HPV status and favorable outcome in vulvar squamous cancer

Katie Wakeham[1,2], Kim Kavanagh[3], Kate Cuschieri[4], David Millan[5], Kevin G Pollock[6], Sarah Bell[5], Kevin Burton[7], Nick Reed[8] and Sheila V Graham[9]

[1] Sussex Cancer Centre, Brighton and Sussex University Hospital, [2]Institute of Cancer Sciences, University of Glasgow, [3]Department of Mathematics and Statistics, University of Strathclyde, [4]Scottish HPV Reference Laboratory, Royal Infirmary of Edinburgh, [5]Department of Pathology, The Queen Elizabeth University Hospital, NHS Greater Glasgow and Clyde, [6]Vaccine Preventable Diseases, Health Protection Scotland, [7]Department of Gynaecological Oncology Glasgow Royal Infirmary, [8]Beatson West of Scotland Cancer Centre, Glasgow and [9]MRC-University of Glasgow Centre for Virus Research, University of Glasgow

[1] Corresponding author: Dr Katie Wakeham

FRCR PhD
Sussex Cancer Centre
Brighton and Sussex University Hospital
Eastern Road
Brighton
BN2 5BE
katiewakeham@nhs.net
Tel: 07523004569

Impact statement

The epidemiology of vulvar cancer (VSCC) is changing. Our data reveal that the age at which women are diagnosed with VSCC is falling. High-risk (HR) human papillomavirus (HPV) infection is very common in vulvar intraepithelial neoplasia (VIN) and present in just over 50% of VSCC. HR-HPV positivity was associated with lower progression rates from VIN to VSCC and improved progression-free survival of VSCC.

Short Title: HPV positivity correlates with improved outcome in invasive vulvar cancer.

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Key words: HPV, VIN, vulvar cancer

Abbreviations:

HPV, human papillomavirus

HR-HPV, high risk- human papillomavirus

VSCC, Vulvar squamous cell cancer

VIN, Vulvar intraepithelial neoplasia,

CIN, cervical intraepithelial neoplasia

OPSCC, oropharyngeal squamous cell cancer

LSIL low grade squamous intraepithelial lesion

HSIL high grade squamous intraepithelial lesion

(FFPE), formalin fixed, paraffin-embedded

NOS, not otherwise specified

NA, nucleic acid

SIMD, Scottish Index of Multiple Deprivation

OS, overall survival

PFS, progression free survival

Abstract

It is universally accepted that high-risk human papillomavirus (HR-HPV) is the cause of cervical dysplasia and cancer. More recently it has been shown that HPV is also a marker of clinical outcome in oropharyngeal cancer. However, contemporary information is lacking on both the prevalence of HPV infection in vulvar cancer (VSCC), its precursor lesion, vulvar intraepithelial neoplasia (VIN) and the influence of HPV-status on the prognosis of this malignancy. We have conducted a detailed population-based study to examine rates of progression of VIN to VSCC, type-specific HPV prevalence in vulvar disease and the influence of HPV status on clinical outcome in VSCC. We observed that the age at which women are diagnosed with VSCC is falling and there is a significant time gap between first diagnosis of VIN and progression to invasive disease. HR-HPV infection was detected in 87% (97/112) cases of VIN and 52% cases (32/62) of VSCC. The presence of HR-HPV in squamous intraepithelial lesion was associated with lower rates of progression to invasive cancer (hazard ratio, 0.22, p=0.001). In the adjusted analysis, HR-HPV was associated with improved progression-free survival of VSCC compared to those with HPV negative tumours (hazard ratio, 0.32, p=0.02).

Introduction

Squamous cell cancer of the vulva accounts for about 3-4% of genital cancers among women with an estimated 30,000 new cases diagnosed per year worldwide. [1] There is evidence that vulvar squamous cell cancer (VSCC) and its precursor lesion, vulvar intraepithelial neoplasia (VIN) is increasing. Recently, we reported that the incidence of vulvar cancer in Scotland has increased by about 64% in the past three decades, particular among young women. [2, 3] The reasons behind the changing epidemiology of vulval disease are unclear but it may be driven by a secular increase in HPV infection and/or changed behaviors that have facilitated the transmission.

The estimates of HPV prevalence in VIN reported in an international meta-analysis were 84%.[4] VIN is challenging to manage - surgical or topical treatment can be associated with significant morbidities. Natural history studies indicate that around 10% of VIN will progress to cancer although comprehensive, global data are relatively sparse when compared to other HPV-associated neoplasms.[5] For cervical intraepithelial neoplasia (CIN) the estimated spontaneous regression rate is between 30 and 50%, with about 10 to 40% progressing to invasive cancer if untreated.[6] Factors that predict progression of VIN to invasive disease are poorly characterised.

HPV status is considered a marker of favorable clinical outcome in oropharyngeal squamous cell cancer (OPSCC) compared to HPV-negative tumours.[7] As nearly all cervical cancers are positive for HPV, few studies have

looked at HPV status and clinical outcome, although there is some evidence to suggest that cancers where HPV is not detectable have a worse prognosis.[8] Although the global data on HPV type-specific epidemiology in VSCC are less comprehensive compared to cervical cancer and OPSCC, current estimates indicate that approximately 20% of VSCC are associated with HPV. [9] However, data on the influence of HPV-status on the prognosis of patients with vulvar cancer is either lacking or conflicting.

Two recent publications have reported that among women with VSCC the presence of HPV-DNA in tumour tissue is an independent prognostic factor associated with a favourable outcome.[10, 11] Studies have also reported tumours expressing p16, a biomarker associated with HPV positivity[12] are less likely to recur.[10, 13] In contrast, Alonso et al investigated presence of HPV DNA with response to radiotherapy; in multivariable analysis only the presence of lymph nodes metastasis was associated with outcome.[14] A similar finding was reported by another study[15], that in the presence of lymph node metastasis, HPV status if the vulvar lesion was not predictive of outcome.

Given the aforementioned knowledge gaps, the aim of the present study was to characterize VIN and VSCC in a population-based series of patients with respect to (1) progression of VIN to VSCC (2) type-specific HPV prevalence and (3) influence of HPV status on clinical outcome/prognosis in VSCC. Scotland Is an ideal location for a study of a relative rare cancer such as VSCC due to the almost complete population uptake of health care through the National Health Service [16] and consistent national coverage of collection of

health service data.[17]

Materials and Methods

Case selection and identification

All cases of VIN and VSCC diagnosed between 01/01/2001 and 31/12/2014 were identified using the National Health Service, Greater Glasgow and Clyde pathology database, a prospectively gathered record of all routine pathology reports that links to a bio-repository archive of all processed tissue. The Health Board covers the following locations: West Dunbartonshire; East Dunbartonshire; East Renfrewshire; Glasgow City; Inverclyde and Renfrewshire and covers a population of approximately 1.1 million people with about 50% of these being women.[18] Health services in Scotland are financed almost entirely out of general taxation and available to all inhabitants, with only a very small independent health care sector.[16] VIN lesions were graded as part of routine National Health Service management as not otherwise specified (NOS), 'low grade' (LSIL/VIN1) or 'high grade' (HSIL/VIN2 or 3) dysplasia. Women often had multiple biopsies taken of a disease episode over a short time period and so the study population was divided into the following four groups to reduce misclassification of concurrent diagnosis as sequential disease: 1. All VIN lesions identified within 365 days of each other were treated as a single episode of VIN; 2. VIN recurrence was defined as two positive biopsies separated by 365 days or more; 3. VSCC defined as an incident case of cancer diagnosed within the study period; and 4. VIN progressing to VSCC, where 365 days or more separated the diagnoses of VIN and VSCC.

Identification, retrieval and review of cases

To allow pathology review and HPV genotyping, tissue was retrieved from the National Health Service, Greater Glasgow and Clyde pathology biobank for cases of VIN and non-metastatic VSCC diagnosed between 01/01/2008 and 31/12/2009. Detailed pathology review including classifying cases using the 2015 International Society for the Study of vulvovaginal disease (ISSVD) terminology of vulvar squamous intraepithelial lesions into low-grade squamous intraepithelial lesion (LSIL) and high-grade SIL (HSIL) and differentiated vulvar intraepithelial neoplasia (dVIN). To enable analysis of VIN recurrence or VSCC progression and associated pathological and viral factors in this cohort, any samples taken between 01/01/2001 and 31/12/2014 from these women were also reviewed and sent for HPV genotyping. All retrieved tissue underwent pathology review by two consultant pathologists.

VIN and VSCC case management

For the duration of this study patients were managed within the National Health Service, Greater Glasgow and Clyde, tertiary gynaecology oncology referral centre, which offered dedicated vulval clinics for patients with VIN and VSCC. For VIN and VSCC cases the treatment philosophy was based on surgery during this time frame, aiming for a 1cm margin based on clinical findings at the time of surgery. Where positive margins were identified after the primary excision then re-excision would be considered to achieve clear margins. However, this decision would be taken on an individual case basis. All patients were followed up in the tertiary gynaecology oncology referral centre at least twice per year with a physical examination and radiological procedure in cases of VSCC.

HPV genotyping

Formalin fixed, paraffin-embedded blocks were selected and a 10 μm section obtained for subsequent nucleic acid (NA) extraction and HPV genotyping. NA extraction was performed.[19] Subsequently, HPV genotyping was performed using the Optiplex HPV Genotyping assay (Diamex, Heidelberg, Germany). This assay detects 24 HPV types including all established oncogenic or high risk (HR)-types and, as a check for specimen adequacy, incorporates a cellular housekeeping control (beta globin). Immunohistochemical staining for p16 was carried out using BD Pharmingen p16 (Cat No51-1325GR). Cases were coded negative if staining was negative or focal and positive if staining diffuse.

Annotation of cases with clinical information

The demographic and clinical data/variables collected were age, FIGO cancer stage,[20] treatment modality, immunosuppression, smoking status, date and cause of death and Scottish Index of Multiple Deprivation (SIMD) quintiles which identifies small area concentrations of multiple deprivation.[21] Date of diagnosis was taken as date of pathology sample collection.

Analysis

Analysis of factors associated with HR-HPV infection was carried out in Stata12 (StataCorp LP, College Station, TX). Kappa scores for agreement between HR-HPV genotype status and p16 positivity were calculated. Associations between HPV-status and clinical variables were estimated using the Pearson chi-squared test. Both unadjusted and adjusted odds ratios (ORs) for HPV positivity

and 95% confidence intervals (CIs) were calculated using logistic regression modeling. For women with VIN, rates of recurrent or progressive disease by HPV status and co-factors were estimated accounting for differing person years of contribution for each individual. The univariate association between recurrent and progressive disease and each of the clinical variables were assessed using Cox proportional-hazards models.

In those with VSCC, rates of overall survival and progression-free survival were estimated by means of the Kaplan–Meier method and Cox proportional-hazards models were used to estimate unadjusted hazard ratios for all of the clinical variables. A fully adjusted model was created using all variables and then backwards selection used to remove insignificant variables at each stage resulting in final adjusted hazard ratios to estimate the difference in survival by HR HPV- status. All Cox proportional-hazards models were run using R version 3.2.3.

All P-values were 2-sided and we considered $P < 0.05$ to be statistically significant.

Governance

Permission was gained from National Health Service Greater Glasgow and Clyde (Biorepository Reference Number 96).

Results

Prevalence of VIN and VSCC between 2001-2014 and association with age at diagnosis

There were 6666 pathology specimens of benign and malignant vulvar tissue and of these, there were 949 individual women with either VIN or VSCC identified. Incident cases during the selected time period of VSCC accounted for 43% (404/949) of samples and VIN, 57% (545/949). Table 1 describes the cases pathological categories and age. The median age of diagnosis of VSCC decreased from 76 years (IQR 64-89) in 2001 to 64 years (IQR 50-78) in 2014. The median age at VSCC diagnosis was significantly older compared to women presenting with a diagnosis of pre-invasive disease (65 Vs 47 years [$p<0.001$]).

Underlying pathology of VIN and VSCC and rates of progression between 2001-2014

In this cohort for samples collected between 2001 and 2014, the prevalence of recurrent VIN and VIN progressing to VSCC was 18% (96/545) and 6% (32/545), respectively. The median time that women had recorded episodes of recurrent VIN (with no documented pathology of VSCC) was 3.1 years (range 1.7-14.3 years). The median time from first recorded VIN to VSCC was 2.8 years (range 1.1 to 9.1 years). When using the pathology scores recorded for National Health Service management, no association was found between grade of lesion (low grade versus high grade) and risk of VIN recurrence ($p=0.9$) or progression to cancer ($p=0.7$).

VIN and VSCC pathology for detailed subset between 2008 and 2009

A subset of the above cases diagnosed between 01/01/2008 and 31/12/2009 were selected for HPV genotyping (Table 1). This time period was chosen on the basis complete case ascertainment of pathology specimens and to allow length of time of follow-up for survival analysis. Two cases were associated with immunosuppressive drug administration to prevent transplant rejection.

The five-year overall survival for women less than 60 years, 60 years to 79 years, and 80 years and older was 90%, 50% and 15% respectively. Stage of cancer was also related to 5-year overall survival with FIGO Stage 1, 2 and 3/4a having survival rates of 79%, 40% and 38% respectively.

HPV type specific distribution in VIN

For cases of VIN, valid results were obtained for 112/118 samples; two results were considered invalid when tests were negative for both HPV and beta globin, and four results were not available due to sampling/operational reasons. HPV infection was identified in 87% cases (97/112) and HR-HPV types in 85% (95/112). Of cases where HPV was detected, single infections with 16, 18, 33, 42, 45 and 51 were identified in 79, 2, 6, 2, 1 and 1 cases respectively. The occurrence of multiple infections of 16/18, 16/33, 16/33/53, 16/42, 18/6 and 16/6/11 all occurred in a single case each. The level of agreement between HR-HPV genotype and p16 staining was strong (79%, P=0.001).

In the adjusted multivariable analysis (Table 2) the odds of high-risk HPV-positivity was higher in women with HSIL (p=0.04) compared to women with

LSIL or dVIN. Women with VIN staining positive for the biomarker p16 were also more likely to have pre-invasive disease associated with HR-HPV (p=0.04).

Type specific persistence of HPV in women with recurrent VIN

A total of 34/36 cases of VIN diagnosed between 01/01/2008 and 31/12/2009 met study criteria for recurrent VIN and had valid HPV results. The HR-HPV was detected in 88% (30/34) of the first recorded samples and type specific infections were HPV 16 alone in 25/34 cases and 16/18, 16/33/53, 16/6/11, 33 and 51 in one case each. Additional samples from these 34 cases, also separated by at least one year, were sought from the larger (01/01/2001 and 31/12/2014) database. There were 65 such samples identified, with each woman having between 3 and 8 samples. The prevalence of HR-HPV positivity in the first biopsy sample for each case of recurrent VIN did not differ significantly from women with a single episode of VIN (88% [30/34] Vs 89% [58/65], p=0.7). Of the 34 recurrent VIN cases, the detection of HR-HPV was retained and did not change HPV sub-type in 28 cases, never detected in 3 cases, lost from 2 cases and gained HPV in one. All gained and lost HPV types were 16.

Type specific persistence of HPV in women with progressive VIN

There were 13/14 women identified between 01/01/2008 and 31/12/2009 that met study criteria for VIN progressing to VSCC and had valid HPV results at

baseline. As above, additional samples for these women were also extracted from the larger (01/01/2001 and 31/12/2014) database. Cases progressing from VIN to VSCC had between 1 and 9 biopsies (median=4) prior to a diagnosis of invasive cancer. The presence of HR-HPV in the baseline sample was less common in VIN from women whose disease progressed to VSCC compared to VIN that did not transform to cancer (89% [88/99] Vs 54% [7/13], p<0.001). Among VIN cases that progressed to VSCC all HPV-positive cases (n=7) at baseline were associated with type 16 HPV alone. Two HPV 16 positive cases progressed to a HPV negative tumour. Nine of the fourteen cases had high-grade dysplasia reported at other perineal anatomical sites.

Rates if recurrence and progression of VIN

We next investigated the rate of recurrence VIN or VIN progressing to cancer by HR-HPV status and other clinical variables (Table 3). VIN associated with HR-HPV had a lower rate of progression to cancer (HR 0.02, p=0.001). In addition, HSIL lesions compared to dVIN disease were less likely to progress to invasive disease (HR 0.17, p=0.003). There was also evidence that women older than 70 years of age had a higher rate of disease progressing to cancer (age 70-79 year HR 4.46, p=0.01 and women 80 years and over HR 20.92, p=0.001). Women with dVIN tended to be older than woman with HSIL or LSIL (Table 1) and were less likely to be associated with HR-HPV (Table 2).

HPV type specific distribution in VSCC

Of the 66 cases of VSCC, valid HPV results were obtained from 62/66; three were classed invalid by testing both HPV negative and negative for beta globin and 1 was not available for operational reasons. HPV infection was present in

52% cases (32/62) and all types identified were high-risk. HPV 16 was identified in 30 cases and type 31 and 33 in one case each. Only one case had dual HPV types; 16 and 42. There was almost perfect agreement between HR-HPV genotype and P16 positivity (82%, P<0.001). In the adjusted multivariable analysis (Table 4) the presence of VIN surrounding a tumour remained an independent predictor of HR-HPV positivity (p=0.003), as did positive staining for p16 (p=0.006).

Influence of HPV status on survival in patients with VSCC

The mean follow-up time from incident diagnosis was 5.8 years (range 55 days to 14 years). The 5-year rates of OS were 78% in the HR-HPV-positive group and 49% in the HPV-negative group. The 5-year rates of progression free survival (PFS) were 87% in the HR-HPV positive group and 47% in the negative group.

In the unadjusted analysis, women with HR-HPV positive VSCC had better over-all survival (p=0.005, and progression-free survival (p=0.001) (Tables 5 and 6). After adjustment for age and cancer stage, patients with HPV-positive tumours had a 51% reduction in risk of death but the association was not statistically significant ((hazard ratio, 0.43, p=0.09) (Table 6). However, there was a statistically significant 70% reduction in risk of disease progression (hazard ratio, 0.32, p=0.02) compared to those with HPV negative tumours (Table 5).

Discussion

We have conducted a detailed population-based study to examine rates of progression of VIN to VSCC and influence of clinical factors. Moreover, we have

determined type-specific HPV prevalence in vulvar disease and the influence of HPV status on clinical outcome in VSCC. Our data reveal key novel aspects of VIN and VSCC. In particular, the age at which women are diagnosed with VSCC is falling and there is a significant time gap between first diagnosis of VIN and progression to invasive disease. HR-HPV infection was very common in VIN and present in just over 50% of VSCC. Most importantly, detection of HR-HPV was associated with a reduction in risk of progression from VIN to cancer and associated with improved progression-free survival of VSCC.

We have reported that the incidence of vulvar cancer in Scotland has increased by about 64% in the past three decades, particularly among young women.[2, 3] The above data indicate that the epidemiology of vulvar disease may be changing – with the diagnosis of VSCC occurring at an earlier age according to this series. These findings are consistent with previous work in Scotland which showed that the European age-standardised ratio for VSCC between 1972–1976 and 2007–2011, increased from 3.3 per 100,000 to 4.2 per 100,000 and that this increase was most prominent among younger women.[3] These data also reconcile with the US study, which assessed VSCC data from nine US Cancer registries for the period 1973-2004 and showed that VIN and VSCC increased 3.5% and 1.0% respectively, each year with the increase observed in all categories but the largest increase occurred in women aged 54 or less.[22]

Reasons for this phenomenon are unclear at present. It is feasible that changed clinical practices for the ascertainment and management of vulval disease may be influential in the case of VIN, but we do not believe this would be the case

for VSCC as cancer related symptoms and access to care have not appreciably changed between 2000 and 2014 in Scotland. Changed sexual behaviour(s), including age at first intercourse, which in turn facilitates transmission of HR-HPV could result in increased incidence of disease. Evidence of a secular increase in HR-HPV prevalence has been reported elsewhere[23] and the significant increase in HPV-associated oropharyngeal cancer over the last two decades may be due to an epidemic of highly transmissible HR-HPV.[24] In addition, increased incidence of HR-HPV-positive oropharyngeal cancer has been partly attributed to changed sexual practices.[25] Sexual behaviours associated with risk for vulval infection are considered similar to those for cervical infection and include age, single versus in partnership, age at first intercourse and number of sexual partners. [26] However, given that we did not specifically quantify or account for sexual behaviours in this study we are not able to address this directly and in fact there are few analyses on sexual behaviours and practices in women with VIN and VSCC – particularly when compared to those undertaken in those with cervical or increasingly, head and neck cancer. In terms of risk of HPV acquisition and tobacco use, smoking prevalence in Scotland remains higher than both England and Wales, there has been a downward trend from the mid-1970's to the present time. [27]

Clearly, it is of interest to promptly identify which women exhibit a greater chance of recurrence, which has a prevalence of about one-fifth of cases in this study. The pathological grading of VIN as defined by National Health Service local practice, showed no association with recurrence and this may support the concept there is not a continuum with VIN lesions leading to cancer as

demonstrated for CIN and cervical cancer.[28] Using the ISSVD terminology, cases of dVIN were more likely to recur or progress compared to LSIL and HSIL, but numbers were small. While no other clinical factor studied identified risk of progression or recurrence of VIN, identifying the small number of women with dVIN, who tend to be older and HR-HPV negative for close active monitoring may be important.

Another crucially important group to identify are those who have VIN that will progress to VSCC. In our series, the number of VIN cases that progressed to VSCC was between 6% and 10%. This figure is similar to that described in a recent analysis of 1094 Dutch women with VIN where the cumulative 20 year incidence was 8.9%.[29] VIN associated with HR-HPV and categorised as HSIL had a lower rate of progression to cancer. Younger women also appeared less likely to progress compared to women in older age groups. The results presented here suggest that VIN regression rates in HR-HPV associated high-grade disease may be high. This is important to characterised to prevent young women having extensive morbid surgical management of pre-invasive disease, which may regress. Low-grade cervical disease is characterised by transient infection while high-grade disease is due to subsequent persistent HPV infection. Our data suggest that in many cases, VIN progression to VSCC may not follow this pathway. Our data was limited due to small numbers and use of a retrospective cases series. A study using a much larger patient group will be necessary in future to extend and clarify these findings.

The development of VSCC after VIN treatment is relatively high compared to progression after treatment of CIN. [30] This may be due to widespread field change over the perineal area; in the group that underwent HPV testing nine of the 14 cases that progressed had severe dysplasia at another perineal/genital site. In addition, technically it is more challenging to treat vulvar disease compared to cervical disease. Unfortunately, unlike cervical disease, there is no equitable screening and recall system for VIN. Our study indicates that development of such a system would be advantageous because of the significant length of time taken for progression from VIN to VSCC and the opportunity for an intervention to prevent VSCC. Cellular biomarkers which have been interrogated in the vulvar context have included chemokine receptor proteins [31], p16 and p53 [10] and ROCK1 protein.[32] Charting the loss or gain of viral biomarkers [33] may shed significant light on the unusual aetiology of VIN and progression to VSCC.

With respect to HPV status, HPV infection was identified in 87% of VIN cases with HPV type 16 and/or 18 present in 78% of these. Comparatively, HPV was detected in 52% of VSCC cases with HPV 16 identified in 48% of cases. VSCC surrounded by VIN was much more likely to be HR-HPV positive and may represent widespread perineal/genital HPV infection and field change caused by the virus. There is a paucity of contemporary data in the UK on type-specific prevalence of HPV in vulval disease. The limitation of this and other studies is that vulvar disease is relatively rare. Consequently, these data are important to help inform the potential impact of the prophylactic HPV vaccines on vulval disease, particularly as vaccine uptake rates in the UK have been consistently

high (>90%) since introduction in 2008.[34] HPV status was significantly associated with age in our study. Our data indicates that the prophylactic HPV vaccine will prevent about half the cases of VSCC under the age of 69 years. It should be noted that a number of cases of VIN were associated with HPV 33, which would be prevented by immunisation with bivalent vaccine through cross-protection but would not be prevented by the quadrivalent vaccine currently used as part of the national programme.[35]

One key finding of this study was that women who had HPV-positive VSCC had a significantly improved prognosis compared to those who had HPV negative tumours, and that this observation was maintained after adjustment for age at diagnosis and tumour stage. This observation reconciles with the recent study of Hay et al (2016) who assessed the impact of HPV status (through HPV specific PCR and p16 staining) and p53 mutation status in series of 92 VSCC diagnosed between 1998-2007.[10] Notably HPV positive (and p16 positive) patients were less likely to have recurrence and there were no VSCC related deaths whereas p53-mutatant positive patients had a greater probability of recurrence and were significantly more likely to die from VSCC. While we did not measure p53 status in the present study – the observation that HPV status confers a favourable outcome is consistent. It is now well established that HPV positive OPSCC is associated with improved clinical outcomes and annotation of OPSCC for HPV status to provide clinicians with insight into a patients' prognosis is becoming more widespread. [7] Furthermore, although the relative proportions are small – cervical cancers where no HR-HPV is detected have been shown to have a worse outcome. [8] The drivers as to why HPV

associated neoplasms are associated with improved outcomes compared to their HPV negative counterparts are not fully established and are complicated by influential confounders, particularly smoking which is often challenging to document accurately. However the higher levels of host-mutation associated with non HR-HPV cancers may explain the differential outcome at least in part.

To conclude, the management of VIN remains a challenge. While HPV immunisation will significantly impact on this disease, the benefits realisation will take time and will not protect against all cases – including HPV-negative cases of VSCC, which appear to have a worse prognosis. HPV testing should be considered routine for VSCC management, to guide prognosis and to drive development of novel approaches in treatment. While much has been invested in the development of biomarkers for the improved management of pre-invasive cervical disease there is a comparative lack of studies in the VIN context – more are needed to inform and support the improved management of this complex, disease which appears to be increasing in incidence.

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Author contributions

Katie Wakeham (study design, data collection, data analysis, data interpretation, literature search, generation of figures, writing of the manuscript),

Kim Kavanagh (data analysis, data interpretation, literature search, generation of figures, writing of the manuscript), Kate Cuschieri (study design, data interpretation, literature search, writing of the manuscript), David Millan (study design, data collection, writing of the manuscript), Kevin G Pollock (data interpretation, literature search, writing of the manuscript), Sarah Bell (data collection, data interpretation, literature search, writing of the manuscript), Kevin Burton (data interpretation, writing of the manuscript), Nick Reed (data collection, data interpretation, writing of the manuscript), and Sheila Graham (study design, data interpretation, literature search, writing of the manuscript).

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Table 1: Number of vulvar cases by pathological category and age

Period of diagnosis 2001-2014		% (number)	Median age (IQR)
	Vulvar intraepithelial neoplasia (VIN)		
	VIN not otherwise specified	3% (16)	45 (42-60)
	Low-grade squamous VIN	6% (32)	53 (43-67)
	High-grade squamous VIN	91% (497)	47 (38-56)
	Total VIN cases	100% (545)	47 (38-56)
	Cancer	100% (404)	65 (50-78)
Detailed subset			
	Vulvar intraepithelial neoplasia (VIN)		
	Differentiated VIN	4% (5)	69 (67-80)
	Low-grade squamous VIN	6% (7)	59 (44-66)
	High-grade squamous VIN	90% (106)	47 (38-54)
	Cancer	100% (66)	62 (49-72)

IQR (inter-quartile range)

Table 2: Factors associated with high-risk human papillomavirus (HR-HPV) among vulvar intraepithelial neoplasia (VIN) cases

	Cases of VIN with valid HPV genotype result (n=112)*						
Characteristics	Number	HR-HPV positive (N=95) % (n)	HR-HPV negative (N=17) % (n)	Unadjusted OR (95% CI)	Unadjusted P value	Adjusted OR (95% CI)*	Adjusted P value*
Age							
<50	65	88% (57)	12% (8)	1			
50-59	25	92% (23)	8% (2)	1.61 (0.32-8.18)	0.6		
60-69	12	83% (10)	16% (2)	0.70 (0.13-3.80	0.7		
70-79	8	62% (5)	38% (3)	0.23 (0.05-1.17)	0.08		
80 and over	2	0% (0)	100% (2)				
Age in years (Range 19-87)	112			0.96 (0.92-0.99)	0.03 [trend]	0.98 (0.48-1.02)	0.4 [trend]
Smoking status							
No	48	88% (42)	13% (6)	1			
Yes	52	81% (42)	19% (10)	0.6 (0.20-1.80)	0.4		
Missing	*12*						
Scottish Index of Multiple deprivation							
1 (most deprived)	35	83% (29)	17% (6)	1			
2	24	88% (21)	13% (3)	1.45 (0.32-6.46)	0.6		
3	14	79% (11)	21% (3)	0.76 (0.16-3.57)	0.7		
4	13	77% (10)	23% (3)	0.69 (0.14-3.29)	0.6		
5 (least deprived)	14	93% (13)	7% (1)	2.69 (0.30-24.66)	0.4		
Missing	*12*						
Immunosuppression status							
No	105	85% (89)	15% (16)	1			
Yes	7	86% (6)	14% (1)	1.07 (0.12-9.57)	0.9		
p16 status							
Negative	23	65% (15)	35% (8)	1		1	
Positive	89	90% (80)	10% (9)	4.74 (1.58-14.25)	0.006	3.70 (1.07-12.70)	0.04
Pathological type of vulvar intraepithelial neoplasia (VIN)							
Differentiated VIN	5	20% (1)	80% (4)	1		1	
Low-grade squamous VIN	7	86% (6)	14% (1)	24.00 (1.14-505.19)	0.04	8.77 (0.36-211.66)	0.1
High-grade squamous VIN	100	88% (88)	12% (12)	29.33 (3.02-284.72)	0.004	12.82 (1.10-149.65)	0.04
Lichen Sclerosis							
No	105	13% (14)	87% (91)	1		1	
Yes	7	43% (3)	57% (4)	0.21 (0.04-1.02)	0.05	0.57 (0.08-4.02)	0.6

P values for association with exposure for outcome of high-risk HPV positivity, P values for heterogeneity unless stated as for trend

*Valid HPV genotype results available for 112/118 cases

** Adjusted for age, p16 status, pathological category and lichen sclerosis status

OR, odds ratio; CI, confidence interval

Table 3: Rates of recurrance and progression of vulvar intraepithelial neoplasia (VIN)

Cases of VIN with valid HPV genotype result (n=112)*																			
Variable	Level	Number	Number with recurrance or progression	Person years follow up	Rate per 100 person years	95% CI Lower	95% CI Upper	HR	Lower 95% CI	Upper 95% CI	p value	Number with progression to cancer	Person years follow up	Rate per 100 person years	95% CI Lower	95% CI Upper	HR	Lower 95% CI	Upper 95% CI	p value
HR-HPV status	Negative	17	12	80.28	14.95	7.72	26.11	1	-	-	-	8	117.59	6.80	2.94	13.41	1	-	-	-
	Positive	95	46	521.52	8.82	6.46	11.77	0.80	0.42	1.55	0.51	11	760.13	1.45	0.72	2.59	0.22	0.09	0.56	0.001
Age	<50	65	35	347.92	10.06	7.01	13.99	1	-	-	-	9	527.84	1.71	0.78	3.24	1.00	-	-	-
	50-59	25	10	144.68	6.91	3.31	12.71	0.71	0.35	1.43	0.33	3	203.64	1.47	0.30	4.31	0.89	0.24	3.31	0.87
	60-69	12	5	68.73	7.27	2.36	16.98	0.76	0.30	1.96	0.58	1	95.07	1.05	0.03	5.86	0.61	0.08	4.80	0.64
	70-79	8	6	35.90	16.71	6.13	36.38	1.41	0.59	3.38	0.44	4	46.61	8.58	2.34	21.97	4.55	1.39	14.96	0.01
	80 and over	2	2	4.56	43.82	5.31	158.30	3.67	0.82	16.39	0.09	2	4.56	43.82	5.31	158.30	20.92	3.60	121.68	0.0007
Smoking status	No	48	27	231.43	11.67	7.69	16.97	1	-	-	-	9	353.04	2.55	1.17	4.84	1	-	-	-
	Yes	52	29	273.26	10.61	7.11	15.24	0.89	0.53	1.51	0.67	10	411.88	2.43	1.16	4.47	0.96	0.39	2.37	0.93
	Missing	*12*	2	97.12	2.06	0.25	7.44	0.18	0.04	0.77	0.02	0	112.80	0.00	0.00	3.27	-	-	-	-
Scottish Index of Multiple	1 (most deprived)	35	18	173.61	10.37	6.14	16.39	1	-	-	-	7	264.52	2.65	1.06	5.45	1	-	-	-
deprivation	2	24	13	121.63	10.69	5.69	18.28	1.07	0.52	2.19	0.85	5	171.75	2.91	0.95	6.79	1.08	0.34	3.41	0.90
	3	14	9	68.83	13.08	5.98	24.82	1.22	0.54	2.72	0.64	1	123.29	0.81	0.02	4.52	0.32	0.04	2.62	0.29
	4	13	8	66.09	12.11	5.23	23.85	1.06	0.46	2.46	0.89	2	113.33	1.76	0.21	6.37	0.67	0.14	3.26	0.62
	5 (least deprived)	14	8	74.54	10.73	4.63	21.15	1.07	0.46	2.46	0.88	4	92.02	4.35	1.18	11.13	1.64	0.48	5.63	0.43
	Missing	*12*	2	97.12	2.06	0.25	7.44	0.21	0.05	0.89	0.03	0	112.80	0.00	0.00	3.27	-	-	-	-
Immunosuppression status	No	105	53	571.47	9.27	6.95	12.13	1	-	-	-	18	813.41	2.21	1.31	3.50	1	-	-	-
	Yes	7	5	30.33	16.48	5.35	38.47	1.98	0.78	4.98	0.15	1	64.31	1.55	0.04	8.66	0.76	0.10	5.72	0.79
p16	Negative	23	10	139.88	7.15	3.43	13.15	1.00	-	-	-	6	167.25	3.59	1.32	7.81	1	-	-	-
	Positive	89	48	461.92	10.39	7.66	13.78	1.63	0.82	3.24	0.16	13	710.47	1.83	0.97	3.13	0.54	0.20	1.42	0.21
Pathological type	Differentiated VIN	5	5	25.42	19.67	6.39	45.91	1	-	-	-	4	33.16	12.06	3.29	30.88	1	-	-	-
	Low-grade squamous VIN	7	52	522.63	9.95	7.43	13.05	0.68	0.26	1.76	0.43	0	58.29	0.00	0.00	6.33	-	-	-	-
	High-grade squamous VIN	100	1	53.75	1.86	0.05	10.36	0.13	0.02	1.17	0.07	15	786.27	1.91	1.07	3.15	0.16	0.05	0.53	0.003
Lichen Sclerosis	No	105	55	559.54	9.83	7.40	12.79	1	-	-	-	16	835.46	1.92	1.09	3.11	1	-	-	-
	Yes	7	3	42.26	7.10	1.46	20.74	0.74	0.23	2.37	0.61	3	42.26	7.10	1.46	20.74	3.16	0.92	10.89	0.07

*Valid HPV genotype results available for 112/118 cases

HR, Hazard ratio; CI, confidence interval

VIN recurrence was defined as two positive biopsies separated by 365 days or more with no reported invasive vulvar cancer event between 01/01/2001 and 31/12/2014

VIN progressing to vulvar cancer, where 365 days or more separated the diagnoses of VIN and vulvar cancer

Table 4: Factors associated with high-risk human papillomavirus (HR-HPV) among vulvar squamous cancer cases (VSCC)

Characteristics	Number	HR-HPV positive (N=32) % (n)	HR-HPV negative (N=30) % (n)	Unadjusted OR (95% CI)	Unadjusted P value	Adjusted OR (95% CI)**	Adjusted P value**
		Cases of VSCC with valid HPV genotype result (N=62)*					
Age							
<50	16	63% (10)	38% (6)	1		1	
50-59	13	77% (10)	23% (3)	2.00 (0.39-10.31)	0.4	1.89 (0.08-46.7)	0.7
60-69	16	56% (9)	44% (7)	0.78 (0.19-3.17)	0.7	4.95 (0.21-116.5)	0.3
70-79	9	33% (3)	67% (6)	0.30 (0.05-1.67)	0.1	0.25 (0.01-5.60)	0.4
80 and over	8	0% (0)	100% (8)			na	
Age in years (Range 30-89)	62			0.93 (0.89-0.97)	0.001 [trend]	0.96 (0.91-1.02)	0.2 [trend]
Smoking status							
No	29	31% (9)	69% (20)	1		1	
Yes	29	66% (19)	34% (10)	4.22 (1.40-12.66)	0.008	4.12 (0.47-36.49)	0.2
Missing	*4*						
Scottish Index of Multiple deprivation							
1 (most deprived)	21	48% (10)	52% (11)	1			
2	15	53% (8)	47% (7)	1.26 (0.33-4.75)	0.7		
3	9	44% (4)	56% (5)	0.88 (0.18-4.23)	0.9		
4	8	50% (4)	50% (4)	1.10 (0.22-5.61)	0.9		
5 (least deprived)	4	25% (1)	75% (3)	0.37 (0.03-4.12)	0.4		
Missing	*5*						
Immunosuppression status							
No	52	48% (25)	52% (27)	1			
Yes	5	40% (2)	60% (3)	0.72 (0.11-4.67)	0.7		
Missing	*5*						
FIGO stage							
1	*33*	*59% (19)*	*42% (14)*	*1*			
2	*12*	58% (7)	42% (5)	0.96 (0.24-3.68)	0.9		
3	*13*	38% (5)	62% (8)	0.43 (0.11-1.60)	0.2		
4	*2*	*0% (0)*	*100% (2)*	na			
Missing	*2*						
Lymph node metastasis							
No	*46*	*57% (26)*	*43% (20)*	1			
Yes	*14*	*36% (5)*	*64% (9)*	0.43 (0.12-1.48)	0.2		
Missing	2						
VSCC surrounded by VIN							
No	25	12% (3)	88% (22)	1		1	
Yes	37	78% (29)	22% (8)	26.58 (6.31-111.97)	<0.001	8.24 (1.20-56.49)	0.03
p16							
Negative	34	26% (9)	74% (25)	1		1	
Positive	25	92% (23)	8% (2)	32.0 (6.23-163.60)	<0.001	28.79 (2.68-308.75)	0.006
Missing	3						
Lichen Sclerosis							
No	49	59% (29)	41% (20)	1		1	
Yes	13	23% (3)	77% (10)	0.21 (0.05-0.85)	0.03	0.68 (0.01-0.97)	0.8
Tumour assocciated with differentiated VIN (dVIN)							
No	52	60% (31)	40% (21)	1		1	
Yes	10	10% (1)	90% (9)	0.07 (0.009-0.63)	0.02	0.61 (0.04-9.26)	0.7
Treatment							
Surgery alone	50	58% (29)	42% (21)	1			
Surgery and chemoradiotherapy	5	60% (3)	40% (2)	1.10 (0.17-7.08)	0.9		
Chemoradiotherpay alone	4	0% (0)	100% (4)	na			
Radiotherpy alone	3	0% (0)	100% (3)	na			

P values for association with exposure for outcome of high-risk HPV positivity, P values for heterogeneity unless stated as for trend

** Valid HPV genotype results available for 62/66 cases

* Adjusted for age, smoking status, VSCC surrounded by VIN, p16 status, lichen sclerosis and presence of dVIN

OR, odds ratio; CI, confidence interval; na, not applicable

Table 5. Hazard ratios for progression free survival

Progression-free survival (N=61*)												
Variable	Level	Number	Number recur or die	Person years follow up	Unadjusted HR	Lower 95% CI	Upper 95% CI	p value	Reduced model adjusted HR**	Lower 95% CI	Upper 95% CI	p value
HR-HPV status	Negative	30	22	129.14	1	-	-	-	1	-	-	-
	Positive	31	7	203.67	0.24	0.10	0.56	0.001	0.32	0.12	0.84	0.02
FIGO stage	1	32	7	208.61	1	-	-	-	1	-	-	-
	2	11	8	56.04	3.61	1.29	10.11	0.01	1.01	0.18	5.66	0.99
	3	13	10	48.84	5.55	2.10	14.68	0.001	1.32	0.45	3.88	0.61
	4	2	2	0.53	34.79	5.84	207.35	0.0001	1.93	0.54	6.93	0.31
	Missing	3	2	18.79	3.13	0.65	15.14	0.16	3.65	1.00	13.32	0.05
Age	<50	16	6	109.98	1	-	-	-	1	-	-	-
	50-59	13	2	84.67	0.43	0.09	2.17	0.31	3.78	1.33	10.71	0.01
	60-69	16	8	79.51	1.62	0.56	4.68	0.37	6.63	2.26	19.42	0.001
	70-79	8	6	44.80	2.20	0.71	6.86	0.17	11.53	1.70	78.45	0.01
	80 and over	8	7	13.84	5.86	1.89	18.15	0.002	2.10	0.37	12.04	0.41
VSCC surrounded by VIN	No	25	19	97.15	1	-	-	-	1	-	-	-
	Yes	36	10	235.66	0.26	0.12	0.57	0.001	1.04	0.31	3.52	0.95
Scottish Index of Multiple deprivation	1 (most deprived)	21	11	111.90	1	-	-	-				
	2	15	6	90.97	0.77	0.28	2.09	0.61				
	3	9	5	43.87	1.12	0.39	3.23	0.83				
	4	7	5	28.75	1.87	0.64	5.44	0.25				
	5 (least deprived)	4	2	22.94	0.89	0.20	4.07	0.88				
	Missing	5	0	34.38	-	-	-	-				
Smoking status	No	28	17	141.87	1	-	-	-				
	Yes	29	12	163.07	0.65	0.31	1.36	0.25				
	Missing	4	0	27.87	-	-	-	-				
p16	Negative	33	20	157.11	1	-	-	-				
	Positive	25	6	167.48	0.31	0.12	0.76	0.01				
	Missing	3	3	8.21	2.29	0.67	7.87	0.19				
Treatment	Surgery alone	49	18	307.95	1	-	-	-				
	Surgery and chemoradiotherapy	5	4	21.51	3.64	1.19	11.08	0.02				
	Chemoradiotherapy alone	4	4	2.41	13.35	3.78	47.22	0.0001				
	Radiotherapy alone	3	3	0.95	23.83	5.18	109.70	0.00005				

* 1 individual removed from PFS analysis as cause of death noted as cancer but date of recurrance unknown.
** Adjusted for all statistically significant variables in the table with the exception of p16 and treatment. p16 is highly associated with HPV status and treatment is highly associated with stage. These variables are therefore not included in the adjusted model to avoid collinearity.
HR, hazards ratio; CI, confidence interval; PFS, progression-free survival

Table 6. Hazard ratios for overall survival

Overall-survival (N=62)												
Variable	Level	Number	Number dead	Person years follow up	Unadjusted HR	Lower 95% CI	Upper 95% CI	p value	Reduced model adjusted HR**	Lower 95% CI	Upper 95% CI	p value
HR-HPV status	Negative	30	19	165.0	1	-	-	-	1	-	-	-
	Positive	32	7	226.8	0.28	0.12	0.68	0.005	0.43	0.16	1.17	0.10
FIGO Stage	1	32	6	234.9	1	-	-	-	1	-	-	-
	2	12	7	75.5	3.45	1.15	10.30	0.027	3.26	1.06	9.98	0.04
	3	13	9	57.9	5.58	1.97	15.79	0.001	6.39	2.05	19.92	0.001
	4	2	2	3.3	16.31	3.16	84.13	0.001	4.66	0.76	28.52	0.10
	Missing	3	2	20.1	3.56	0.71	17.69	0.12	2.04	0.35	11.78	0.43
Age	<50	16	4	127.5	1	-	-	-	1	-	-	-
	50-59	13	2	93.4	0.69	0.12	3.83	0.67	1.52	0.24	9.65	0.66
	60-69	16	7	97.4	2.16	0.63	7.42	0.22	1.77	0.51	6.13	0.37
	70-79	9	6	52.3	3.32	0.93	11.83	0.06	3.40	0.84	13.78	0.09
	80 and over	8	7	21.2	8.04	2.26	28.65	0.001	5.41	1.28	22.86	0.02
VSCC surrounded by VIN	No	25	16	127.3	1	-	-	-	1			
	Yes	37	10	264.5	0.33	0.15	0.72	0.006	1.05	0.29	3.74	0.94
Scottish Index of Multiple	1 (most deprived)	21	10	131.5	1	-	-	-				
deprivation	2	15	6	102.4	0.84	0.30	2.32	0.74				
	3	9	4	58.0	0.92	0.29	2.95	0.89				
	4	8	4	36.8	1.43	0.44	4.61	0.55				
	5 (least deprived)	4	2	25.1	1.05	0.23	4.86	0.95				
	Missing	5	0	37.9	0.00	-	-	1.00				
Smoking status	No	29	17	174.8	1	-	-	-				
	Yes	29	9	186.4	0.51	0.23	1.15	0.10				
	Missing	4	0	30.7	0.00	-	-	1.00				
p16 Status	Negative	34	18	198.0	1	-	-	-				
	Positive	25	6	182.1	0.38	0.15	0.97	0.04				
	Missing	3	2	11.7	1.69	0.39	7.32	0.49				
Treatment	Surgery alone	50	15	358.5	1	-	-	-				
	Surgery and chemoradiotherapy	5	4	26.2	3.58	1.17	10.94	0.03				
	Chemoradiotherapy alone	4	4	3.4	18.72	5.14	68.20	0.00001				
	Radiotherapy alone	3	3	3.8	15.24	3.97	58.46	0.00007				

* Adjusted for all statistically significant variables in the table with the exception of p16 and treatment. P16 is highly associated with HPV status and treatment is highly associated with stage. These variables are therefore not included in the adjusted model to avoid collinearity.

HR, hazards ratio; CI, confidence interval; PFS, progression-free survival

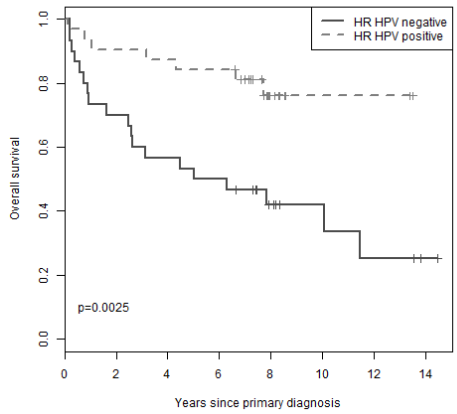
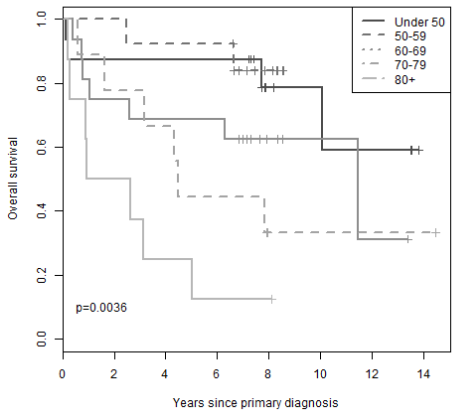
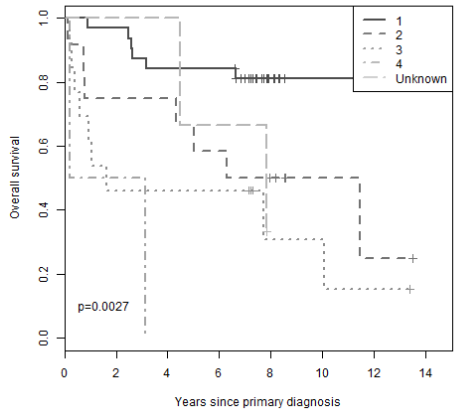
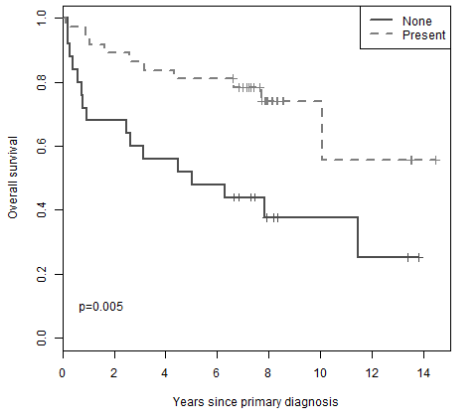
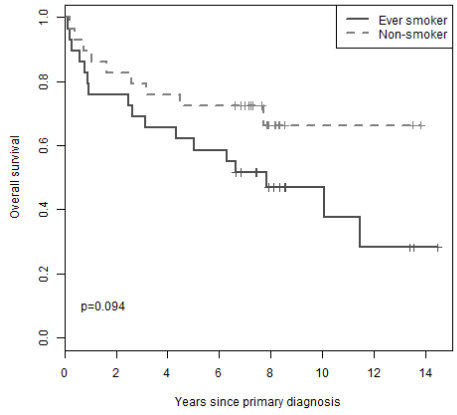
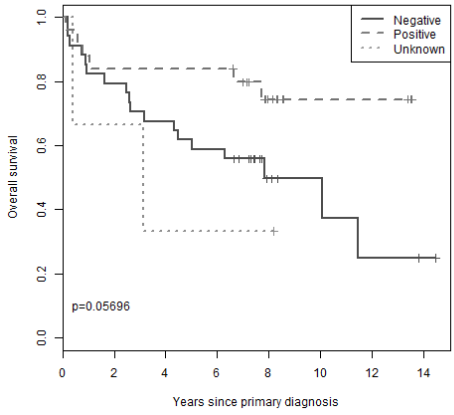
A. Overall survival according to high-risk HPV status	B. Overall survival according to age at diagnosis
	
C. Overall survival according to tumour FIGO stage	D. Overall survival according to the presence of tumour-associated VIN (none vs. present)
	
E. Overall survival according to smoking status	F. Overall survival according to p16 status
	

Figure 1. Kaplan-Meier estimates for Overall survival among women with VSCC.

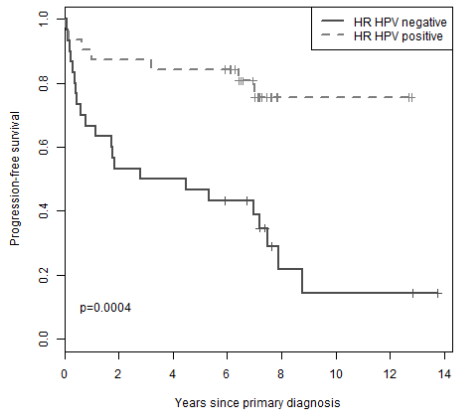
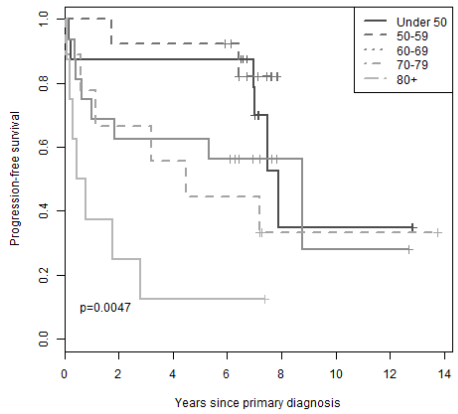
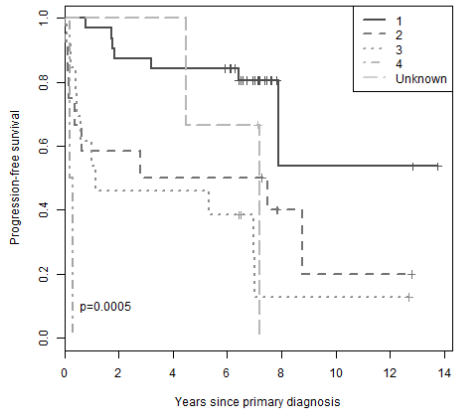
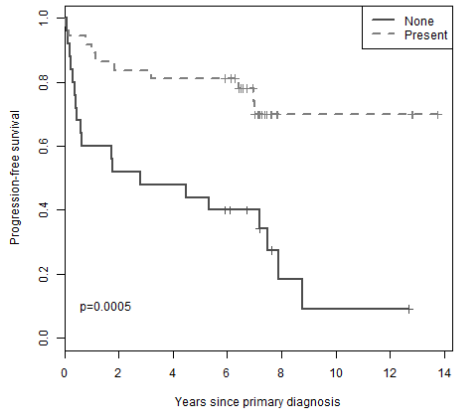
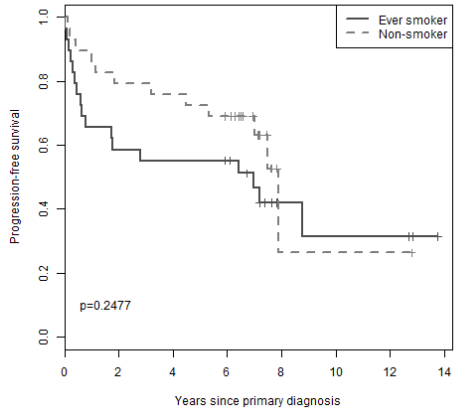
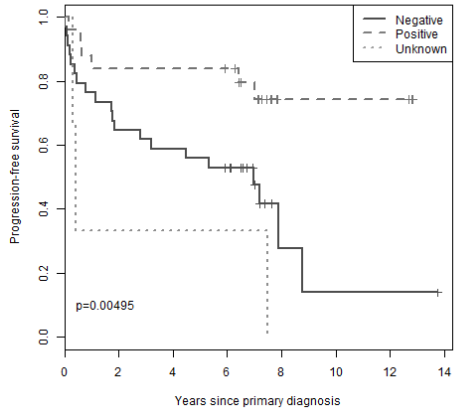
A. Progression-free survival according to high-risk HPV status	B. Progression-free survival according to age at diagnosis
	
C. Progression-free survival according to tumour FIGO stage	D. Progression-free survival according to the presence of tumour-associated VIN (none vs. present)
	
E. Progression-free survival according to smoking status	F. Progression-free survival according to p16 status
	

Figure 2. Kaplan-Meier estimates for progression-free survival among women with VSCC.