 patients was 62.5 years (SD 13.9 years), significantly higher compared to VaIN2 patients (53.1 years, SD 12.9, $p < 0.0001$). Among postmenopausal women, 69.8% had VaIN3, a significantly higher percentage than premenopausal women (34.6%, $p < 0.0001$). None of the patients included was vaccinated with any HPV vaccine.

Overall, 20.4% (46) of our cohort had undergone hysterectomy: 13.0% of VaIN2 and 39.7% of VaIN3 ($p < 0.0001$). Reasons for hysterectomy were cervical HSIL or invasive cancer in 52.2% of patients, benign disease in 41.3%, and endometrial cancer in 6.5%. The median time from hysterectomy to VaIN diagnosis was 54.9 months (IQR: 20.5–216.7).

44.6% of patients (79/178) had an associated concurrent or previous cervical HSIL: previous to VaIN diagnosis in 36 patients and a concomitant in 43.

A previous vulvar HSIL was present in eight patients (3.5%). The presence of vulvar or cervical HSIL was more frequent in VaIN3 patients compared to VaIN2 (cervical HSIL in 62.1% of VaIN3 vs. 40.0% of VaIN2, $p = 0.0159$, vulvar HSIL in 6.4% of VaIN3 vs. 2.4% of VaIN2, $p = 0.2222$).

HPV DNA genotyping was available in 178 patients, of whom 168 (94.4%) were positive. HPV DNA positivity increased significantly with increasing the severity of the lesion, from 93.7% in VaIN2 up to 96.1% in VaIN3 ($p \leq 0.001$). Multiple infection was observed in 26.8% patients (45), with two genotypes detected in 20.8% cases (35), three genotypes in 4.8% cases (8), and four genotypes in 1.2% (2), as detailed in Table 1. Considering only HR HPV genotypes, one or more HR HPV was detected in 81.5% of VaIN2/3 cases, in particular in 78.1% of VaIN2 and in 89.8% of VaIN3.

Detailed HPV genotyping distribution in VaIN2 and VaIN3 is shown in Figure 1. The most frequent genotypes reported were HPV 16 (51.8%), HPV 58 (8.3%), HPV 31 (5.4%), and HPV 73 (5.4%). HPV 16 was significantly more prevalent in VaIN3 (77.5%) than in VaIN2 (41.2%, $p < 0.0001$).

TABLE 1 HPV genotyping results by single or multiple infections.

	All ($n = 168$)	VaIN2 ($n = 119$)	VaIN3 ($n = 49$)	p Value
Single	123 (73.2)	83 (69.7)	40 (81.6)	0.2657
2	35 (20.8)	29 (24.4)	6 (12.3)	
3	8 (4.8)	5 (4.2)	3 (6.1)	
4	2 (1.2)	2 (1.7)	0 (0.0)	
Number of HPV				
1	123 (73.2)	83 (69.7)	40 (81.6)	0.1139
>1	45 (26.8)	36 (30.3)	9 (18.4)	
HR HPV positive[a]	137 (81.5)	93 (78.1)	44 (89.8)	0.0843

Abbreviations: HPV, human papillomavirus; VaIN, vaginal intraepithelial neoplasia.

[a]High-Risk HPV, HR HPV, genotypes considered are HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 according to International Agency for Research on Cancer (IARC).[11]

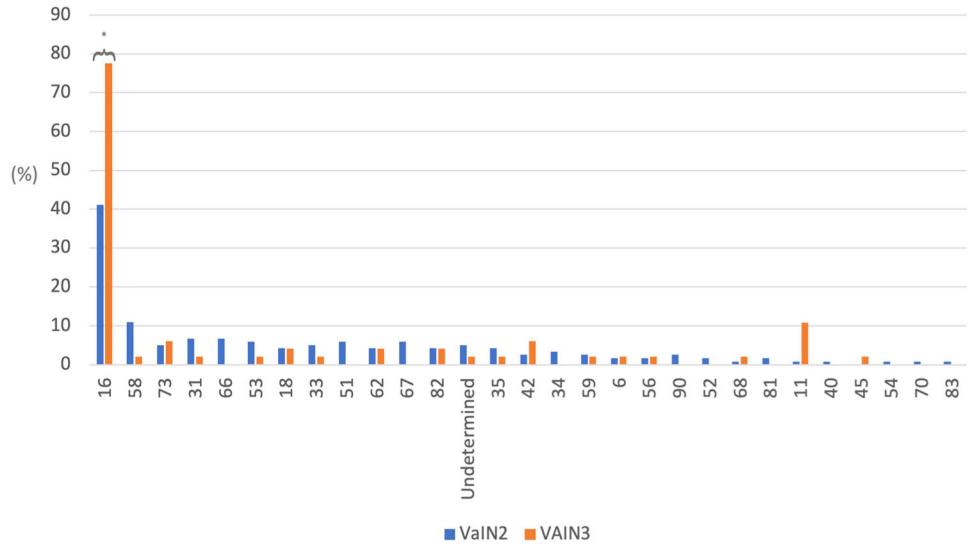

FIGURE 1 Distribution of HPV genotype in VaIN2 and VaIN3. *$p < 0.0001$. HPV, human papillomavirus; VaIN, vaginal intraepithelial neoplasia.

Regarding HPV genotypes included in the 9-valent HPV vaccine (HPV 6, 11, 16, 18, 31, 33, 45, 52, and 58), 69.1% (85/123) single infections and 73.3% (33/45) multiple infections had at least one or more HPV of 9-HPV vaccine.

4 | DISCUSSION

HR HPV genotypes have been linked to VaIN oncogenesis in many cohort studies,[3,10,12] however, no information are available concerning the contribution of HPV to VaIN development in Italy. In our study, we provide the largest HPV genotyping cohort of VaIN2/3 collected from Northern Italy during 2003–2022.

HPV DNA in VaIN2/3 was searched using a sensitive HPV genotyping assay that covers all oncogenic and probably/possibly oncogenic types, as well as many low-risk genotypes. The overall prevalence of HPV DNA was 94.4% and it increased with increasing the grade of the lesions (93.7% in VaIN2 and 96.1% in VaIN3, $p < 0.0001$). This finding complies with the other large VaIN genotyping study from China,[10] accounting an overall positivity of 92.5% in VaIN2 and 96.8% in VaIN3, and with an European study that reports 96% HPV DNA positivity in VaIN2/3.[12] A metanalysis showed an 87.1% (95% confidence interval [CI]: 78.7–93.7) HPV prevalence in 821 VaIN2/3 cases,[3] with wide variation due to different detection methods and geographical origin, ranging from 20% to 100%. Specifically, Europe showed the lower pooled HPV prevalence (80.8%, 95% CI: 60.6–94.9), while South America had the highest (92.5%, 95% CI: 84.4–97.2). Some suggestion regarding what caused this difference, besides the low number of cases included in the meta-analysis, HPV DNA detection method, type of sample used, and PCR primer type and behavioral and social characteristic of the examined population.

HPV DNA genotyping showed HPV16 as the most frequent genotype identified, (51.8%, 41.2% of VaIN2, and 77.6% of VaIN3 in our cohort), followed by HPV 58, 31, 73, 53, and 66. It is interesting to note that the fourth most frequent genotype in our analysis is considered a low-risk HPV genotype (HPV 73), with a prevalence similar to another European study,[12] while it is far rarer in the Chinese study.[10] Remaining HPV16 the predominant genotype, other leading genotypes were 52, 58, 53, 56 from China,[10] and 18, 52, 73, and 33 from Europe.[12] Furthermore, a metanalysis showed a HPV 16 pooled prevalence of 53.1% (95% CI: 39.2–66.9), followed by HPV 33 (5.7%, 95% CI: 2.8–9.5), HPV 52 (5.2%, 95% CI: 2.7–8.5) and HPV 18 (4.7%, 95% CI: 2.3–7.9).[3]

Detection of multiple infections decreased from VaIN2 (30.3%) to VaIN3 (18.4%), suggesting that selection of the most carcinogenic HPV genotypes has occurred, along with clearance of the least carcinogenic ones during the oncogenic process.[13] This is in line with other reports,[10,12] and also with other HPV-related intraepithelial neoplasms and cancers such as vulva and anus.[14–16]

Since one of the HPV types included in the HPV 9-valent vaccine was present in 70.2% of VaIN2/3 cases, these lesions could have been likely prevented by 9-HPV vaccination, underlying the importance of this intervention in appropriate age. Recent cohort studies from Denmark[17] indicated that VaIN incidence decreased significantly after 2006 among women under 30 years of age, with an annual decrease of almost 16% per year, that is after HPV vaccine approval. Also, women 30–39 years of age showed a modest, but not statistically significant, decrease.[17] Another report from Denmark highlighted lower rates of vaginal HSIL in vaccinated (adjusted hazard ratio: 0.3, 95% CI: 0.13–0.68) compared to unvaccinated women, being the effect the strongest when vaccination occurred <16 years (adjusted hazard ratio: 0.16, 95% CI: 0.04–0.55).[18] Thus, it can be assumed that prophylactic HPV vaccination effectiveness will have beneficial effects also against non-cervical HPV-associated malignancies and the strongest effect can be expected in early vaccinated women.

We also reported the high burden of other associated HPV-related intraepithelial neoplasm of the lower genital tract that are more frequent in higher grade VaIN, with 44.6% of VAIN2/3 having an associated previous or concurrent cervical HSIL and 3.2% an associated vulvar HSIL. These data support the importance of a thorough examination of the cervix, and the vulva[19–21] during vaginal colposcopic examination of patients affected by VaIN and highlights once again the strong HPV field effect, in particular for HPV 16.[22,23]

The strengths of the study are the high number of enrolled patients and the centralized processing and histological assessment. Furthermore, we used a robust and specific assay to increase sensitivity: the assay provided extended genotyping of all HR HPV and a wide range of low risk HPV genotypes (overall, 41 genotypes), further increasing the added value of the present work. The two centers involved are also highly representative and cover a large proportion of the regional population. Furthermore, mainly for the mean age of the patients, none of the patient was vaccinated against HPV, which could have affected HPV prevalence distribution.[24,25]

Among potential limitations, the cross-sectional retrospective nature of the study and unavailability of some clinical information, which limited further analysis of potentially relevant factors such as smoking, immunodepression, or location of lesions.

5 | CONCLUSIONS

Most VaIN2/3 cases are HPV positive, with HPV 16 being the most frequent genotype reported. VaIN3 has a higher likelihood of having HPV 16 infection and presence of multicentric lesions. The 9-HPV vaccine could have hypothetically prevented more than 70% of VaIN2/3 cases of our cohort and early vaccination shouldbe encouraged.

AUTHOR CONTRIBUTIONS

Conceptualization: Mario Preti and Renzo Boldorini. *Methodology*: Chiara Airoldi. *Formal analysis*: Chiara Airoldi and Fulvio Borella. *Investigation*: Niccolò Gallio, Cristina Cavagnetto, and Luca Bertero. *Data curation*: Chiara Airoldi and Niccolò Gallio. *Writing—original draft preparation*: Niccolò Gallio and Mario Preti. *Writing—review and editing*: Fulvio Borella, Niccolò Gallio, Mario Preti, Raffaella Ribaldone, Paola Cassoni, Chiara Benedetto, Elena Pisapia, Enrica Bovio and Valentino Remorgida. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

This research received no external funding.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Preti M, Boldorini R, Gallio N, et al. Human papillomavirus genotyping in high-grade vaginal intraepithelial neoplasia: a multicentric Italian study. *J Med Virol*. 2024;96:e29474. doi:10.1002/jmv.29474