TITLE PAGE

AGE-RELATED DISTRIBUTION OF UNCOMMON HPV GENOTYPES IN CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE 3

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ABSTRACT

Aim: Cervical cancer prevention guidelines include Human Papillomavirus (HPV) test, cytology, and HPV-16/18 typing for triage to determine the risk of cervical intraepithelial neoplasia (CIN) grade 3 as the best proxy of cervical cancer risk. In doing that, they do not consider how age can modify the type-specific risk of CIN3. The present study aimed to evaluate the age-related distribution of HPV genotypes affecting the risk-assessment in cervical cancer screening programs: non-screening-type-HPV and non-HPV-16/18 in unvaccinated women with CIN3.

Methods: Retrospective multi-institutional study, including HPV genotyped women with CIN3 on cone histology treated between 2014-2019. The sample was divided into three categories of age: <30, 30-44, ≥45. HPV genotypes were grouped in non-screening-type-HPV (not-including genotypes 16/18/31/33/35/39/45/51/52/56/58/59/66/68) and non-HPV-16/18. Associations and trends between different age-groups and HPV genotypes were measured.

Results: 1332 women were analyzed. Non-screening-type-HPV CIN3 were 73 (5.5%). Non-HPV-16/18 were found in 417 participants (31.3%). Women over 45 associated with non-screening-type HPV [odds ratio (OR)=1.87, 95% confidence interval (CI) 1.07–3.25; p=0.027]. Non-screening-type-HPV prevalence increased significantly with age (3.9% vs 5.1% vs 9.0%, p=0.016). Women under 30 showed a lower rate of non-HPV-16/18 (OR=0.65, 95% CI 0.47–0.89; p=0.007). There was a positive trend with age of non-HPV-16/18 CIN3 (23.6% vs 32.1% vs 38.0%, p=0.0004)

Conclusion: The proportion of CIN3 lesions unrelated to genotypes detected by primary screening tests increased with age. This implies that age probably modifies the risk of CIN3 and possibly of

cancer associated with HPV types. The risk-based recommendation should take into consideration age to define the management of HPV positive women.

Keywords: Human Papillomavirus; non-screening type-HPV; non-HPV-16/18; age, cervical intraepithelial neoplasia.

1. INTRODUCTION

Age is the main clinical variable on which cervical screening and vaccine policies are based [1, 2]. Both cervical cancer screening and Human Papillomavirus (HPV) vaccination are recommended for specific target age groups [3]. The cervical cancer screening program aims to prevent cervical cancer incidence by detecting and treating precancerous lesions [3, 4]. Several trials showed that testing for high-risk (hr) HPV (genotypes 16/18/31/33/35/39/45/51/52/56/58/59) in women between 30-65 years of age is more effective than testing for cytological abnormalities in preventing cancers [5]. Due to the HPV test's high sensitivity, women with a negative screening result should be tested at least after five years [6-8]. Moreover, given the elevated protection of HPV vaccines and the high negative predictive value of the HPV testing, it was hypothesized that just two screening tests over a lifetime might be enough in the post-vaccination era [9].

While the assumptions above could represent the future direction of screening strategies, growing evidence shows that HPV genotype distribution in cervical cancer (CC) changes with age [10-12]. Previous studies showed that with increasing age, a proportion of cancers appeared to be associated with non-hr or negative HPV [10, 13]. More conflicting results with less significance were found in pre-invasive cervical diseases about the correlation between HPV genotype distribution and age. Some authors showed inconsistent results about the trend of HPV 16/18 genotypes with age in cervical intraepithelial neoplasia (CIN) 2, 3, and cancer [14]. More recent studies showed a negative correlation between HPV 16 and age in CIN3 [15, 16].

A further aspect to be considered concerns the impact of HPV vaccination on genotype prevalence changes. We will likely see fewer cervical lesions due to HPVs targeted by vaccines, but a similar number of CINs linked to other uncommon HPV genotypes [17]. However, assessing HPV vaccine impact across age groups, including older age, will be a topic for years to come.

To date, risk-based guidelines include HPV test, cytology, and HPV 16/18 typing for triage to determine the risk of having or developing in the next future a CIN3 as the best proxy of cervical cancer risk [8]. In doing that, they do not consider how age can modify the type-specific risk of CIN3.

The present study aimed to assess the age-related distribution of HPV genotypes affecting the risk-assessment in cervical cancer screening programs: non-screening-type HPV and non-HPV 16/18 in unvaccinated women with CIN3 lesions.

2. MATERIAL AND METHODS

2.1.Study design and setting

This retrospective multi-institutional study included unvaccinated women with a histological diagnosis of CIN3 on cone specimens. All participants underwent loop electrosurgical excision procedure between January 2014 and January 2019. Women with previous conization, immunological disease, pregnancy, or unavailable HPV genotyping before surgery were excluded. The study design provided that the procedures and data of interest were performed or acquired according to routine clinical practice before starting the study.

The approval of the Ethics Committee (CERM) was obtained (Prot. 373/2020). Given the retrospective study design and according to Italian law, the Ethic committee authorized patient data without their specific consent if it was impossible to contact them [18].

The Departments participating in the study were the following:

- Woman's Health Sciences Department, Gynecologic Section, Polytechnic University of Marche, Ancona;
- Gynecological Oncology Unit, Fondazione IRCCS - Istituto Nazionale Tumori, Milano;
- IRCCS S. Matteo Foundation, Department of Clinical, Surgical, Diagnostic and Paediatric Sciences, University of Pavia, Pavia;
- Gynaecology Unit, Fondazione IRCCS - Ca' Granda Ospedale Maggiore Policlinico, Milan;
- Azienda Usl Toscana Nord-Ovest, U.O.C. Ostetricia e Ginecologia, Ospedale Apuane, Massa, Italy.

The participating Departments are research Centers managing women included in both opportunistic and organized cervical cancer screening programs. Usually, in these Centers, HPV genotyping is performed to aid clinical decision making every 12 months during follow-up, or before surgery when excisional treatment is decided.

2.2.Variables

Based on previous studies [13], the women were divided into increasing age categories: <30, 30–44, and ≥45. Based on HPV genotype classifications [19, 20], HPV genotyping outcomes were classified in the following categories: 1) non-screening-type HPV (not-including genotypes 16/18/31/33/35/39/45/51/52/56/58/59/66/68); 2) non-HPV 16/18. Moreover, non-vaccine-type HPV (not-including genotypes 6/11/16/18/31/33/45/52/58), possibly carcinogenic HPVs (genotypes 26/30/53/67/70/73/82/85), low-risk HPVs (genotypes 6/11/40/42/43/44/54/55/61), negative HPV and not classified subtypes (genotypes other than 16/18/31/33/35/39/45/51/52/56/58/59/66/68/26/30/53/67/70/73/82/85/6/11/40/42/43/44/54/55/61)

were also measured. Finally, the rate of hr-HPVs, HPV 16/18, and multiple HPV infections was also reported.

According to previous studies [21, 22], we used a hierarchical attribution estimate in multiple HPV infection cases. In this regard, CIN3 was attributed to the genotype most associated with high-grade cervical lesions. For example, a lesion was attributed to non-screening-type HPV genotypes only if screening-type HPVs were not present (genotypes 16/18/31/33/35/39/45/51/52/56/58/59/66/68). Likewise, a lesion was attributed to HPV genotypes not included in the vaccine only if HPV genotypes included in the vaccine was not present (HPV 16/18/31/33/45/52/58). The same hierarchical criterion was used in multiple infections, including possibly carcinogenic, low-risk, or other HPV subtypes. The prevalence of each HPV genotype was reported considering its presence in both single and multiple infections.

2.3.Data sources/measurements

All data were retrieved from the electronic database used in our Clinics and anonymized before analysis. Usually, cytologic samples were collected with an endocervical swab and Thin Prep (TP) (Hologic, Marlborough, MA, USA). Afterward, DNA extraction and HPV typing were made according to locals protocols using the HPV Sign® Genotyping Test (Qiagen, Hilden, Germany), or INNO-LiPA® HPV Genotyping Extra assay (Innogenetics, Ghent, Belgium), or CLART® HPV2 PCR (Genomica, Madrid, Spain). The procedures have been described in detail previously [16, 23-25].

2.4.Sample size calculation

Based on previous data [13, 22], we expected a non-screening-type HPV CIN3 lesions rate of approximately 6%. Likewise, we expected a percentage of non-HPV 16/18 CIN3 lesions of about

30%. With a confidence level of 95%, and confidence interval width (2-sided) equal to 6 (± 3%), the minimum required sample size should include 250, or 894 women with CIN3, respectively.

2.5.Statistical methods

The associations and trends between age strata and different HPV groups were reported according to the distribution of specific HPV genotypes in the three age-groups. Given that HPV genotype distribution may also be affected by ethnicity or the HPV genotyping method, these variables were included in the analysis.

Categorical variables were expressed as numbers and percentages. The Chi-squared test was used to compare categorical variables (e.g., age groups and ethnicity or HPV genotyping methods). When the data originated from ordered categories, we used the chi-squared test for trend to test the relationship between two classification factors (e.g., age and HPV genotype groups). It is more potent than the unordered independence test when a classification table has two columns and three or more rows (or two rows and three or more columns) [26]. The association [Odds ratio (OR)] between distinct age strata and different HPV groups (non-screening-type HPV, non-HPV-16/18, non-vaccine-type HPV, low-risk HPV, possibly carcinogenic HPV, and negative HPV) were measured using logistic regression analysis. Age groups represented categorical independent variables, while HPV groups represented the dependent variable.

All statistical analyses were performed using MedCalc Statistical Software (MedCalc® Statistical Software version 19.5.3; MedCalc Software Ltd, Ostend, Belgium; https:// www.medcalc.org; 2020). No formal statistical test of hypothesis is performed; therefore, we did not set a significance threshold. P-values should be considered as continuous variables reporting the probability that a difference would be observed under the null hypothesis.

3. RESULTS

During the study period, 1708 consecutive women with CIN3 on cone specimens were recruited. After excluding 376 cases, 1332 women were analyzed (Figure 1).

Patient characteristics are reported in Table 1. Seventy-three participants (5.5%) were negative for screening-type HPV, whereas 120 women (9.0%) showed HPV genotypes not included in the vaccines. Non-HPV 16/18 were found in 417 women (31.3%). Different age-groups included the following categories: < 30 years (280 women) [median age: 26.0 (interquartile range 25.0-28.0)], 30-44 years (831 women) [median age: 36.0 (interquartile range 33.0-40.0)], ≥45 years (221 women) [median age: 51.0 (interquartile range 47.0-59.0)].

The majority of women were Italian (about 90%), and ethnicity did not show differences between the three age-groups [Italian women <30 years=253 (90.4%), 30-44 years=727 (87.5%), ≥45 years=196 (88.7%), p=0.122]. Likewise, the HPV genotyping methods showed no differences in HPV distribution between study groups [HPV Sign® Genotyping Test <30 years=80 (28.6%), 30-44 years=298 (35.9%), ≥45 years=70 (31.7%); INNO-LiPA® HPV Genotyping Extra assay <30 years=107 (38.2%), 30-44 years=277 (33.3%), ≥45 years=70 (31.7%); CLART® HPV2 PCR <30 years=93 (33.2%), 30-44 years=256 (30.8%), ≥45 years=81 (36.7%), p=0.113].

Among non-screening-type HPV (73/1332 women), the most common possibly carcinogenic HPV was genotype 53 (23.3%), followed by HPV-73 (12.3%), HPV-70 and HPV-82 (2.74%) (Figure 2). Twenty-one women out of 73 included in non-screening-type HPVs (28.76%) were HPV negative (Figure 2). The most common hr-HPV was genotype-16 (63.1%), followed by HPV-18 (15.8%), HPV-52 (14.0%), HPV-33 (13.0%), HPV-31 (11.8%) (Figure 3).

Non-screening-type HPVs showed the following age-trend: 3.9%, 5.1%, 9.0% in women < 30, 30-44, ≥45 years of age, respectively (p=0.016) (Figure 4). Non-HPV 16/18 had the following age-

trend: 23.6%, 32.1%, 38.0% in women < 30, 30-44, ≥45 years of age, respectively (p=0.0004) (Figure 4). HPV genotypes not included in the vaccines showed the following age-trend: 5.7%, 8.5%, 14.9% in women < 30, 30-44, ≥45 years of age, respectively (p=0.0005) (Figure 4).

Logistic regression analysis showed a significant association between women ≥45 years of age and non-screening-types HPV in high-grade cervical lesions (OR = 1.87, 95% confidence interval (CI)) 1.07–3.25; p=0.027 (Table 2). Women under 30 showed a lower rate of non-HPV 16/18 (OR vs ≥45=0.65, 95% CI 0.47–0.89; p=0.007). Women aged 45 years and over also showed an increased prevalence of lesions attributable to HPV genotypes not included in the vaccines (OR=1.88, 95% CI 1.20–2.92; p=0.005) (Table 2).

4. DISCUSSION

The present study's main findings showed significant associations and trends between advanced age and HPV genotypes not included in primary screening tests. In a large population, 9% of the CIN3 were related to non-screening-type HPV in women ≥45 years. Interestingly, half of non-hr HPV CIN3 lesions over 45 were linked to possibly carcinogenic HPVs (4.5%). Non-HPV 16/18 precancerous lesions showed a significant positive trend with age amounting to 38% after 45 years. Finally, 15% of the precancer lesions in women aged 45 and over were related to non-vaccine-type HPV.

Previous studies investigated the age-related changes of HPV genotype distribution in preinvasive cervical lesions with conflicting results. Carozzi et al. showed a decrease in the proportion of HPV-16/18 genotypes in 144 CIN2 and 193 invasive cancers, but not in 385 CIN3 [14]. Other authors showed a negative trend with increasing age in HPV-16 CIN3 lesions [11, 15, 16]. A recent study, including 503 unvaccinated women with high-grade histological lesions,

showed an age-related HPV genotype distribution [13]. The authors reported a higher rate of non-vaccine-type or negative type HPV CIN2+ in advanced age. In a sample of 77 women aged 45 years and over, the authors found 8 (10%) non-hr HPV-related lesions. Likewise, they showed just over 15% of HSIL not-related to HPV genotypes included in the vaccines [13]. These results were in line with our percentages. They also reported 3.4% of HPV-negative high-grade cervical lesions (17/503) [13]. Our study had a lower rate of HPV-negative CIN3 (1.6%), probably reflecting the absence of glandular lesions in our sample. We also know that finding negative HPVs can depend on the test's sensitivity [23, 27]. A recent paper reported that 50% of negative HPV high-grade cervical lesions revealed the presence of possibly carcinogenic genotypes at other HPV genotyping tests [28].

This age-related polarization of HPV genotype distribution in precancer cervical lesions has already been the subject of possible hypotheses. Rositch et al. reported that, with increasing age, most HPV infections were due to viral reactivation rather than new sexual partners [29]. In advanced age, immune changes due to "immunosenescence" could affect the acquisition and reactivation of some HPV infections [30]. It is likely that quickly cleared HPV genotypes at a young age may persist in advanced age and lead to some cervical lesions' progression. It has been highlighted that HPV-induced cancerogenesis is a multistep process characterized by a small probability of progression. A lower likelihood of passage from one step to another would bring to a longer time required to reach an appreciable proportion of high-grade lesion and cancers [14]. This is consistent with the younger age at onset of HPV16/18 cancers, compared with other HPV types [12]. Nevertheless, to date, the reason for this different HPV genotype distribution is unclear.

To understand the real impact of precancerous cervical lesions missed by the primary HPV screening test on cervical cancer prevention tools, we should know the real oncogenic potential of these lesions. We know from several indirect observations on HPV types in cancers [12, 31] and

prospective studies [32] that non-screening targeted HPV types have a low cancerogenic potential. On the other hand, we know that the proportion of CIN3 that, if not treated, will progress to cancer in older women is higher [33, 34]. Any consideration about the role of these CIN3 should start from the fact that incidence of cancers after a negative HPV-test is a much rarer event than after a negative Pap test [5, 35], but become virtually absent after two or three negative hr-HPV tests [32].

Previous studies reported that delaying the onset of screening age in the post-vaccination era may have its rationale as about 95% of preinvasive lesions in women under 30 are related to high-risk HPVs targeted by the vaccines [36]. Our results suggest that also for CIN3, the non-16/18 and non-9-valent-vaccine type would be few below 30, supporting the conclusions of the recent recommendations proposed for Italian screening programs in vaccinated women [37]. As reported by a recent paper [17], we will likely observe a significant decrease in high-grade lesions due to HPV genotypes included in the vaccines and an almost constant prevalence of cervical lesions due to non-vaccine HPV types. To date, these findings are mainly evident in the younger age groups, but we also expect to see a decline in the older age group in the future as more vaccinated people age in these groups [17]. Although the most significant impact of these changes affects vaccinated women, it also includes unvaccinated women (herd immunity) [38]. However, future studies designed with this clear goal will need to confirm these data further.

Risk-based guidelines for managing the HPV positive women recently issued by all the leading American scientific societies in this field use the information on HPV type to stratify women's risk [8]. In particular, women with HPV16 or 18 infections should be referred immediately to colposcopy, while for women infected with other hr-HPV types, a cytology triage is recommended [8]. Our study does not allow us to calculate the PPV of type-specific-HPV infection since we only have CIN3 cases and not HPV infections without lesions. Still, it suggests that the risk of CIN3 underlying non-HPV16/18 infections could change with age. Therefore, assess how the risk of

CIN3 linked to different HPV types changes with age, screening round, and vaccine status is crucial to develop a sound risk-based recommendation to manage HPV positive women. This age-dependent HPV distribution raises the issue of the final screening test if the exit age is anticipated in the near future. A recent study showed that a negative hr-HPV test or a negative co-test in unvaccinated women after 55 years of age correlated with a shallow risk of developing cervical cancer [39].

The present study has several limitations. 1) Its retrospective nature does not allow to estimate the real risk of CIN3 in women positive to a specific HPV type in a given age; 2) the time elapsed between the last HPV test and conization is not precisely known; 3) the use of different genotyping tests; 4) we do not know the reason for referring the women to colposcopy guided biopsy, (e.g., if women were referred for cytology positive, HPV positive or both). It should be emphasized that in the Departments included in the study, usually, the time interval between decision and conization does not exceed four weeks. We can state that the HPV testing before surgery should have been performed within four weeks with a good approximation. Moreover, although the HPV genotyping procedures were not the same for all women, two out of three methods (HPV Sign® Genotyping Test and INNO-LiPA® HPV Genotyping Extra assay) showed an overall agreement rate of 85.1% [23].

The study's strengths include the large sample size of HPV genotyped women affected by the true precancerous cervical lesion (CIN3). It does not have the histopathological reproducibility limitations of CIN2 [40]. Finally, the reliability of the histological reference standard represented by the cone specimens instead of cervical biopsies.

4.1. **Conclusions**

To conclude, in an extensive series of women, the proportion of CIN3 lesions unrelated to genotypes detected by primary screening tests and non-HPV 16/18 increased with age. This implies that age probably modifies the risk of CIN3 and possibly of cancer associated with HPV types. The risk-based recommendation should take into consideration age to define the management of HPV positive women.

Author Contributions

A.C., L.G., P.G.R., conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft; G.D.C., J.D.G., S.I., G.B., B.G., E.M., A.G., C.A.L., E.R., data curation, investigation, and methodology; A.C., A.S., F.R., P.V. data curation, investigation, and supervision. All authors have read and agreed to the published version of the manuscript.

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No funding was received for this study.

Conflicts of Interest

The authors declare no conflict of interest.

Figure Legends:

Figure 1. Study flow-chart.

Figure 2. Genotype prevalence in 73 non-screening-type HPV CIN3 (sample size = 1332).

Figure 3. High-risk HPV genotype prevalence in 1332 CIN3.

Figure 4. Age-related trend of specific HPV genotypes in 1332 CIN3.

Table Legends:

Table 1. Patient characteristics.

Table 2. Logistic regession analysis showing associations between age and HPV genotypes groups in CIN3.

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