



Universiteit
Leiden
The Netherlands

Towards a tailored therapeutic approach for vulvar cancer patients

Kortekaas, K.E.

Citation

Kortekaas, K. E. (2021, May 27). *Towards a tailored therapeutic approach for vulvar cancer patients*. Retrieved from <https://hdl.handle.net/1887/3180650>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/3180650>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <https://hdl.handle.net/1887/3180650> holds various files of this Leiden University dissertation.

Author: Kortekaas, K.E.

Title: Towards a tailored therapeutic approach for vulvar cancer patients

Issue Date: 2021-05-27

4

VULVAR CANCER SUBCLASSIFICATION BY HPV AND P53 STATUS RESULTS IN THREE CLINICALLY DISTINCT SUBTYPES

K.E. Kortekaas, E. Bastiaannet, H.C. van Doorn, P.J. de Vos van
Steenwijk, P.C. Ewing-Graham, C.L. Creutzberg, K. Akdeniz, L.S.
Nooij, S.H. van der Burg, T. Bosse, M.I.E. van Poelgeest

ABSTRACT

Objective

There is great need for better risk stratification in vulvar squamous cell carcinoma (VSCC). Our aim was to define the prognostic significance of stratifying VSCC based on p16- and p53 immunohistochemistry (IHC) as surrogate markers for HPV and *TP53* mutations.

Methods

A large retrospective cohort of surgically treated women with primary VSCC was used. VSCC were classified into three subtypes: HPV-associated (HPVpos), HPV-independent/p53 mutant (HPVneg/p53mut), and HPV-independent/p53 wildtype (HPVneg/p53wt). Overall survival (OS), relative survival (RS), and recurrence-free period (RFP) were depicted using the Kaplan-Meier method and survival curves for relative survival; associations were studied using univariable and multivariable Cox proportional hazard models.

Results

Of the 413 VSCCs, 75 (18%) were HPVpos, 63 (15%) HPVneg/p53wt, and 275 (66%) HPVneg/p53mut VSCC. Patients with HPVneg/p53mut VSCC had worse OS and RS (HR 3.43 (95%CI 1.80–6.53), and relative excess risk (RER) of 4.02 (95%CI 1.48-10.90), respectively), and worse RFP (HR=3.76, 95%CI 2.02-7.00). HPVpos VSCC patients showed most favorable outcomes. In univariate analysis, the molecular subtype of VSCC was a prognostic marker for OS, RS and RFP ($p=0.003$, $p=0.009$, $p<0.001$, respectively) and remained prognostic for RFP even after adjusting for known risk factors ($p=0.0002$).

Conclusion

Stratification of VSCC by p16- and p53-IHC has potential to be used routinely in diagnostic pathology. It resulted in the identification of three clinically distinct subtypes and may be used to guide treatment and follow-up, and in stratifying patients in future clinical trials.

INTRODUCTION

Clinical management of vulvar cancer raises several challenges for the oncological gynecologist. With an incidence of 1-2 per 100.000 women and a median age of 70, vulvar cancer is a rare cancer of elderly women, although the disease has shown rising incidence rates with concurrently decreasing median age at onset over the past few decades.¹⁻³ The great majority of vulvar cancers are squamous cell carcinomas.⁴

Vulvar squamous cell carcinoma (VSCC) is a heterogeneous disease with an overall 5-year survival rate of 70%.⁴ Current treatment is highly dependent on anatomical extent of disease, as represented in the International Federation of Gynecology and Obstetrics (FIGO) 2009 staging system.⁵ Treatment for early-stage VSCC includes groin surgery in addition to wide local tumor resection according to international treatment recommendations.⁶ The presence and number of lymph node metastasis is the main prognostic factor for recurrence and survival: 5-year disease-specific survival ranges between 70% and 95% in patients with negative inguino-femoral lymph nodes and decreases to 25% to 41% if groin nodes are affected.^{4, 7} In addition to surgery, further treatment modalities such as adjuvant (chemo)radiotherapy, especially for locally advanced and metastatic disease, are recommended.⁶ Treatment for VSCC often leads to substantial and long-term morbidity in affected patients due to loss of function of adjacent vital structures (bladder, anus and/or clitoris), lymphedema, sexual and psychological dysfunction and wound healing disorders.⁸ Therefore, there must be a careful balance between adequate treatment and morbidity. Due to the rarity of the disease randomized controlled trials in VSCC are lacking; and criteria for different treatment modalities therefore remain controversial with low levels of evidence. There is a critical need to improve the identification of high-risk patients who may benefit from more intensive or novel therapies. On the other hand, it is necessary to identify patients at low-risk for recurrence or death for whom less aggressive therapy might be safe.

Recent evidence has provided novel insights into the molecular heterogeneity of VSCC.⁹ ¹⁰ Two intrinsically different VSCC subtypes have been identified; one is initiated by a high-risk human papilloma virus (HPV) infection and accounts for ~20% of VSCC ¹⁰. The more common VSCC subtype however is HPV-independent and most likely initiated by *TP53* mutations.^{9, 11-13} There is increasing evidence that HPV-associated VSCC have favorable clinical outcomes compared to HPV-independent VSCC^{9, 10, 14-18}. Clinical outcomes of HPV-independent VSCC vary widely and further prognostic stratification by *TP53* mutation status has been recently suggested.^{9, 19, 20} HPV-independent VSCC with wildtype *TP53* was poorly defined, since sequencing is not routinely performed. Assessment of *TP53* status by p53 immunohistochemistry (IHC) is thought to be suboptimal.²¹ However, we recently reported on a pattern-based p53-IHC assessment for VSCC, allowing near-perfect prediction of *TP53* mutational status ^{19, 20} with a high level of interobserver agreement. This improved

approach towards p53-IHC in VSCC, now allows us to study the potential clinical relevance of stratifying HPV-independent VSCC by *TP53* status. In addition to p53-IHC, p16-IHC is an excellent surrogate marker for HPV-presence²² which indicates that two IHCs can be used to classify VSCC into three molecular subtypes.

In this study, we tested the hypothesis that molecular classification in VSCC is associated with significant differences in prognosis. We used an easy clinically applicable molecular classification tool based on p16- and p53-IHC in a large, well-characterized retrospective cohort of VSCC. This classification may designate patients into less heterogeneous and more appropriate risk groups. As a consequence, better prediction for clinical outcome and guidance for personalized treatment protocols can be envisioned.

MATERIAL AND METHODS

Institutional approval for this study was obtained from each of the participating centers. Primary VSCCs treated between January 2000 and December 2015 with clinical follow-up data were collected from two Dutch university hospitals (Leiden University Medical Center (LUMC) and ErasmusMC Rotterdam (EMC)). All patients were treated according to the Dutch national guideline (www.oncoline.nl) consisting of a wide local excision or vulvectomy and a sentinel lymph node procedures or inguinofemoral lymphadenectomy. The standard follow-up schedule was; 6 weeks postoperative, the first two years after initial treatment every 3 months, in the fourth and fifth year every 6 months and thereafter once a year. Inclusion criteria were surgical treatment with curative intent and availability of formalin-fixed paraffin-embedded (FFPE) blocks from resection specimens. An exclusion criterium was previous (chemo)radiotherapy in the pelvic area. Clinicopathological variables were collected from the electronic patient file. These comprised FIGO 2009 staging, tumor size, depth of invasion, lymphovascular space involvement (LVSI), number and localization of involved lymph nodes, treatment characteristics, pathological resection margins, date and location of recurrent disease, and/or date of last follow-up or death. Survival data were linked to the Dutch Cancer Registries till October 2017. All FFPE blocks were cut in 4µm slides and stained with hematoxylin and eosin (HE) and were checked for the presence of invasive VSCC by a pathologist with gynecological expertise (TB). In case of absence of the tumor, the patient was excluded from the study.

P16 immunohistochemistry

We performed p16-IHC (CINtec p16ink4A histology; 1:25 dilution; clone E6H4) on all cases.⁹ Two observers (KEK and TB) scored p16-IHC. Presence of the integration of high-risk HPV was defined as “block-type” p16 expression according to consensus recommendations.²³ Upon block-type p16 expression, we confirmed the presence of HPV by HPV-PCR (LiPa genotyping, Innogenetics, Gent, Belgium) including the determination of the HPV genotype on FFPE material. This group was referred to as HPV-associated VSCC (HPVpos VSCC). Absent

or patchy expression of p16 was classified as HPV-independent VSCC (HPVneg VSCC). In case of discordance between HPV-PCR and p16-IHC, cases were excluded from the study since these tumors might influence the outcome of the predefined subtypes.²⁴

P53 immunohistochemistry

Subsequently, HPVneg VSCC were categorized based on the pattern of p53-IHC expression. P53-IHC was performed with Dako Ominis FLEX+ detection system (p53 antibody, clone DO-7, Dako, mouse monoclonal, ready-to-use)¹⁹, and was assessed by two observers (KEK and TB). Six different p53-IHC patterns were described, of which two p53-IHC patterns (scattered and mid-epithelial) were classified as final class 'p53-IHC wildtype' (HPVneg/p53wt VSCC), and the remaining four patterns (basal, parabasal to diffuse, cytoplasmic, and absent) as final class 'p53-IHC mutant' (HPVneg/p53mut VSCC).^{19, 20} The agreement on final class p53-IHC (wildtype versus mutant) using this approach was substantial ($k=0.71$, $p<0.001$) and resulted in a sensitivity of 95% and specificity of 100%. The accuracy of p53-IHC to predict *TP53* mutations was 97%.¹⁹ In addition, tumors were excluded when one of the assays failed, because a molecular subtype could not be assigned to the particular case.

Statistical analysis

End points of the study were overall survival (OS), relative survival (RS), and recurrence-free period (RFP). The OS was defined as the time from the date of primary surgery to date of death of any cause. Because VSCC mainly affects elderly women¹, it is likely that these patients could die from non-cancer related causes. Therefore, we calculated the RS which takes into account the risk of dying from other causes than VSCC. The RS is based on a ratio of the observed survival in our cohort and the survival that would have been expected based on the corresponding general population, matched for age, gender, and year of incidence.²⁵ The RS is preferred above the disease-specific survival in this study, to avoid errors resulting from misclassification of the cause of death.²⁶ The RFP was defined as the time period from surgery to any recurrence as event or end of follow-up. In order to exclude cases with potential residual disease, patients with tumor-positive surgical margins who did not undergo re-excision were excluded in the RFP analysis.

For data analysis and illustration of the graphs and figures, the statistical software package SPSS 23.0 (SPSS Inc., Chicago, IL), or STATA/SE 12.0 was used. The baseline characteristics between the groups were calculated by the chi-square test (categorical data) or Mann-Whitney U test (numerical data). The RS was calculated by the Ederer II method^{27, 28} for which national life tables from www.mortality.org were used to estimate expected survival. Relative excess risks (RERs) of death were estimated using an adjusted generalized linear model with a poisson distribution, based on collapsed relative survival data, using exact survival times.

We used a univariate analysis to identify prognostic variables for each clinical outcome, and a Cox proportional Hazards model to determine independent predictive variables for the corresponding outcome. The global deviance test was calculated in both uni- and multivariate analysis for all variables irrespective of individual parameters. Two sided p -values <0.05 and an alpha of <0.05 were considered significant.

RESULTS

Clinicopathological characteristics

A total of 553 patients with primary VSCC were treated at the LUMC ($n=179$) and EMC ($n=374$) between January 2000 and December 2015. We excluded 107 patients based on the exclusion criteria (supplemental figure 1). Thirty-three patients were excluded due to absence of VSCC or failed IHC ($n=4$). Three cases were excluded due to discordancy between p16-IHC and HPV-PCR (figure 1, supplemental figure 1). The baseline clinical and histopathological characteristics of the 413 included patients stratified by molecular subtype are shown in table 1. Median follow-up time for the cohort was 30 months (range 0-201).

The majority of the tumors (275/413, 66%) were HPVneg/p53mut VSCC, 75/413 (18%) were HPVpos VSCC, and 63/413 (15%) were HPVneg/p53wt VSCC (figure 1, table 1). HPVpos VSCC were mostly caused by hr-HPV type 16 (71%), followed by type 33 (20%), and type 18 (8%). Age, FIGO stage, depth of invasion, LVSI, and treatment modality were all significantly associated with molecular subtype. Age at surgery for women with HPVpos VSCC (median 59 years) was lower than in the HPVneg/p53wt VSCC and HPVneg/p53mut VSCC (73 versus 75 years, respectively). The majority of patients with HPVpos VSCC (59/75, 79%) or HPVneg/p53wt VSCC (51/63, 81%) tumors presented with stage I/II disease, compared to 156/275 (57%) HPVneg/p53mut VSCC. The latter were more likely to have an advanced stage (III) disease and receive adjuvant (chemo)radiotherapy. Eighty-one percent of patients with stage III disease had HPVneg/p53mut VSCC tumors. HPVpos VSCC and HPVneg/p53wt VSCC were similar with respect to all measured clinicopathological parameters except for age (supplemental table 1).

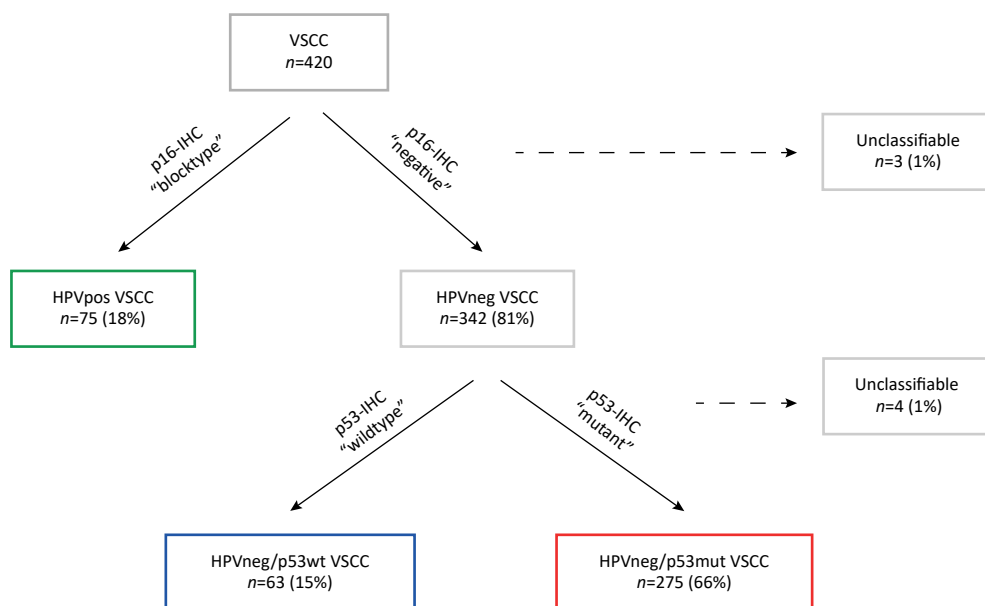


Figure 1. Decision tree for molecular vulvar squamous cell carcinoma subtyping using surrogate markers. FWE performed p16-IHC on all cases, and categorized tumors with “blocktype” p16-IHC expression as hrHPV-dependent VSCC (HPVpos VSCC), which was subsequently confirmed by HPV-PCR. VSCC with patchy or absent expression of p16 were referred to as hrHPV-independent (HPVneg VSCC) tumors. HPVneg VSCC were categorized based on p53-IHC expression. Both scattered and mid-epithelial p53 expression were classified as ‘p53-IHC wildtype’ (HPVneg/p53wt VSCC). In case of basal, parabasal to diffuse, absent or cytoplasmic p53 expression, tumors were classified as ‘p53-IHC mutant’ (HPVneg/p53mut VSCC). HPVpos VSCC = HPV-positive vulvar carcinoma, HPVneg VSCC = HPV-negative VSCC, HPVneg/p53wt VSCC = HPV-negative and p53 wildtype VSCC, HPVneg/p53mut VSCC = HPV-negative and p53 mutant VSCC, IHC = immunohistochemistry, VSCC= vulvar squamous cell carcinoma.

Table 1. Association between molecular subtype and clinicopathological variables.

	HPVpos VSCC (n=75, 18%)	HPVneg/p53wt VSCC (n=63, 15%)	HPVneg/p53mut VSCC (n=275, 66%)	p-value
Age				
yr (median, range)	59 (19-92)	73 (35-91)	75 (23-98)	<0.0001
FIGO 2009 (n, %)				<0.0001
IA	9 (12.0%)	3 (4.8%)	2 (0.5%)	
IB	50 (66.7%)	46 (73.0%)	143 (52.0%)	
II	0 (0.0%)	2 (3.2%)	11 (4.0%)	
III*	1 (1.3%)	2 (3.2%)	57 (20.7%)	
IIIA	13 (17.3%)	6 (9.5%)	13 (4.7%)	
IIIB	1 (1.3%)	4 (6.4%)	42 (15.3%)	
IIIC	1 (1.3%)	0 (0.0%)	7 (2.6%)	
Size in cm				0.25
(median, range)	19 (1-72)	25 (1.9-120)	25 (2-110)	
≤2 (n, %)	39 (52.0%)	24 (39.1%)	100 (36.4%)	
2-4 (n, %)	23 (30.7%)	23 (36.5%)	102 (37.1%)	
≥4 (n, %)	11 (14.7%)	15 (23.8)	70 (26.6%)	
Missing	2 (2.7%)	1 (1.6%)	3 (1.1%)	

Table 1. Continued

	HPVpos VSCC (n=75, 18%)	HPVneg/p53wt VSCC (n=63, 15%)	HPVneg/p53mut VSCC (n=275, 66%)	<i>p</i> -value
Depth of invasion in mm				0.001
(median, range)	3 (0.4-35)	4.5 (0.5-27)	6 (1-69)	
≤4 (n, %)	46 (61.3%)	29 (46.0%)	95 (34.6%)	
>4 (n, %)	29 (38.7%)	34 (54.0%)	179 (64.0%)	
Missing	0 (0.0%)	0 (0.0%)	1 (0.4%)	
LVSI (n, %)				0.03
No	58 (77.3%)	51 (81.0%)	219 (79.6%)	
Yes	7 (9.3%)	6 (9.5%)	44 (16.0%)	
Missing	10 (13.3%)	6 (9.5%)	12 (4.4%)	
Tumor positive margins (n, %)				0.26
No	70 (93.3%)	59 (93.7%)	243 (88.4%)	
Yes	5 (6.7%)	4 (6.4%)	32 (11.6%)	
Missing	0 (0.0%)	0 (0.0%)	0 (0.0%)	
HPV genotyping				
Type 16	53 (70.7%)			
Type 18	6 (8.0%)			
Type 33	9 (20.0%)			
Other	4 (5.3%)			
Treatment modality (n, %)				0.005
Surgery	60 (80.0%)	52 (82.5%)	174 (63.3%)	
Surgery & (chemo)radiotherapy	15 (20.0%)	11 (17.5%)	101 (36.7%)	
Recurrence (n, %)				<0.0001
No	64 (85.3%)	47 (74.6%)	156 (56.7%)	
Yes**	11 (14.7%)	16 (25.4%)	119 (43.3%)	
Local (first recurrence)	10 (13.3%)	13 (20.6%)	94 (34.2%)	
Locoregional (first recurrence)	1 (1.3%)	3 (4.8%)	25 (9.1%)	
Distant (first recurrence)	N/A	N/A	N/A	
Number of recurrences (n,%)				0.48
1	8 (72.7%)	8 (50.0%)	73 (63.9%)	
≥2	3 (27.3%)	8 (50.0%)	46 (38.7%)	
Time to first recurrence in months (median, range)***	18 (8-145)	37.5 (8-103)	19 (1-125)	0.14
Survival status (n, %)				<0.001
Alive	61 (81.3%)	22 (34.9%)	146 (53.1%)	
Dead	14 (18.7%)	41 (65.1%)	129 (46.9%)	

All significant *p*-values are shown in bold. HPV = human papilloma virus, LVSI = lymphovascular space invasion.

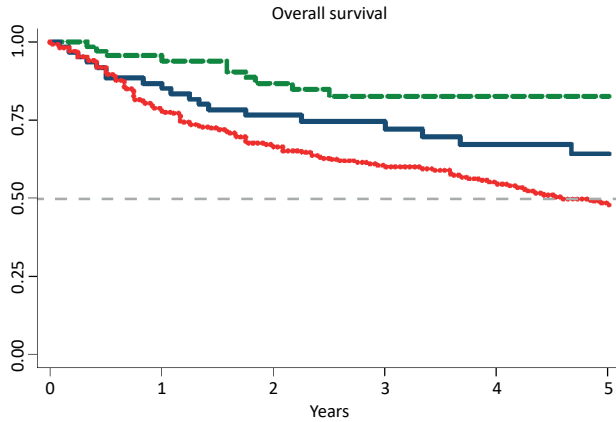
*Not all patients were eligible for lymphadenectomy, therefore the stage of disease was at least FIGO III based on imaging and/or lymph-node puncture.

**Recurrences were scored based on pathology or imaging reports. The depth of invasion, LVSI, perineural growth were scored based on the pathology reports.

***Two patients had a follow-up period of <1 month.

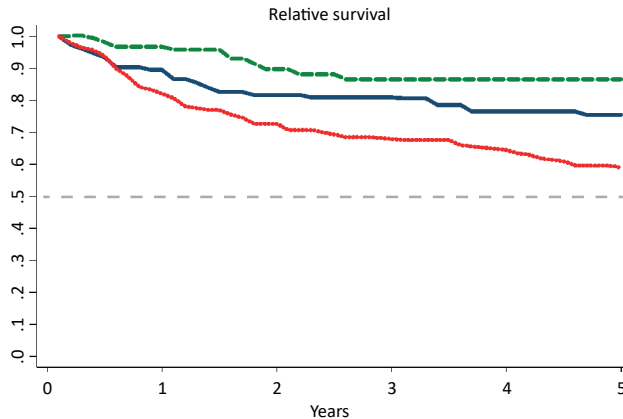
Clinical outcomes

HPVneg/p53mut VSCC have worse outcomes than other molecular subtypes, with HPVpos VSCC representing the group with best overall and relative survival (figure 2). The 5-years overall survival was 83% (95%CI 69.9-90.3), 64% (95%CI 48.9-75.9), and 48% (95%CI 41.5-55.0) for HPVpos VSCC, HPVneg/p53wt VSCC, and HPVneg/p53mut VSCC, respectively (figure 2A). A similar pattern was seen for RS, where HPVpos VSCC showed survival benefit (5-year RS 91.1% (95%CI 77.1-99.5)) compared to HPVneg/p53wt VSCC (77.8% (95%CI 59.8-91.6)) and HPVneg/p53mut VSCC (59.7% (95%CI 51.2-67.8), figure 2B). Comparison between the different molecular subtypes, revealed that patients with HPVpos VSCC had better OS at 5-years than patients with HPVneg/p53wt VSCC (HR=2.16 (95%CI 1.00-4.64), $p=0.049$) and HPVneg/p53mut VSCC (HR=3.43 (95%CI 1.80-6.52), $p<0.001$), HPVneg/p53wt VSCC also had 5-years OS benefit over HPVneg/p53mut VSCC (HR=0.63 (95%CI 0.39-1.02), $p=0.06$). The relative survival of HPVpos VSCC and HPVneg/p53wt VSCC was comparable (RER=2.26 (95%CI 0.69-7.37); $p=0.18$), but HPVneg/p53mut VSCC showed worst RS (RER=4.02 (95%CI 1.48-10.90); $p=0.006$). The relative survival between HPVneg/p53wt VSCC and HPVneg/p53mut VSCC did not differ (RER=0.56 (95%CI 0.28-1.15); $p=0.11$). Univariate analysis demonstrated that molecular subtype was associated with OS ($p=0.003$), RS ($p=0.009$), and RFP ($p<0.001$), as were age, FIGO stage, tumor size, depth of invasion, and treatment modality (table 2). LVSI and positive margins were associated with OS ($p<0.001$) and RS ($p=0.001$) but not with RFP ($p=0.36$ and $p=0.32$, respectively). In multivariable analyses that included clinicopathological factors associated with outcome¹⁰, age ≥ 70 years, FIGO stage \geq IIIA, and size >2 cm remained significant for OS ($p<0.001$, $p<0.001$, and $p=0.03$, respectively). For RS, age ($p=0.02$), FIGO stage \geq IIIA ($p=0.001$), and size >2 cm ($p=0.04$) were independent predictive factors.

A

Number at risk	0	1	2	3	4	5
HPVpos VSCC	75	59	46	35	26	23
HPVneg/p53wt VSCC	63	51	44	32	27	21
HPVneg/p53mut VSCC	275	201	161	123	97	72

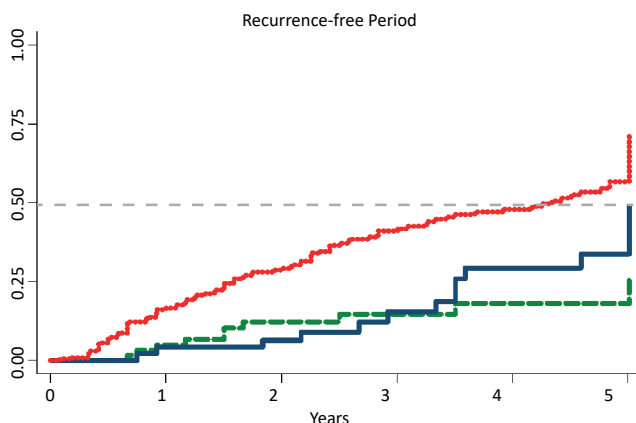
HPVpos VSCC (REF) versus HPVneg/p53wt VSCC HR 2.16 (1.00-4.64); $p=0.049$
 HPVpos VSCC (REF) versus HPVneg/p53mut VSCC HR 3.43 (1.80-6.53); $p<0.001$
 HPVneg/p53wt VSCC (REF) versus HPVneg/p53mut VSCC HR 0.63 (0.39-1.02); $p=0.06$

B

HPVpos VSCC (REF) versus HPVneg/p53wt VSCC RER 2.26 (0.69-7.37); $p=0.18$
 HPVpos VSCC (REF) versus HPVneg/p53mut VSCC RER 4.02 (1.48-10.90); $p=0.006$
 HPVneg/p53wt VSCC (REF) versus HPVneg/p53mut VSCC RER 0.56 (0.28-1.15); $p=0.11$

Figure 2. Significant differences in clinical outcome for the three molecular subtypes of vulvar squamous cell carcinoma. HPVpos VSCC showed best clinical outcome in overall survival (OS, panel A), relative survival (RS, panel B), and recurrence-free period (RFP, panel C), in contrast to HPVneg/p53mut VSCC which showed worst clinical outcome. HPVneg/p53wt VSCC showed an intermediate risk for both OS and RS, but shows no difference compared to HPVpos VSCC for RFP. Patients at risk could not be determined for relative survival, due to the nature of the calculated outcome. HPVpos VSCC = HPV-associated vulvar squamous cell carcinoma, HPVneg VSCC = HPV-independent vulvar squamous cell carcinoma, HPVneg/p53wt VSCC = HPV-independent and p53 wildtype vulvar squamous cell carcinoma, HPVneg/p53mut VSCC = HPV-independent and p53 mutant vulvar squamous cell carcinoma, HR = hazard ratio, OS = overall survival, REF = reference, RS = relative survival, RFP = recurrence-free period.

C



Number at risk	74	56	42	30	24	23
HPVpos VSCC	74	56	42	30	24	23
HPVneg/p53wt VSCC	60	46	41	26	19	13
HPVneg/p53mut VSCC	251	169	169	83	62	39

HPVpos VSCC (REF) versus HPVneg/p53wt VSCC	HR 1.64 (0.74-3.61); $p=0.22$
HPVpos VSCC (REF) versus HPVneg/p53mut VSCC	HR 3.76 (2.02-7.00); $p<0.001$
HPVneg/p53wt VSCC (REF) versus HPVneg/p53mut VSCC	HR 0.43 (0.25-0.76); $p=0.003$

4

Figure 2. Continued

Among the 275 HPVneg/p53mut VSCC there were 119 recurrences (43%) compared to 16/63 (25%) and 11/75 (15%) in the HPVneg/p53wt VSCC and HPVpos VSCC, respectively ($p<0.0001$, table 1). The median time to first recurrence did not differ between the subtypes. Recurrences were local in the great majority of cases (91% for HPVpos VSCC, 81% for HPVneg/p53wt VSCC, and 79% for HPVneg/p53mut VSCC). HPVneg/p53wt VSCC and HPVneg/p53mut VSCC more often had multiple (≥ 2) recurrences, however this was not significant. The hazard ratios for RFP for HPVpos VSCC compared to HPVneg/p53wt VSCC was 1.64 (95%CI 0.74-3.61, $p=0.22$) and compared to HPVneg/p53mut VSCC 3.76 (95%CI 2.02-7.00, $p<0.001$) (figure 2C). Interestingly, there was no difference between HPVpos VSCC and HPVneg/p53wt VSCC regarding RFP, whereas HPVneg/p53mut VSCC showed significantly worse RFP compared to the other molecular subtypes. In a multivariate analysis for RFP, age ≥ 70 years ($p=0.005$), and molecular subtype ($p=0.0002$) remained of significant impact

Table 2. Univariate analysis of all vulvar squamous cell carcinoma patients (n=413).

Factor	Overall survival		Relative survival		Recurrence-free period	
	HR (95%CI)	p-value	RER (95%CI)	p-value	HR (95%CI)	p-value
Age (years)						
<70	1.0		1.0		1.0	
≥70	3.33 (2.29-4.85)	<0.001	2.43 (1.53-3.85)	<0.001	1.83 (1.34-2.67)	<0.001
Molecular subtype		0.003*		0.009*		<0.001*
HPVpos VSCC	1.0		1.0		1.0	
HPVneg/p53wt VSCC	2.16 (1.00-4.64)	0.049	2.26 (0.69-7.37)	0.18	1.64 (0.74-3.61)	0.22
HPVneg/p53mut VSCC	3.43 (1.80-6.52)	<0.001	4.02 (1.48-10.90)	0.006	3.76 (2.02-7.00)	<0.001
FIGO stage**						
I/II	1.0		1.0		1.0	
III/IIIA/IIIB	2.32 (1.61-3.34)	<0.001	3.10 (1.81-5.31)	<0.001	1.01 (0.67-1.52)	0.94
IIIC	4.76 (3.14-7.21)	<0.001	7.32 (4.21-12.75)	<0.001	1.89 (1.14-3.14)	0.01
Size (cm)						
≤2	1.0		1.0		1.0	
2-4	2.26 (1.47-3.46)	<0.001	3.54 (1.61-7.76)	0.002	1.22 (0.84-1.77)	0.30
≥4	4.55 (2.95-7.03)	<0.001	8.06 (3.71-17.48)	0.001	1.72 (1.07-2.77)	0.02
Unknown	0.76 (0.10-5.58)	0.79	1.34 (0.11-16.13)	0.82	0.93 (0.23-3.80)	0.92
Depth of invasion (mm)***						
≤4	1.0		1.0		1.0	
>4	2.36 (1.64-3.41)	<0.001	3.37 (1.85-6.15)	<0.001	1.65 (1.16-2.33)	0.005
LVSI						
No	1.0		1.0		1.0	
Yes	2.0 (1.37-2.94)	<0.001	2.27 (1.39-3.70)	0.001	1.27 (0.48-3.70)	0.36
Unknown	2.5 (1.16-5.56)	0.02	2.70 (0.97-7.69)	0.058	1.79 (0.78-4.17)	0.17
Positive margin						
No	1.0		1.0		1.0	
Yes	2.10 (1.37-3.20)	0.001	2.43 (1.43-4.12)	0.001	1.40 (0.71-2.77)	0.32
Treatment						
Surgery	1.0		1.0		1.0	
Surgery & (chemo)radiotherapy	2.33 (1.70-3.21)	<0.001	3.03 (1.95-4.71)	<0.001	1.46 (1.02-2.09)	0.036

HPV = human papillomavirus, HPVpos VSCC = HPV-positive vulvar carcinoma, HPVneg VSCC = HPV-negative VSCC, HPVneg/p53wt VSCC = HPV-negative and p53 wildtype VSCC, HPVneg/p53mut VSCC = HPV-negative and p53 mutant VSCC, HR = hazard ratio, LVSI = lymphovascular space invasion, RER = relative excess risk.

*Global deviance test without individual parameters

**We combined the FIGO stages for the analysis into three categories: 1) FIGO stage I and II, 2) III, IIIA, IIIB and 3) IIIC due to limitations by sample size.

***One patient unknown, excluded from analysis.

Table 3. Multivariate analysis of all VSCC patients (n=413).

Factor	Overall survival		Relative survival		Recurrence-free period	
	HR (95%CI)	p-value	RER (95%CI)	p-value	HR (95%CI)	p-value
Age (years)						
<70	1.0		1.0		1.0	
≥70	2.59 (1.74-3.85)	<0.001	1.75 (1.07-2.85)	0.02	1.67 (1.17-2.40)	0.005
Molecular subtype		0.23*		0.49*		0.0002*
HPVpos VSCC	1.0		1.0		1.0	
HPVneg/p53wt VSCC	1.56 (0.71-3.40)	0.27	1.47 (0.52-4.15)	0.46	1.37 (0.61-3.08)	0.44
HPVneg/p53mut VSCC	1.79 (0.91-3.51)	0.09	1.67 (0.72-3.90)	0.23	3.04 (1.59-5.81)	0.001
FIGO stage**						
I/II	1.0		1.0		1.0	
III/IIIA/IIIB	2.35 (1.46-3.78)	<0.001	3.19 (1.62-6.20)	0.001	0.72 (0.42-1.23)	0.24
IIIC	4.56 (2.52-8.24)	<0.001	7.28 (3.34-15.88)	<0.001	0.91 (0.44-1.86)	0.80
Size (cm)						
≤2	1.0		1.0		1.0	
2-4	1.74 (1.06-2.84)	0.03	2.16 (1.02-4.55)	0.04	0.93 (0.60-1.43)	0.75
≥4	2.76 (1.60-4.77)	<0.001	3.94 (1.79-8.69)	0.001	1.07 (0.61-1.87)	0.81
Unknown	1.41 (0.18-10.83)	0.74	1.79 (0.13-25.24)	0.67	1.64 (0.39-6.89)	0.50
Depth of invasion (mm)***						
≤4	1.0		1.0		1.0	
>4	0.99 (0.62-1.57)	0.95	1.10 (0.56-2.17)	0.79	1.37 (0.90-2.10)	0.14
LVI						
No	1.0		1.0			
Yes	1.22 (0.81-1.85)	0.34	1.09 (0.63-1.89)	0.77		
Unknown	0.99 (0.44-2.22)	0.97	0.85 (0.30-2.44)	0.75		
Positive margin						
No	1.0		1.0			
Yes	1.35 (0.85-2.15)	0.19	1.49 (0.83-2.68)	0.18		
Treatment						
Surgery	1.0		1.0		1.0	
Surgery & (chemo)radiotherapy	0.75 (0.47-1.20)	0.23	0.66 (0.36-1.23)	0.19	1.48 (0.84-2.59)	0.17

Only 29 patients were diagnosed with locoregional disease, and were therefore not included in the univariate analysis. Significant p-values are shown in bold. HPV = human papillomavirus, HPVpos VSCC = HPV-positive vulvar carcinoma, HPVneg VSCC = HPV-negative VSCC, HPVneg/p53wt VSCC = HPV-negative and p53 wildtype VSCC, HPVneg/p53mut VSCC = HPV-negative and p53 mutant VSCC, HR = hazard ratio, LVI = lymphovascular space invasion, RER = relative excess risk.

*Global deviance test without individual parameters

**We combined the FIGO stages for the analysis into three categories: 1) FIGO stage I and II, 2) III, IIIA, IIIB and 3) IIIC due to limitations by sample size.

***One patient unknown, excluded from analysis.

irrespective of other clinicopathological factors. The effect of molecular subtype on RFP was driven by HPVneg/p53mut VSCC (HR=3.04 (95%CI 1.59–5.81; $p=0.001$, table 3).

DISCUSSION

Clinical management of women with vulvar cancer is highly challenging for oncological gynecologists, because current treatment is associated with significant short- and long-term morbidity²⁹, and high recurrence rates.^{4,30} In the era of precision medicine, there is a great need to improve assessment of individual risks for recurrence and death by for instance the use of clinicopathological and molecular features of tumors. The question addressed in this study was whether molecular VSCC subtypes are of prognostic significance. We performed a molecular classification using the relatively straightforward interpretation of the widely available p16- and p53-IHC. We applied the molecular classification tool to a large cohort of VSCC for which we obtained detailed clinicopathologic data and outcomes. We were able to define three molecular subgroups in VSCC; HPVpos VSCC (18%), HPVneg/p53wt VSCC (15%), and HPVneg/p53mut VSCC (66%). HPVpos VSCC were associated with early-stage disease, and with younger age compared to the HPVneg VSCC subtypes. The three subtypes showed significant different clinical outcomes for both OS, RS, and RFP with worst prognosis for HPVneg/p53mut VSCC. Importantly, within the HPVneg VSCC, tumors with wildtype p53 had similar clinicopathological parameters as HPVpos VSCC except for age, and showed better overall survival and a longer RFP compared to HPVneg/p53mut VSCC.

Current treatment decisions for VSCC are based on a clinicopathologic risk stratification strategy with all patients with the same FIGO stage receiving the same treatment.³¹ This staging system is however not highly predictive of the outcome⁵, and this may be due to the heterogeneity of VSCC. Our data showed that among VSCCs, women with tumors classified as HPVneg/p53mut VSCC had worst outcomes compared to women with HPVneg/p53wt VSCC and HPVpos VSCC, suggesting that intrinsically these tumors have a more aggressive biology. OS, RS, and RFP were all significantly reduced for the HPVneg/p53mut VSCC in an univariate analysis. The majority of the recurrences were local recurrences in all subgroups. The minority of the recurrences in the HPVpos VSCC group were locoregional recurrences (9%), followed by 19% for HPVneg/p53wt VSCC, and 21% for HPVneg/p53mut VSCC. Although the sample size is limited, the percentage of locoregional recurrences is higher in HPVneg VSCC which is associated with worse overall survival due to the involvement of the lymph nodes.^{7,32} This could be an explanation for the apparent mismatch between the prognostic variables for OS and RFP. In our analysis, we pooled local recurrences and locoregional recurrences while the development of local recurrent disease does not necessarily impact overall survival. Another explanation for the discrepancy between RFP and OS is that some local recurrences may not be true recurrences but second primary tumors that may not be equally distributed among the molecular subtypes. Despite the fact

that 101 of 275 HPVneg/p53mut VSCC (37%) received adjuvant (chemo)radiotherapy, this group showed the highest risk of recurrence and death. This strongly suggests that (neo) adjuvant treatment and/or alternative approaches for extended surgery should be explored in patients with HPVneg/p53mut VSCC.

Presurgical assessment of molecular subtype on biopsies using p16- and p53-IHC has been shown to be feasible, and therefore may be used to direct future (surgical) trials.^{22, 33} HPVneg/p53mut VSCC might also benefit from a more intensified follow-up schedule and self-examination in order to detect suspicious lesions earlier.³⁴ Interestingly, women with HPVneg/p53wt VSCC showed more indolent disease compared to those with HPVneg/p53mut VSCC, as reflected by better survival and longer RFP. Although HPVneg/p53wt VSCC had similar clinical characteristics compared to the HPVneg/p53mut VSCC HPVneg/p53wt VSCC presented with less advanced stage of disease and had lower risk for recurrent disease. It may be that other oncogenic pathways are involved in HPVneg/p53wt VSCC⁹, and therefore these tumors may benefit from other targeted therapies.

Patients with HPVpos VSCC had the most favorable clinical outcomes, as observed across several studies including our own.^{9, 10, 18} Among the 75 HPVpos VSCC in this cohort, fifteen (20%) received adjuvant (chemo)radiotherapy which is to be expected given the fact the 21% of women in this group had FIGO stage III disease. At present, the cause for the improved survival for HPVpos VSCC^{9, 10, 18} is unknown, and may be attributable to the combined effects of therapy and/or less aggressive biological behavior. It has been shown that HPVpos VSCC are more sensitive to radiotherapy³⁵⁻³⁷ and, as has been suggested recently, have a more favorable immune infiltrate³⁸ that may be further stimulated by treatment(s). In our study, no data were available on the adjuvant radiation in terms of the dose and fields that were used. Therefore, no conclusions could be drawn on the association between local recurrence risk and radiosensitivity of HPVpos VSCC. The prognostic value of these variables in relation to molecular subtype, needs to be validated in a larger cohort including patients with locally advanced or metastatic disease. Given the suggested higher radiosensitivity in HPVpos VSCC³⁵⁻³⁷, the prognostic relevance of molecular subtypes in locally advanced VSCC may even be more pronounced than presented in the current cohort.

The excellent prognosis for HPVpos VSCC might be independent of treatment. The patients showed even after radical surgery sometimes followed by (chemo)radiotherapy with no survival benefit. HPVpos OPSCC receive de-intensified adjuvant treatment compared to the HPV-independent counterpart.³⁹ In addition, clinical trials in HPVpos OPSCC are focused on the use of HPV vaccination and checkpoint inhibition⁴⁰, because these tumors seem to have more benefit from (neo)adjuvant immunotherapy than HPVneg OPSCC.⁴¹ Whether these treatment strategies may also be applicable to HPVpos/p53wt VSCC should to be explored given the good clinical outcome in this group.

The use of p16- and p53-IHC as easily accessible, inexpensive, and accurate surrogate markers for HPV status and *TP53* mutational status respectively, has also been shown by others.^{19, 20, 22} Molecular classification using these assays was successful for 413/420 (98%) of VSCCs evaluated, stressing the clinical applicability of this approach. Only one out of 413 VSCC (0.2%) showed a double positive result, as it was HPV-associated (type 18) and showed a mutant p53 immunostaining pattern (strong diffuse nuclear overexpression). We classified this case as HPVpos VSCC based on a predefined flowchart. Given the apparent rarity of this scenario of double positivity larger clinically annotated cohorts will be required to inform correct assignment. The patient was diagnosed with FIGO stage IIIA and no adjacent precursor lesions was identified. Treatment consisted of a wide local excision and adjuvant radiotherapy, after which the patient did not develop any recurrent disease and survived 11 years until the accrual time of the study. Our current interpretation is that the *TP53* mutation may have been acquired during tumor progression of this particular HPVpos VSCC tumor.

The current study included patients with primary VSCC who were surgically treated with curative intent, irrespective of adjuvant treatment given. This resulted in a large but clinically heterogeneous retrospective study cohort. Therefore, we cannot draw any definitive conclusions with respect to the putative effect of adjuvant treatment in the different VSCC subtypes. Molecular analysis of a study cohort from a prospective clinical trial, such as the GROINSS-V studies⁴², would be of interest in exploring the effects of adjuvant radiotherapy given after sentinel node (SN) biopsy.

Although we were able to include a high number of cases for this rare cancer type, we were not able to reliably obtain other influencing factors (e.g., comorbidity, smoking). Due to the retrospective character of the study, data for the included variables were not complete in all individuals. Other clinicopathological variables associated with outcomes¹⁰ should not be neglected. The combination of clinicopathological, immunological, and molecular parameters may be an improvement upon either system alone, but this needs to be evaluated in future studies.

In conclusion, we have demonstrated clinicopathological, molecular and survival differences within VSCC. In addition, we have shown that molecular classification based on two IHCs is easy and applicable to FFPE tumor tissue, and results in the identification of three distinct prognostic VSCC subtypes. The association was maintained after correction for other prognostic clinicopathological parameters. Our work suggests that molecular classification of VSCC may improve risk prediction and aids in decisions regarding adjuvant therapies. We would like to emphasize that a subgroup of HPVneg VSCC with a better prognosis can be easily identified by p53-IHC. We postulate that the molecular analyses performed for this

large VSCC cohort, could be easily implemented at most medical centers since the assays are already in clinical use. Prospective validation of our findings in an independent cohort and further assessment of treatment efficacy within specific molecular subgroups will be an important first step to personalized medicine and improved outcomes for women with VSCC.

ACKNOWLEDGEMENTS

We would like to thank Enno Dreef, Natalja ter Haar, and Isabelle Gordijn for their technical support, and Heleen Rogaar for her help with collecting the clinical data of the cohort.

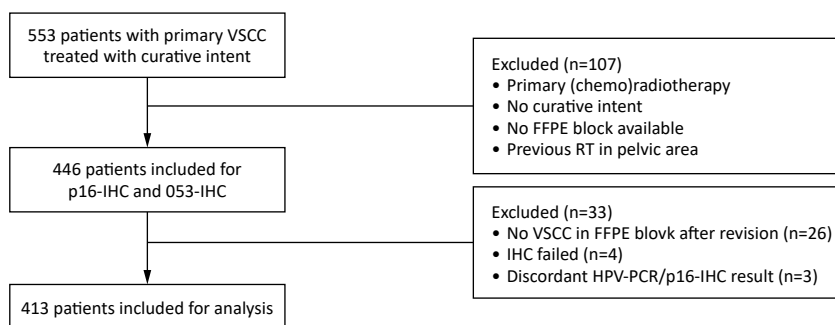
REFERENCES

1. Schuurman, M.S., et al., *Trends in incidence and survival of Dutch women with vulvar squamous cell carcinoma*. Eur J Cancer, 2013. 49(18): p. 3872-80.
2. Judson, P.L., et al., *Trends in the incidence of invasive and in situ vulvar carcinoma*. Obstetrics & Gynecology, 2006. 107(5): p. 1018-1022.
3. Eva, L.J., et al., *Trends in HPV-dependent and HPV-independent vulvar cancers: The changing face of vulvar squamous cell carcinoma*. Gynecol Oncol, 2020.
4. Gadducci, A., et al., *Old and new perspectives in the management of high-risk, locally advanced or recurrent, and metastatic vulvar cancer*. Crit Rev Oncol Hematol, 2006. 60(3): p. 227-41.
5. Hacker, N.F., *Revised FIGO staging for carcinoma of the vulva*. Int J Gynaecol Obstet, 2009. 105(2): p. 105-6.
6. Oonk, M.H.M., et al., *European Society of Gynaecological Oncology Guidelines for the Management of Patients With Vulvar Cancer*. Int J Gynecol Cancer, 2017. 27(4): p. 832-837.
7. Woelber, L., et al., *Prognostic role of lymph node metastases in vulvar cancer and implications for adjuvant treatment*. Int J Gynecol Cancer, 2012. 22(3): p. 503-8.
8. Gaarenstroom, K.N., et al., *Postoperative complications after vulvectomy and inguinofemoral lymphadenectomy using separate groin incisions*. Int J Gynecol Cancer, 2003. 13(4): p. 522-7.
9. Nooij, L.S., et al., *Genomic Characterization of Vulvar (Pre)cancers Identifies Distinct Molecular Subtypes with Prognostic Significance*. Clin Cancer Res, 2017. 23(22): p. 6781-6789.
10. Hinten, F., et al., *Vulvar cancer: Two pathways with different localization and prognosis*. Gynecol Oncol, 2018. 149(2): p. 310-317.
11. van der Avoort, I.A., et al., *Vulvar squamous cell carcinoma is a multifactorial disease following two separate and independent pathways*. Int J Gynecol Pathol, 2006. 25(1): p. 22-9.
12. Trietsch, M.D., et al., *Genetic and epigenetic changes in vulvar squamous cell carcinoma and its precursor lesions: a review of the current literature*. Gynecol Oncol, 2015. 136(1): p. 143-57.
13. Olivier, M., M. Hollstein, and P. Hainaut, *TP53 mutations in human cancers: origins, consequences, and clinical use*. Cold Spring Harb Perspect Biol, 2010. 2(1): p. a001008.
14. Lindell, G., et al., *Presence of human papillomavirus (HPV) in vulvar squamous cell carcinoma (VSCC) and sentinel node*. Gynecol Oncol, 2010. 117(2): p. 312-6.
15. Larsson, G.L., et al., *Human papillomavirus (HPV) and HPV 16-variant distribution in vulvar squamous cell carcinoma in Sweden*. Int J Gynecol Cancer, 2012. 22(8): p. 1413-9.
16. van de Nieuwenhof, H.P., et al., *The etiologic role of HPV in vulvar squamous cell carcinoma fine tuned*. Cancer Epidemiol Biomarkers Prev, 2009. 18(7): p. 2061-7.
17. Hacker, N.F., P.J. Eifel, and J. van der Velden, *Cancer of the vulva*. Int J Gynaecol Obstet, 2012. 119 Suppl 2: p. S90-6.

18. McAlpine, J.N., et al., *Human papillomavirus (HPV)-independent vulvar squamous cell carcinoma has a worse prognosis than HPV-associated disease: a retrospective cohort study.* *Histopathology*, 2017. 71(2): p. 238-246.
19. Kortekaas, K.E., et al., *Performance of the Pattern Based Interpretation of p53 Immunohistochemistry as a Surrogate for TP53 Mutations in Vulvar Squamous Cell Carcinoma.* *Histopathology*, 2020. 77(1): p. 92-99.
20. Tessier-Cloutier, B., et al., *Major p53 immunohistochemical patterns in in-situ and invasive squamous cell carcinomas of the vulva and correlation with TP53 mutation status.* *Mod Pathol*, 2020.
21. de Graeff, P., et al., *Modest effect of p53, EGFR and HER-2/neu on prognosis in epithelial ovarian cancer: a meta-analysis.* *Br J Cancer*, 2009. 101(1): p. 149-59.
22. de Sanjose, S., et al., *Worldwide human papillomavirus genotype attribution in over 2000 cases of intraepithelial and invasive lesions of the vulva.* *Eur J Cancer*, 2013. 49(16): p. 3450-61.
23. Darragh, T.M., et al., *The Lower Anogenital Squamous Terminology Standardization project for HPV-associated lesions: background and consensus recommendations from the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology.* *Int J Gynecol Pathol*, 2013. 32(1): p. 76-115.
24. Rasmussen, J.H., et al., *Risk profiling based on p16 and HPV DNA more accurately predicts location of disease relapse in patients with oropharyngeal squamous cell carcinoma.* *Ann Oncol*, 2019. 30(4): p. 629-636.
25. Lambert, P.C., et al., *Estimating the crude probability of death due to cancer and other causes using relative survival models.* *Stat Med*, 2010. 29(7-8): p. 885-95.
26. Sarfati, D., T. Blakely, and N. Pearce, *Measuring cancer survival in populations: relative survival vs cancer-specific survival.* *Int J Epidemiol*, 2010. 39(2): p. 598-610.
27. Seppa, K., T. Hakulinen, and A. Pohrel, *Choosing the net survival method for cancer survival estimation.* *Eur J Cancer*, 2015. 51(9): p. 1123-9.
28. Louwman, W.J., et al., *Clinical epidemiology of breast cancer in the elderly.* *Eur J Cancer*, 2007. 43(15): p. 2242-52.
29. Gaarenstroom, K., et al., *Postoperative complications after vulvectomy and inguinofemoral lymphadenectomy using separate groin incisions.* 2003. 13(4): p. 522-527.
30. Coulter, J. and N. Gleeson, *Local and regional recurrence of vulvar cancer: management dilemmas.* *Best Pract Res Clin Obstet Gynaecol*, 2003. 17(4): p. 663-81.
31. Rogers, L.J. and M.A. Cuello, *Cancer of the vulva.* *Int J Gynaecol Obstet*, 2018. 143 Suppl 2: p. 4-13.
32. Burger, M.P., et al., *The importance of the groin node status for the survival of T1 and T2 vulvar carcinoma patients.* *Gynecol Oncol*, 1995. 57(3): p. 327-34.
33. Singh, N., et al., *p53 Immunohistochemistry is an Accurate Surrogate for TP53 Mutational Analysis in Endometrial Carcinoma Biopsies.* *J. Pathol.*, in press.
34. Oonk, M.H., et al., *The value of routine follow-up in patients treated for carcinoma of the vulva.* *Cancer*, 2003. 98(12): p. 2624-9.

35. Proctor, L., et al., *Association of human papilloma virus status and response to radiotherapy in vulvar squamous cell carcinoma*. Int J Gynecol Cancer, 2020. 30(1): p. 100-106.
36. Dohopolski, M.J., et al., *The Prognostic Significance of p16 Status in Patients With Vulvar Cancer Treated With Vulvectomy and Adjuvant Radiation*. Int J Radiat Oncol Biol Phys, 2019. 103(1): p. 152-160.
37. Horne, Z.D., et al., *Human papillomavirus infection mediates response and outcome of vulvar squamous cell carcinomas treated with radiation therapy*. Gynecol Oncol, 2018. 151(1): p. 96-101.
38. Kortekaas, K.E., et al., *High numbers of activated helper T cells are associated with better clinical outcome in early stage vulvar cancer, irrespective of HPV or p53 status*. J Immunother Cancer, 2019. 7(1): p. 236.
39. Amin, M., S. Edge, and F. Greene, *AJCC cancer staging manual 8th edition*. New York: Springer, 2017.
40. Massarelli, E., et al., *Combining Immune Checkpoint Blockade and Tumor-Specific Vaccine for Patients With Incurable Human Papillomavirus 16-Related Cancer: A Phase 2 Clinical Trial*. JAMA Oncol, 2019. 5(1): p. 67-73.
41. Pan, C., N. Issaeva, and W.G. Yarbrough, *HPV-driven oropharyngeal cancer: current knowledge of molecular biology and mechanisms of carcinogenesis*. Cancers Head Neck, 2018. 3: p. 12.
42. Oonk, M.H., et al., *Size of sentinel-node metastasis and chances of non-sentinel-node involvement and survival in early stage vulvar cancer: results from GROINSS-V, a multicentre observational study*. Lancet Oncol, 2010. 11(7): p. 646-52.

SUPPLEMENTAL MATERIALS



Supplemental figure 1. Inclusion flowchart of the cohort.

In total, 413 patients were included for analysis in this study of which 161 patients were treated in the LUMC and 252 in the EMC. Of the LUMC patients, clinicopathological variables were obtained from a cohort previously described.⁹ FFPE = formalin-fixed paraffin-embedded, HPV-PCR = human papillomavirus polymerase chain reaction, IHC = immunohistochemistry, RT = radiotherapy, VSCC = vulvar squamous cell carcinoma.

Supplemental table 1. Differences in clinicopathological variables between different molecular subtypes.

	<i>p</i> -value HPVpos VSCC vs HPVneg/p53wt VSCC	<i>p</i> -value HPVpos VSCC vs HPVneg/p53mut VSCC	<i>p</i> -value HPVneg/p53wt VSCC vs HPVneg/p53mut VSCC
Age (years) median, range	<0.0001	<0.0001	0.55
FIGO 2009 all stages	0.13	<0.0001	0.001
Size tumor* (cm) median, range	0.18	0.10	0.99
Depth of invasion* (mm) median, range	0.09	<0.0001	0.11
LVSI * yes/no	0.78	0.009	0.13
Tumor positive margins* yes/no	1.00	0.29	0.26
Treatment modality surgery vs surgery & (chemo) radiotherapy	0.83	0.006	0.003
Recurrence yes/no	0.13	<0.0001	0.01
If yes, 1 or ≥2	0.43	0.53	0.42
Time to first recurrence in months median, range	0.31	0.61	0.05
Survival status yes/no	0.03	<0.0001	0.01

All significant *p*-values are shown in bold.

HPVpos VSCC = HPV-positive vulvar carcinoma, HPVneg VSCC = HPV-negative VSCC, HPVneg/p53wt VSCC = HPV-negative and p53 wildtype VSCC, HPVneg/p53mut VSCC = HPV-negative and p53 mutant VSCC, LVSI=lymphovascular space invasion.

*The depth of invasion, LVSI, perineural growth were scored based on local pathology reports.

