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Time trends in prevalence of p16 positivity and combined HPV/p16 positivity in a large cohort of Danish vulvar cancer patients

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Abstract

Vulvar cancer is rare, but causes substantial morbidity in affected patients. A subset of vulvar cancers is caused by high-risk human papillomavirus (hrHPV), which primarily exerts its oncogenic effect through upregulation of tumor suppressor protein p16. Tumors positive for both hrHPV and p16 (double positive) are assumed to be HPV-driven, but only few large studies have investigated the combined prevalence of hrHPV and p16 positivity in vulvar cancer over time. In this Danish cross-sectional study, we assessed the prevalence of p16 positivity and double positivity for hrHPV and p16 in a large sample of vulvar squamous cell carcinomas (VSCCs) diagnosed during 1990 to 2017. In a nationwide register, we identified VSCCs from 13 hospitals across Denmark, and collected archival tumor tissue for hrHPV testing with INNO-LiPA and immunohistochemical p16 staining. We calculated the prevalence of hrHPV, p16 positivity and double positivity according to time, age and histological.
subtype and evaluated time trends through estimated annual percentage changes. We included 1278 VSCCs. Overall, 35.0% (95% confidence interval [CI]: 32.4-37.6) were positive for p16 and 31.0% (95% CI: 28.4-33.5) were positive for both hrHPV and p16. The prevalence of p16 positivity and double positivity increased over time, both in women aged ≤59 and ≥60 years. The double positive prevalence was higher in nonkeratinizing (60.7%) and warty/basaloid VSCCs (67.5%) than in keratinizing (16.1%) and verrucous VSCCs (5.0%). These results indicate that approximately one-third of vulvar cancers were caused by hrHPV infection, supporting a substantial preventive potential of the HPV vaccine.

KEYWORDS
HPV, p16, time trends, vulvar cancer

What’s new?
A subset of vulvar cancers is caused by high-risk human papillomavirus (HPV), which primarily exerts its oncogenic effect through upregulation of tumor suppressor protein p16. How the proportion of HPV-driven vulvar cancers has changed over time remains largely unexplored. This Danish cross-sectional study estimated HPV prevalence and p16 positivity in more than 1200 vulvar cancer cases diagnosed in 1990 to 2017. The proportion of HPV-driven tumors (ie, positive for both HPV and p16) increased to 35.7% in the most recent period. The findings support the potential of HPV vaccination to substantially prevent vulvar cancer.

1 | INTRODUCTION

Vulvar cancer occurs at an incidence rate of 0.4 to 1.7 per 100 000 women,1 amounting to ~3% of all gynecological cancers.2 The incidence of vulvar cancer has been increasing in recent years,3-5 especially among younger women.6-8 Histologically, the majority of vulvar cancers are classified as squamous cell carcinomas (VSCCs). Several morphological subgroups are recognized and of these, keratinizing SCCs and verrucous carcinomas are more often associated with chronic inflammatory diseases. On the other hand, warty/basaloid SCCs and nonkeratinizing SCCs are more often associated with high-risk human papillomavirus (hrHPV) infection.9,10

The oncogenic potential of infection with hrHPVs can mainly be attributed to the two oncoproteins E6 and E7. Upon integration of hrHPV DNA into the host genome, E6 and E7 are overexpressed. E6 exerts its effect in the cell by targeting the tumor suppressor protein p53 for degradation, leading to loss of cell cycle control.11 E7 binds to and promotes degradation of retinoblastoma protein (pRb), and this in turn leads to the upregulation and overexpression of the tumor suppressor protein p16,12 and the subsequent further disruption of cell cycle control. Therefore, p16 overexpression has been used as a surrogate marker for hrHPV infection in some studies,13 but others have argued that p16 overexpression should be used as an ancillary marker for the transforming actions of hrHPV infection.14 As such, detection of hrHPV with no overexpression of p16 can be interpreted as a transient hrHPV infection, which may not have a role in carcinogenesis.

Few previous studies have investigated the prevalence of hrHPV and p16 overexpression in VSCCs, and apart from one multicountry study,7 they have all been limited by small populations (<200 vulvar cancers)15 and most studies only investigated either HPV or p16.16-19

We have recently published on the prevalence and time trends of hrHPV and specific HPV types in a large study of Danish VSCC patients.20 In the present study, our aim was to assess the prevalence of p16 positivity, as well as the combination of hrHPV and p16 positivity in a large number of VSCCs. Furthermore, we wanted to investigate the changes in the prevalence of p16 positivity and combined hrHPV- and p16 positivity over time.

2 | MATERIALS AND METHODS

2.1 | Population

The study design and methods have previously been described in detail.20 In brief, from the Danish Pathology Register,21 we identified all women with a diagnosis of primary VSCC between 1 January 1990 and 31 August 2017 at one of the following hospitals covering all five regions of Denmark: Herlev, Hillerød, Gentofte, Hvidovre, Naestved, Roskilde, Slagelse, Nykøbing Falster, Odense, Svendborg, Vejle, Aarhus and Aalborg. The Danish Pathology register is a national register containing data on diagnosis, date of diagnosis and topography and morphology codes for all cytological and histological evaluations performed throughout the country. The register is considered complete from 1997, but most pathology departments have also added
information on examinations going back to the early 1970s. Furthermore, tissue samples and histological slides from diagnostic and treatment procedures are stored in repositories at local hospitals throughout Denmark.

2.2 | Pathology review

We collected a formalin-fixed paraffin-embedded (FFPE) tumor specimen from all identified VSCC cases. Surgical specimens were selected when available, and otherwise the diagnostic biopsy was used. The corresponding hematoxylin and eosin (H&E) stained slide was evaluated by a pathologist to confirm the diagnosis of vulvar SCC. Additionally, in accordance with the World Health Organization’s classification of vulvar tumors at the time of study we attempted to classify each sample into one of the following histological subcategories: keratinizing, nonkeratinizing, warty, basaloïd, verrucous and SCC not otherwise specified (NOS). Keratinizing SCC was defined as substantial areas of the tumor showing keratinization and simultaneous presence of keratin pearls, whereas nonkeratinizing SCC was defined as complete absence of keratinization in the tumor. In cases where these criteria were not precisely fulfilled, the tumor was classified as SCC NOS. Upon pathology review, tumor specimens were sent to a centralized laboratory at Vejle Hospital for HPV testing and p16 immunohistochemical (IHC) staining.

2.3 | HPV test method

The methods employed for extraction, detection and typing of HPV DNA in our study have previously been described. Briefly, HPV DNA was extracted from FFPE tissue samples using the Maxwell 16 FFPE Plus LEV DNA Purification kit and the corresponding Maxwell 16 robot (Promega, Madison, WI). Detection and typing of HPV DNA were performed using the INNO-LiPA HPV assay (Fujirebio, Belgium), which identifies the following 32 HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68, 70, 73, 81, 82, 83 and 89.

2.4 | p16 immunohistochemistry

For IHC staining, 3-μm-thick sections were cut and placed onto slides. The IHC staining for p16 was performed on Benchmark Ultra automatic instrument (Ventana Medical systems, Roche, Tucson, AZ) according to the manufacturer’s protocol. Appropriate positive and negative controls were included for each run. Using the CINtec p16 Histology Kit (Roche, Tucson, AZ), which contains a monoclonal primary mouse antibody, a nuclear and/or cytoplasmic staining of the tissue was achieved. The reaction was then visualized using the OptiView DAB IHC Detection Kit (Roche, Tucson, AZ), and the result is visible as a brown staining of the p16 positive areas in the tissue.

2.5 | p16 expression evaluation

Each p16 IHC stained tissue slide was evaluated by two pathologists, who were blinded to each other’s interpretations. p16 overexpression was defined as moderately to intensely expressed p16 in both nuclei and cytoplasm. Distinction was made between absent (<1%) (score = 1), focal (≤25%) (score = 2), multifocal (25%-70%) (score = 3) and diffuse (>70%) (score = 4) p16 staining, with only diffuse staining (score = 4) counted as p16 positivity. These criteria were based on those used by Dong et al. Other studies have employed different thresholds for considering a sample as p16 positive, but we have chosen to employ this one, as it has previously been validated in head and neck cancer. In case of disagreement between the two evaluations, a third evaluation was performed and a majority decision reached where possible. In cases where a majority decision could not be reached, the case was counted as negative if the three evaluations were “absent (score = 1),” “focal (score = 2),” and “multifocal (score = 3);” in all other cases, the sample was excluded.

2.6 | Statistical analysis

We calculated the prevalence of hrHPV and p16 positivity separately, as well as the combined prevalence of hrHPV and p16 positivity (double positivity). HPV types defined as hrHPV were 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. We also estimated the time trends in prevalence of p16 positivity and double positivity by calculating the estimated annual percentage change (EAPC) over the entire study period accompanied by corresponding 95% confidence intervals (95% CIs) employing Poisson regression models with log link. Furthermore, the changes in prevalences of hrHPV, p16 positivity and double positivity over time among women older and younger than 60 years were also evaluated using the same method. Finally, to assess whether the sample type (biopsy vs surgical specimen) impacted our findings, we compared the prevalence of hrHPV, p16 positivity and double positivity according to specimen type. SAS Enterprise Guide 7.1 (Statistical Analysis Software, Cary, NC) was used for all statistical analyses.

3 | RESULTS

3.1 | Study population

We initially identified 1544 women diagnosed with primary VSCC at one of the included hospitals in the period 1990 to 2017 (Figure 1). We excluded women whose samples were autopsy material (n = 7), could not be located in archives (n = 64) or contained too little tumor tissue (n = 157). The remaining 1316 cases were tested for HPV and stained for p16. Of these, we excluded 8 samples (0.6%) which did not produce a valid HPV test result, and 16 (1.2%) cases in which the p16 stained slide was insufficient for evaluation. In 149 cases (11.3%), the two initial p16 scores differed, and a third p16 evaluation was
performed. In 135 of these cases, a majority decision on p16 interpretation was reached, and the remaining 14 were excluded. This left us with 1278 cases included in the present study. The excluded cases (n = 266) were slightly younger at diagnosis than those included in analysis (n = 1278) (median age: 64 vs 72 years), and the proportion of excluded cases was generally higher in the earlier parts of the study period.

3.2 | Population and tumor characteristics

Patient and tumor characteristics are presented in Table 1. The median age was 72 years (range 27-102), and women aged 60 years and older accounted for more than 75% of our study population. The majority of tumors were histologically classified as SCC NOS (67.1%). Keratinizing SCC and nonkeratinizing SCC accounted for 23.3% and 4.8%, respectively. A little more than half of our samples were from biopsy material (51.9%).

3.3 | Prevalence of hrHPV, p16 positivity and double positivity

Table 1 also shows the prevalence of p16 positivity and the combined prevalence of the two biomarkers. The prevalence of p16 positivity was 35.0% (95% CI: 32.4-37.6) whereas the prevalence of hrHPV was 51.9% (95% CI: 49.1-54.6). The prevalence of double positivity, that is, hrHPV positivity in combination with p16 positivity, was 31.0% (95% CI: 28.4-33.5). The type-specific hrHPV prevalences have previously been reported. In the present study, we investigated the type-specific hrHPV prevalence among double positive as well as hrHPV positive/p16 negative cases. We found that the type distribution was
similar in the two groups. The combined prevalence of hrHPV types covered by the four-valent- or nine-valent HPV (4vHPV and 9vHPV) vaccine was also similar (Table S1).

The prevalence of hrHPV, p16 positivity and double positivity according to calendar period are presented in Figure 2. The increasing prevalence of hrHPV over time has been presented previously. The prevalence of p16 positivity showed the same pattern, only at a lower level, ranging from 27.3% during 1990 to 1993 to 41.9% at the end of the study period, corresponding to a statistically significant increase in prevalence with an estimated annual percentage change (EAPC) of 0.57 (95% CI: 0.24-0.91). Similarly, the prevalence of double positivity increased from 24.5% in the earliest period (1990-1993) to 35.7% in the most recent period (2014-2017), with an EAPC of 0.42 (95% CI: 0.10-0.75).

In Figure 3, we present the prevalence of hrHPV, p16 positivity and double positivity for younger women (age ≤ 59; Figure 3A) and older women (age ≥ 60; Figure 3B) over time. The younger women had a higher prevalence of p16 positivity and hrHPV, both separately and in combination, than the older women throughout the study period. In both age groups, an increasing trend in prevalence was observed over time for both p16 positivity and double positivity, with the strongest increase seen for p16 positivity alone.

Figure 4 depicts the prevalence of p16 positivity, hrHPV and double positivity according to histological subgroups. Among women with nonkeratinizing and warty/basaloid SCCs, the prevalence of p16 positivity was similar to that of hrHPV, respectively 65.6% and 75.0%, and the prevalence of double positivity in these two groups was similarly high (60.7% and 67.5%). In contrast, in verrucous and keratinizing SCCs, we observed a hrHPV prevalence around 40% and a much lower prevalence of p16 positivity and double positivity below 20%. For the group of SCC NOS, the hrHPV prevalence was 54.0% with a somewhat lower prevalence of p16 positivity at 36.9% and a prevalence of double positivity of 33.0%.

The prevalence of p16 positivity was somewhat lower in biopsies (31.8%) than in surgical specimens (38.6%), but the prevalence of hrHPV (biopsies: 52.2%; surgical specimens: 51.4%) and double positivity (biopsies: 28.9%; surgical specimens: 33.3%) was similar irrespective of sample type. The proportion of cancers classified as SCC NOS was higher in biopsies (71.1%) than in surgical specimens (62.7%; data not shown).

### 4 DISCUSSION

Our study included 1278 Danish cases of VSCC diagnosed over a period of nearly 30 years, making it the largest national study worldwide. We found overall prevalences of hrHPV, p16 positivity and double positivity of 51.9%, 35.0% and 31.0%, respectively. Our study also showed that these prevalences increased during the last three decades. Younger women generally had higher prevalences than older women and increasing trends over time were observed in both older and younger women for p16 positivity and double positivity.

The largest previous study investigating HPV and p16 prevalence in vulvar cancers from 39 different countries, comprising 1287 vulvar cancer cases, found a crude prevalence of double positivity of 25.1%, which is similar to our result. Also similar to us, they found a higher
Figure 2: Prevalence of hrHPV, p16 overexpression and double positivity* in 1278 Danish vulvar squamous cell carcinoma patients, according to calendar period. *Double positivity = hrHPV positive and p16 positive. The time trends for hrHPV have previously been published. [Color figure can be viewed at wileyonlinelibrary.com]

Figure 3: Prevalence of hrHPV positivity, p16 positivity and double positivity* in 1278 Danish vulvar squamous cell cancers according to calendar period and age at diagnosis (A) among women 59 years and younger, (B) among women 60 years and older. *Double positivity = hrHPV positive and p16 positive. [Color figure can be viewed at wileyonlinelibrary.com]
prevalence in the younger age groups. A Canadian study including 196 VSCC cases found a p16 prevalence of 40.0%, which is comparable to our p16 prevalence of 35.0%. A Polish study found an HPV prevalence of 45.0%, a prevalence of p16 positivity of 41.2% and a double positivity prevalence of 29.4%. Thus, our findings are generally in line with those of previous studies that employed similar test methods and definitions of p16 positivity as ours.

In general, the association between p16 positivity and hrHPV positivity was strong in our study, reflecting the HPV-driven upregulation of p16 overexpression through the pRb pathway. However, we did find 51 cases in which p16 was overexpressed and hrHPV was negative. In these 4% of our cases, the overexpression of p16 might be caused by endogenous genetic disturbances, leading to uncontrolled cell proliferation.

To our knowledge, we are the first to evaluate time trends in p16 positivity and double positivity in VSCC. However, in the multicountry study by de Sanjosé et al, they reported a double positive prevalence of 21.2% in the years 1980 to 1999 and a slightly higher prevalence of 26.7% in the years 2000 to 2011. This is in line with our findings. An increasing HPV prevalence over time has also been shown for oropharyngeal cancer. For example, two Danish studies reported increasing incidence of HPV-positive oropharyngeal cancers over the period 2000 to 2014. However, these studies did not examine p16 overexpression. The increasing trend in incidence of hrHPV-, p16- and double-positive vulvar cancers could be associated with changes in sexual behavior among Danish women over the past decades, where increasing number of lifetime partners as well as lower age at first intercourse have been observed.

In a recently published study, we showed that warty/basaloid and nonkeratinizing SCCs had a higher hrHPV prevalence than verrucous and keratinizing SCCs. In the present study, we furthermore found that in warty/basaloid and nonkeratinizing SCCs, the prevalence of p16 positivity was similar to the hrHPV prevalence, whereas in verrucous and keratinizing SCCs, the prevalence of p16 positivity was markedly lower than the hrHPV prevalence. This could indicate that there was a higher proportion of bystander HPV infections in the keratinizing and verrucous SCCs, whereas the majority of HPV-positive warty/basaloid and nonkeratinizing SCCs were in fact HPV-driven. However, up to 16.1% of keratinizing SCCs were positive for both hrHPV and p16, and thus must be assumed to be HPV-driven, adding to the evidence that histologic diagnosis alone is insufficient to differentiate between HPV-driven and HPV-independent cancers. These findings are also reflected in the latest WHO classification of tumors of the vulva, where vulvar SCC is classified as HPV-associated or HPV-independent, rather than classified based on histomorphologic features.

In Denmark, the 4vHPV vaccine was included in the childhood vaccination program in 2009, where it was offered to girls aged 12, and initial catch-up vaccination of girls up to age 15 was also offered. In 2012, a second catch-up vaccination program was implemented covering women up to the age of 27 years, and finally, the 9vHPV vaccine was implemented into the program in 2017. The 4vHPV and 9vHPV vaccines have shown high clinical efficacy in reducing the incidence of vulvar precancerous lesions causally associated with HPV types covered by the vaccines. Additionally, our group has recently demonstrated in an ecologic study that the incidence of vulvar premalignancies among young Danish women has decreased since the introduction of HPV vaccination. This finding was corroborated in a study of the real-world effectiveness of the 4vHPV vaccine, showing that vaccinated Danish women had significantly reduced risk of vulvar premalignant lesions and cancers compared to unvaccinated women. In the present study, we found that 51.9% of our cases were hrHPV-positive, and 31.0% of our cases were positive for both hrHPV and p16, indicating that nearly one-third of the vulvar cancers in our study population can be assumed to be HPV-driven. Among the double-positive cases, 98.5% were...
positive for types covered in the 9v-HPV vaccine, and 74.5% were positive for types covered by the 4v-HPV vaccine. This provides further support for the substantial potential of the HPV vaccine in preventing vulvar cancer.

Among the strengths of our study is the large population of nearly 1300 Danish women diagnosed over a span of 28 years. This makes our study the largest, national study on prevalence of HPV and p16 positivity in VSCC to date. Furthermore, the nearly three decades long study period gave us the opportunity to investigate changes in prevalence over time, adding new knowledge of the patterns of HPV and p16 positivity over time. The fact that we tested for both hrHPV and p16 overexpression is also an important strength, because detection of hrHPV in a tumor sample without simultaneous detection of p16 positivity could indicate the presence of a transient bystander HPV infection. Therefore, the proportion of HPV-driven tumors could be overestimated by only testing for HPV DNA. Furthermore, we applied centralized and standardized methods for HPV testing and p16 staining, which reduced the risk of inconsistencies in the data and ensured high validity of the results. Finally, the process of two individual pathologists evaluating each p16 stained slide, followed by a third evaluation in case of disagreement, implies a high accuracy and consistency of the p16 results. Nevertheless, some limitations should also be recognized. Some of our samples had been stored for more than 30 years, and the DNA extraction and HPV testing can be more challenging in these older samples. This could possibly contribute to the lower prevalence of hrHPV in the earlier part of our study period. However, samples with degraded DNA would most likely be excluded because of the internal control of the Inno LiPA assay. Since a little more than half of our samples were biopsy material, we must consider the possibility that these tumor samples might not be representative of the entire tumor. This could be a contributing reason for the relatively high proportion of SCC NOS in our study population. However, the prevalence of double positivity for hrHPV and p16 was very similar in biopsies and surgical specimens, indicating that the sample type had limited influence on our results.

In conclusion, the present study shows that the prevalence of hrHPV, p16 positivity and double positivity, respectively, all display increasing trends over the past 28 years in Danish VSCC patients, with a double positive prevalence in the most recent part of our study period of 35.7%. Furthermore, with a double positive prevalence for the entire population of 31.0%, our results indicate that approximately a third of VSCCs were causally associated with hrHPV infection. This supports that the HPV vaccine can potentially prevent a substantial proportion of VSCCs in the future.

AUTHOR CONTRIBUTIONS
Christina Louise Rasmussen: Conceptualization; Data Curation; Formal Analysis; Methodology; Project Administration; Visualization; Writing - Original Draft Preparation. Louise T. Thomsen: Conceptualization; Data Curation; Supervision; Project Administration; Writing - Review & Editing. Louise Baandrup, Maria Benedicte Franzmann, Alexander K. Kjær, Lise Grupe Larsen, Else Mejgaard Madsen, Nadia Margeth Villena Salinas, Doris Schledermann, Birgitte Hjelm Winberg, Dorthe Ørnskov, Marianne Waldstrøm: Data Curation; Investigation; Methodology; Resources; Validation; Writing - Review & Editing. Susanne K. Kjær: Conceptualization; Funding Acquisition; Methodology; Project Administration; Supervision; Validation; Writing - Review & Editing. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICTS OF INTEREST
Susanne K. Kjær has previously received lecture fee from Merck, and research grant through the affiliating institution from Merck. The other authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT
A fully anonymized copy of the data that support the findings of our study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT
Our study was approved by the Scientific Ethics Committee of the Capital Region of Denmark (H-18007908, 23 April 2018). The Scientific Ethics Committee ruled that informed consent was not necessary due to the use of solely archival tissue for analyses. The data processing activities in the study were registered in the internal research register of the Danish Cancer Society Research Center (2019-DCRC-0027).

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

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