

## ANATOMICAL PATHOLOGY

## High tumour mutational burden is associated with strong PD-L1 expression, HPV negativity, and worse survival in penile squamous cell carcinoma: an analysis of 165 cases

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### Summary

Penile squamous cell carcinoma (pSCC) is a rare tumour with a variable prognosis. More prognostic markers linked to mutational signatures and the tumour immune microenvironment are needed. A cohort made up of 165 invasive pSCC was retrospectively analysed using formalin-fixed, paraffin-embedded tumour tissue, focusing on tumour mutational burden (TMB), programmed death ligand 1 (PD-L1) expression, microsatellite instability (MSI), the number of tumour infiltrating lymphocytes (TILs) expressing cytotoxic T-lymphocyte-associated protein 4 (CTLA4), HPV status determined by p16 immunohistochemistry, and several traditional histopathological variables. High TMB (>10 mut/Mb) was associated with high PD-L1 expression (TPS 50–100%), and HPV-negative status. High PD-L1 expression was linked to HPV negativity, a high number of intratumoural CTLA4+ cells, and brisk lymphocytic infiltrate. High TMB was a significant predictor of shorter overall survival (OS) in both univariate and multivariate analysis when using a median cut-off value of 4.3 mut/Mb, but not when using an arbitrary cut-off of 10 mut/Mb. Low CTLA4+ cell infiltration at the tumour invasion front was a marker of shorter OS and cancer-specific survival in both univariate and multivariate analysis. PD-L1 expression had no significant impact on prognosis. Only two cases were MSI high. The results support the hypothesis of two aetiological pathways in pSCC cancerogenesis: (1) SCC linked to HPV infection characterised by low TMB, less common PD-L1 expression, and a lower number of TILs; and (2) SCC linked to chronic inflammation leading to a high number of acquired mutations (high TMB), HPV negativity, increased neoantigen production (i.e., PD-L1), and high immune cell infiltration.

**Key words:** Penile squamous cell carcinoma; tumour mutational burden; PD-L1; CTLA4.

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### INTRODUCTION

Penile cancer is a relatively rare neoplasia, which in 2020 occupied the 30th position on the list of the most common cancers recorded worldwide.<sup>1</sup> With over 36,000 cases and over 13,000 deaths per year, it represents 0.2% of all diagnosed cancers and 0.1% of cancer-related deaths.<sup>2</sup> The vast majority (over 95%) of penile cancer is represented by squamous cell carcinoma. The prognosis of penile squamous cell carcinoma (pSCC) varies from a local disease manageable by surgery to an aggressive malignancy leading to metastatic spread and the patient's death. In the past few decades there has been no significant improvement of morbidity and mortality.<sup>3,4</sup> The presence of regional inguinal lymph node metastases represents a reliable marker of poor prognosis,<sup>5</sup> but it is usually a late sign of advanced disease. Except for the TNM stage, our previous work has shown that high tumour budding and a low number of tumour infiltrating lymphocytes also represent negative prognostic markers which can be determined using haematoxylin and eosin (H&E) stained slides.<sup>6</sup> From molecular alterations, the p53 mutated profile has been documented as a detrimental prognostic sign in our previous work and several other studies.<sup>6–12</sup> Nevertheless, new immunohistochemical and molecular signatures need to be tested in larger datasets to enable effective patient stratification in terms of risk and eventual treatment adjuvant to surgery. Following our previous research on pSCC, we focused on additional genomic and immune microenvironment properties: tumour mutational burden (TMB), microsatellite instability (MSI), programmed death ligand 1 (PD-L1) expression, and the number of T-cells expressing cytotoxic T-lymphocyte-associated protein 4 (CTLA4). These represent widely used

predictive markers in various tumours including squamous cell carcinoma of the head and neck, oesophagus and lung, and new therapeutic options could be associated with these parameters. Additionally, the human papillomavirus (HPV) status determined by p16 immunohistochemistry, as well as traditional clinicopathological variables (stage, grade, tumour budding) were also analysed.

## MATERIAL AND METHODS

### Patient recruitment

A total of 165 cases of surgically resected or biopsied and histologically verified invasive pSCCs from the period 2000–2022 were analysed in the study. The surgical material from excision, circumcision, partial or total penectomy, and lymphadenectomy was included. Representative blocks with formalin-fixed, paraffin-embedded (FFPE) tumour tissue and all available H&E stained slides were obtained from the archives of the involved pathology institutions. Invasive carcinomas of all stages were included, without regard to neoadjuvant/adjuvant therapy. The patients in the cohort had a mean age of 65.5 years [range 31.4–91.8 years, standard deviation (SD) 13.9] at the date of surgery. The follow-up data were obtained from the medical records or exported from the Czech National Oncological Registry. All patients were neoadjuvant therapy naïve. No patient received immune-checkpoint inhibitors. Overall survival (OS) and cancer-specific survival (CSS) were calculated from the date of surgery to either the date of the recorded death or to the last known follow-up date (censoring). The tumour stage was recorded based on the medical records. Stage I–IV was assigned in accordance with the TNM Classification<sup>13</sup> and Union for International Cancer Control (UICC).

### Histopathology evaluation

All available haematoxylin eosin slides were reviewed by two experienced surgical pathologists (JH and ZP) without knowledge of the patient's follow-up. Grade (1–3) was assigned according to the International Society of Urological Pathology<sup>14</sup> as follows: Grade 1 SCC (including verrucous carcinoma) shows extreme differentiation, keratinisation, and maturation; nuclear atypia is minimal or absent. Grade 2 neoplasms are intermediate in their histological features between carcinomas of Grades 1 and 3. These grow in irregular nests with obvious keratinisation and partial cell maturation and the nuclear atypia is moderate. Grade 3 SCCs (including basaloid and sarcomatoid) are usually solid or trabecular. They show scant keratinisation and are predominantly composed of anaplastic cells. There is no maturation. The cells are pleomorphic and show numerous mitoses. Tumour budding was evaluated according to the International Tumor Budding Consensus Conference (ITBCC) 2016 used for colorectal cancer:<sup>15</sup> the number of tumour buds (single tumour cell or cluster of <five tumour cells) was counted in one power field of 20× magnification in the area of the highest budding. The arithmetic mean value from both pathologists was used. The cases were sorted according to the bud number separately using a two-tiered system classifying into low-grade budding (0–4) and high-grade budding (≥5). Intratumoral and peritumoral lymphocytic infiltration was classified according to the scheme used in malignant melanoma:<sup>16</sup> the lymphocytic infiltrate was classified in H&E slides as brisk (present throughout the SCC invasive component or continuously infiltrating the entire base of the invasive tumour), non-brisk (lymphocytes present in one or more foci of the invasive SCC), or absent (no lymphocytes in contact with the invasive tumour, but may be present in perivascular or fibrotic areas). Those cases with differing findings from the observers were reviewed and discussed by both using a multihead microscope with the consensus value being assigned.

### Immunohistochemistry

Representative FFPE tumour tissue blocks were used for the immunohistochemistry. Tissue sections 4 µm thick were stained in the Ventana Benchmark ULTRA autostainer (Ventana Medical Systems, USA). Monoclonal antibodies against PD-L1 (clone SP263, Roche diagnostic kit; Roche, USA), p16 (clone R15-A, ready to use; DB Biotech, Slovakia), and CTLA4 (clone BSB-88, ready to use; Bio SB, USA) were used. The reactions were visualised using the Ultraview Detection System (Ventana Medical Systems),

counterstaining the slides with haematoxylin. Stained slides were dehydrated and covered in a xylene-based mounting medium.

### Immunohistochemistry microscopic evaluation

All anti-PD-L1 stained slides were viewed by three experienced surgical pathologists with routine experience with PD-L1 evaluation (JH, ZP, and RM) without knowledge of other variables and the patient's outcome. PD-L1 was considered positive only when detected as obvious membranous staining. The percentage of positive invasive tumour cells (tumour proportion score, TPS) from the entire available sample was recorded individually by all three pathologists. The cases were sorted according to PD-L1 expression into three subgroups as follows: negative (TPS <1%), positive (TPS 1–49%), and strongly positive (50–100%). In cases of concordant subgroups assigned by all three pathologists, the arithmetic mean of all three TPS values were recorded. Those cases with discordant subgroup assignment by one of the pathologists were subsequently discussed using a multihead microscope and a consensus TPS value was recorded. In cases of PD-L1 positivity, a topographical expression pattern was recorded as either marginal (accented at the tumour invasion front/margin), diffuse, or focal/other. According to p16 immunohistochemistry, the cases were classified as block-positive (cytoplasmic and nuclear staining in ≥75% of viable tumour cells), non-block-positive (<75% of viable tumour cells), and negative (without staining).<sup>17</sup> The discordant findings were discussed at a multihead microscope and the consensus value was recorded (Fig. 1).

### Molecular analysis of TMB and MSI

Total DNA was isolated from 10 µm thick FFPE sections of tumour tissue (>20% tumour cell nuclei in the histological slide) using the MagCore Genomic DNA FFPE One-Step Kit (RBC Bioscience, Taiwan). DNA libraries from tumour tissue were prepared using the KAPA HyperPlus Kit, KAPA Universal UMI Adapters, and UDI Primers Mixes according to the KAPA HyperCap Workflow v3.2 (Roche, Switzerland) with minor modifications (input 300 ng of DNA; enzymatic fragmentation 24 min; 10 PCR cycles). The KAPA custom HyperChoice probes (1163 kbp of target sequence; 951 kbp of coding sequence; genes in the panel are listed in [Supplementary Table 1, Appendix A](#)) were used for enrichment. The enriched libraries were additionally amplified (10 PCR cycles) and sequenced using NextSeq 500 (Illumina, USA) and High Output Kit v2.5 (150 cycles), with the target of >200x average coverage (min=167x; max=1318x; median=465x; mean=506x).

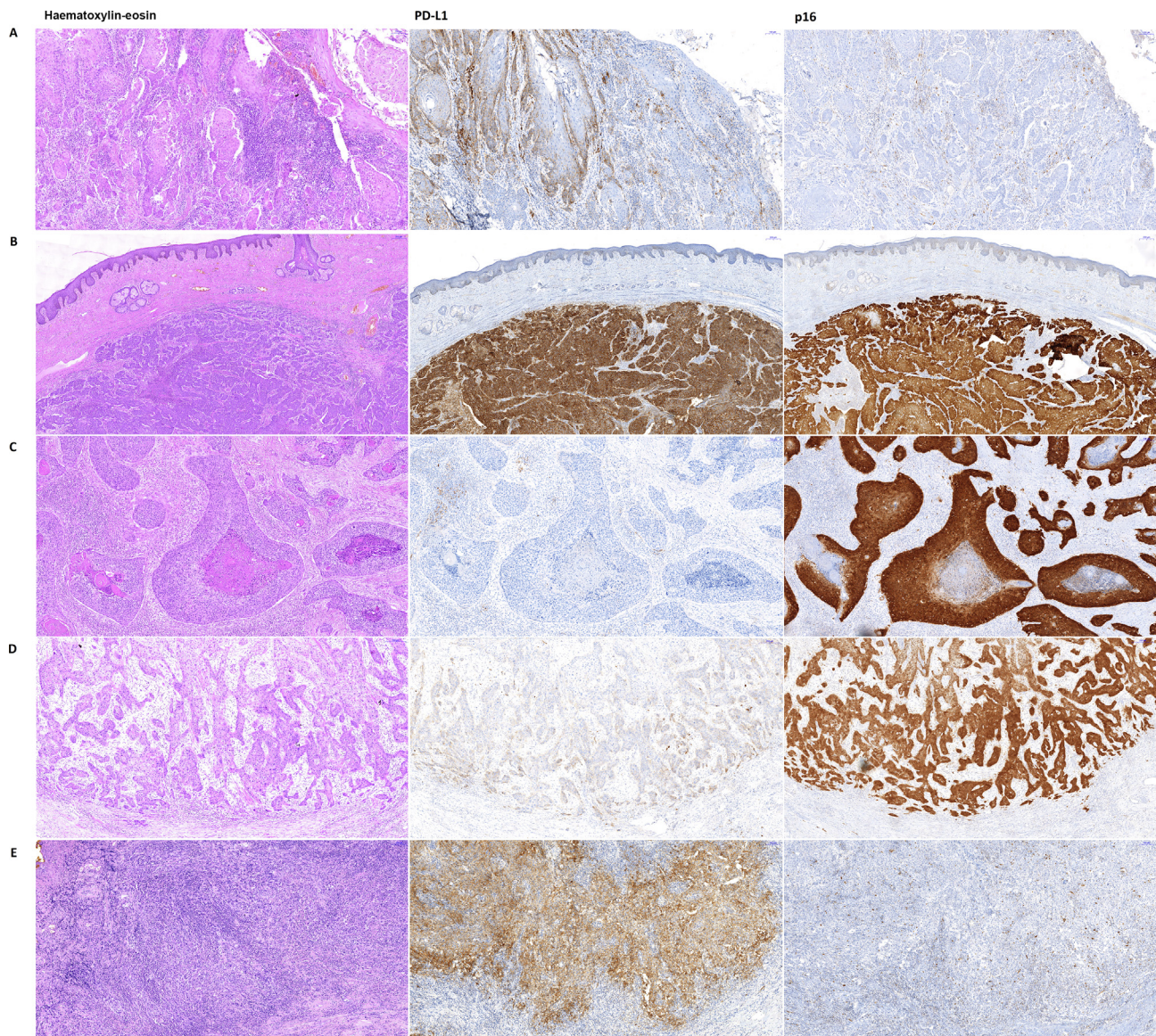
Raw fastq data were analysed using a bioinformatic pipeline including UMI demultiplexing constructed in the CLC Genomics Workbench v23.0.2 software (CLC GW; Qiagen, Germany). The complete bioinformatics workflow with individual module settings is listed in [Supplementary Table 2 \(Appendix A\)](#). The GRCh38 genome build was used for mapping the genome. The ClinVar (230115), Cosmic v95, dbSNP v151, and ExAC databases were used for annotation of the detected variants.

The variants used for TMB calculation were selected to match the filtering criteria applied in the FoundationOneCDx panel approach, with minor changes: (A) all coding single nucleotide (including samesense) and indel variants; (B) mutation allele frequency 5–91%; (C) regions with coverage ≥100x; (D) without record in the dbSNP All database; (E) without record in the ClinVar database; (F) with <3 records in the Cosmic database. All filtered variants were manually confirmed using the Integrative Genomics Viewer (IGV; Broad Institute, USA). The final TMB value was calculated as the number of detected variants divided by the coding area of the used panel (Mb) with coverage >100x (usually >930 kbp).

The MSI score for each sample was calculated using the CLC Genomics Workbench software Detect MSI status module (Qiagen, Germany). This was achieved by comparing the analysis of 51 microsatellite loci within the target sequence to a baseline created from 15 microsatellite-stable samples. Samples with more than 25% unstable loci were classified as MSI-High.

### CTLA4 quantification

The immunohistochemistry slides stained with antibodies against CTLA4 were scanned by the Panoramic Desk II DW device (3DHISTECH, Hungary). The number of positive cells (tumour infiltrating T-lymphocytes) was counted manually per square millimetre of the slide area using CaseViewer software (3DHISTECH). The quantification was performed



**Fig. 1** Microphotographs showing p16– keratinising squamous cell carcinoma with focal weak PD-L1 tumour cell positivity (A). p16+ basaloid squamous cell carcinoma with diffuse strong PD-L1 positivity (B). p16+ PD-L1-negative squamous cell carcinoma (C). p16+ squamous cell carcinoma with marginally accentuated weak PD-L1 tumour cell positivity (D). p16– squamous cell carcinoma with diffuse strong PD-L1 positivity (E).

separately from two representative areas from both the tumour centre and the invasion front seeking the area of the highest positive cell count (Fig. 2). The cohort was binarised according to an optimal cut-point calculated using optimisation of the log-rank test resulting in 38 positive cells/mm<sup>2</sup> in the tumour centre and 490 positive cells/mm<sup>2</sup> in the invasion front being used as the cut-off value. The *surv\_cut-point* function (*survminer*) determined the optimal cut-point using the maximally selected rank statistics from the ‘maxstat’ R package (see below). According to the cut-point, the cohort was classified into CTLA4-high and CTLA4-low in both the tumour centre and the invasion front.

#### Statistical analyses

To assess the impact of the selected variables (TMB, PD-L1, CTLA4) on OS, a univariate Kaplan–Meier analysis was performed with the log-rank test and confidence intervals calculated using the log-log method. Next, a restricted mean survival time (RMST) analysis with a 95% confidence interval (CI) was performed. We used a univariate Cox regression with 95% CIs to calculate the hazard ratio (HR), and a multivariate Cox regression adjusting the analysed variables (TMB, PD-L1, CTLA4) to the patient’s age. In the next step, the survival analyses were performed in terms of cause of death focusing on CSS, and the Aalen–Johansen analysis (cumulative incidence function) was carried

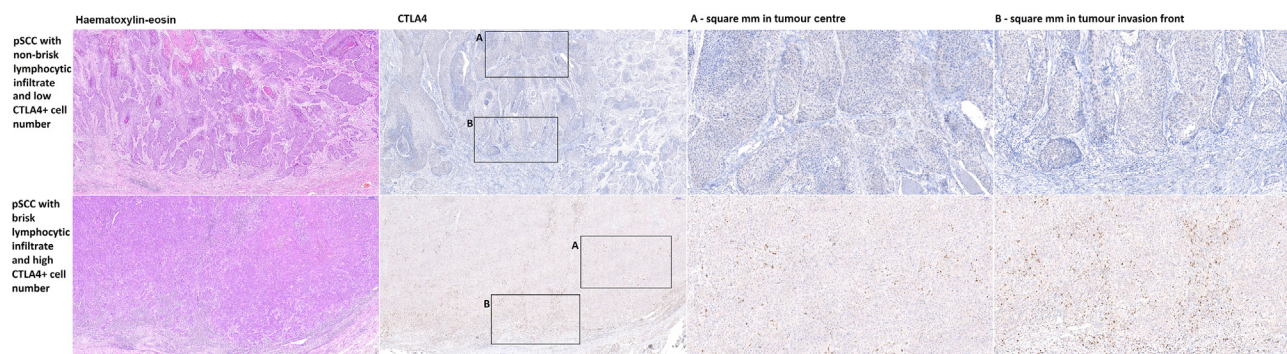
out discriminating between cancer-related cause of death and other/unknown cause of death. To obtain HR separately for cancer-related and other/unknown causes of death, the Fine–Gray regression model was used.

To find associations between the examined variables, Pearson chi squared test/logistic regression was performed for binarised variables as follows: high TMB ( $\geq 10$  mut/Mb) vs low TMB ( $< 10$  mut/Mb); PD-L1 negative+weak vs PD-L1 strong; p16 block-positive vs non-block+negative; CTLA4 tumour centre low vs high; CTLA4 invasion front low vs high; lymphocytic infiltrate brisk vs non-brisk+absent; Grade 1+2 vs 3; pT1+2 vs 3+4; pN0 vs 1+2+3; budding low ( $< 5$  buds/20 $\times$  power field) vs high ( $\geq 5$  buds/20 $\times$  power field).

To identify the independent prognostic factors, OS significance of TMB, PD-L1, and CTLA4 was analysed using multivariate Cox regression adjusting these to the associated variables identified by logistic regression. *p* values  $< 0.05$  were considered statistically significant. All analyses were performed in the R version 4.2.2 (2022-10-31),<sup>18</sup> and survival analysis using package *survival* version 3.4–0.<sup>19</sup>

## RESULTS

All research subjects are listed in Supplementary 3 (Appendix A) and the cohort is described in Table 1. The



**Fig. 2** Squamous cell carcinoma with non-brisk/brisk lymphocytic infiltrate and associated low/high CTLA4+ T-cell number per mm<sup>2</sup>.

**Table 1** Description of the cohort and the numbers of cases with available data in the individual subgroups

	n (%)
Total number	165
Death	
Yes	70 (42.4%)
Caused by pSCC	27 (16.4%)
Other cause	43 (26.1%)
No	95 (57.6%)
Surgery type	
Biopsy	12 (7.3%)
Excision	23 (13.9%)
Circumcision	27 (16.4%)
Partial penectomy	82 (49.7%)
Total penectomy	8 (10.9%)
Penectomy (unspecified)	3 (1.8%)
Tumour site	
Foreskin	42 (25.4%)
Glans	98 (59.3%)
Shaft	6 (3.6%)
Multiple/other	12 (7.2%)
Unknown	7 (4.2%)
pT stage	
pT1a	74 (44.8%)
pT1b	24 (14.5%)
pT2	41 (24.8%)
pT3	24 (14.5%)
pT4	2 (1.2%)
pN stage	
pN0	27 (26.5%)
pN1	9 (8.8%)
pN2	6 (5.9%)
pN3	5 (4.9%)
Unknown	118 (71.5%)
Grade	
1+2	113 (68.5%)
3	52 (31.5%)
Histological subtype	
Usual SCC	117 (70.9%)
Verrucous	19 (11.5%)
Basaloid	16 (9.7%)
Papillary	5 (3.0%)
Warty	3 (1.8%)
Warty-basaloid	2 (1.2%)
Sarcomatoid	2 (1.2%)
Pseudoglandular	1 (0.6%)
PD-L1 expression	
Negative (0%)	52 (31.5%)
Weak positivity (1–49%)	92 (55.8%)
Strong positivity (50–100%)	21 (12.7%)
PD-L1 expression pattern	
Negative	52 (31.5%)
Focal	43 (26.1%)
Marginal	54 (32.7%)
Diffuse	16 (9.7%)
TMB arbitrary cut-off 10 mut/Mb	
Low	119 (72.1%)
High	17 (10.3%)
Unknown	29 (17.6%)

Table 1 (continued)

	n (%)
TMB median cut-off 4.3 mut/Mb	
Low	56 (33.9%)
High	80 (48.5%)
Unknown	29 (17.6%)
CTLA4 tumour centre cut-off 38 cells/mm <sup>2</sup>	
Low	37 (22.4%)
High	106 (64.3%)
Unknown	22 (13.3%)
CTLA4 invasion front cut-off 490 cells/mm <sup>2</sup>	
Low	101 (61.2%)
High	42 (25.5%)
Unknown	22 (13.3%)
p16	
Block	93.0 (56.4%)
Non-block + negative	68.0 (41.2%)
Unknown	4 (2.4%)
Lymphocytic infiltrate	
Brisk	69 (41.8%)
Non-brisk + absent	95 (57.6%)
Unknown	1 (0.6%)
Tumour budding	
Low (<5 buds/20× power field)	97 (58.8%)
High (≥5 buds/20× power field)	67 (40.6%)
Unknown	1 (0.6%)

results of OS analysis are presented in Table 2, the results of CSS analysis in Table 3, and the results of multivariate Cox-regression in Table 4.

### DNA analysis, TMB

The isolation of sufficient quality DNA and NGS panel sequencing was successful in 136 cases from the entire cohort. Only two cases from the cohort (2/136, 1.47%) displayed MSI, and as such were not further statistically analysed. High TMB was a significant predictor of worse OS if binarised according to the median number of 4.3 mutations per megabase as the cut-off value: low TMB <4.3 mut/Mb [ $n=56$ , restricted mean survival time/mean (rmean)=9.03 years] vs high TMB ≥4.3 mut/Mb ( $n=80$ , rmean=5.80 years),  $p=0.047$  (Fig. 3A). In the multivariate Cox-regression, high TMB was a borderline significant prognosticator if adjusted to the patient's age (HR=1.6, 95% CI=0.93–2.76,  $p=0.091$ ).

On the other hand, high TMB did not show any significant prognostic effect if binarised according to the FDA-approved TMB cut-off value of 10 mut/Mb: low TMB <10 mut/Mb ( $n=119$ , rmean=8.81 years) vs high TMB ≥10 mut/Mb ( $n=17$ , rmean=6.19 years),  $p=0.16$ . High TMB (using a median cut-off value of 4.3 mut/Mb) was not a significant

**Table 2** Overall survival (OS) analysis

	<i>n</i>	Deaths	Rmean OS, years	Median OS, years	CI low	CI high	<i>p</i> <sup>a</sup>	HR	95%CI <sup>b</sup>	<i>p</i> <sup>b</sup>	HR <sup>c</sup>	<i>p</i> <sup>c</sup>
<b>TMB</b>												
Low (<10)	119	54	6.92	4.08	2.89	7.54	0.94	1.03	0.47, 2.27	>0.9	1.22	0.6
High (≥10)	17	7	5.30	5.06	1.35	NA						
Low (<4.3)	56	19	9.03	5.27	3.80	NA	<b>0.047</b>		1.0, 2.97	<b>0.049</b>		0.091
High (≥4.3)	80	42	5.80	3.02	1.93	6.85		1.72			1.60	
<b>PD-L1</b>												
Negative	52	26	5.61	3.82	1.93	6.85	0.34	NA				
Weak (1–49%)	92	34	8.47	5.27	2.89	NA						
Strong (>49%)	21	10	7.18	7.81	1.36	NA						
Negative	52	26	5.61	3.82	1.93	6.85	0.23	1.35	0.83, 2.17	0.2	1.47	0.13
Weak+strong	113	44	8.15	5.27	2.89	15.0						
Negative+weak	144	60	7.61	4.62	3.50	7.76	0.6		0.61, 2.34	0.6	1.03	>0.9
Strong	21	10	7.18	7.81	1.36	NA		1.2				
Negative	52	26	5.54	3.82	1.55	6.85	0.38	NA				
Focal positivity	43	17	8.53	7.54	2.14	NA						
Marginal positivity	54	18	8.15	5.27	3.50	NA						
Diffuse positivity	16	9	7.10	7.81	0.50	NA						
<b>CTLA4</b>												
Tumour centre low	37	19	5.36	2.68	1.35	6.85	0.16	1.47	0.85, 2.5	0.2	1.33	0.3
Tumour centre high	106	45	7.69	4.62	3.02	NA						
Invasion front low	101	53	5.73	2.87	1.93	4.62	<b>0.003</b>	2.56	1.33, 5.0	<b>0.005</b>	2.78	<b>0.002</b>
Invasion front high	42	11	10.01	7.81	4.08	NA						

CI, confidence interval; HR, hazard ratio; NA, not applicable; Rmean, restricted mean survival time.

Significant *p* values are in bold.

<sup>a</sup> Kaplan–Meier.

<sup>b</sup> Cox regression.

<sup>c</sup> Age adjusted.

factor in regard to both cancer-related death (HR=1.24, *p*=0.6) and death from another cause (HR=1.86, *p*=0.089) (Fig. 4A).

### PD-L1

PD-L1 expression was identified in 113/165 (68%) cases, with strong expression (50–100% of tumour cells) being present in 21/165 (12.7%) cases. PD-L1 expression displayed no significant impact on OS: rmean=5.61 years in the negative cases (*n*=52), 8.47 years in the weakly positive cases

(*n*=92), and 7.18 years in the strongly positive cases (*n*=21), *p*=0.34 (Fig. 3B). There was no significant effect if the cohort was binarised into negative (*n*=52, rmean=5.61 years) vs positive (*n*=113, rmean=8.15 years), *p*=0.23; or if binarised into negative+weak (*n*=144, rmean=7.61 years) vs strong (*n*=21, rmean=7.18 years), *p*=0.6. Also, no significant effect was observed if the cohort was classified according to the expression pattern as negative (*n*=52, rmean=5.54 years), focal (*n*=43, rmean=8.53 years), marginal/frontal (*n*=54, rmean=8.15 years), and diffuse (*n*=16, rmean=7.1 years), *p*=0.38. Similarly, PD-L1 expression was neither a

**Table 3** Cancer-specific survival analysis

	TMB		PD-L1		CTLA4	
	Low (< 4.3 mut/Mb)	High (≥4.3 mut/Mb)	Negative	Weak+strong	Invasion front low	Invasion front high
<i>n</i>	56	80	52	113	101	42
Cancer-related death						
<i>p</i> (Aalen–Johansen)	0.6			0.29		<b>0.03</b>
HR (Fine–Gray univariate)		1.24	1.54		3.33	
95% CI	0.56, 2.76			0.72, 3.26		1.02, 11.11
<i>p</i> (Fine–Gray)	0.6			0.3		<b>0.046</b>
HR (age adjusted)		1.19	1.54		3.13	
95% CI (age adjusted)	0.54, 2.62			0.72, 3.26		0.95, 10.0
<i>p</i> (age adjusted)	0.7			0.3		0.06
Other cause of death						
<i>p</i> (Aalen–Johansen)	0.09			0.75		0.19
HR (Fine–Gray univariate)		1.86	1.12		1.67	
95% CI	0.91, 3.8			0.61, 2.08		0.8, 3.45
<i>p</i> (Fine–Gray)	0.089			0.7		0.2
HR (age adjusted)		1.6	1.23		1.49	
95% CI (age adjusted)	0.77, 3.32			0.65, 2.38		0.72, 3.03
<i>p</i> (age adjusted)	0.2			0.5		0.3

CI, confidence interval; HR, hazard ratio.

Significant *p* values are in bold.

**Table 4** Multivariate overall survival (OS) Cox regression analysis adjusted for associated variables

Adjusted variables	Variable	<i>n</i>	Hazard ratio	95% CI	<i>p</i> value
TMB/PD-L1	TMB (cut-off 4.3 mut/Mb)	136	1.74	1.0, 3.0	<b>0.048</b>
	PD-L1 (negative+weak vs strong)		1.09	0.51, 2.33	0.8
TMB/p16	TMB (cut-off 4.3 mut/Mb)	132	1.90	1.08, 3.35	<b>0.027</b>
	p16 (block vs non-block+negative)		1.20	0.71, 2.04	0.5
CTLA4 invasion front/pN stage	CTLA4 invasion front (cut-off 490 cells/mm <sup>2</sup> )	41	1.16	0.23, 5.88	0.9
	pN stage (pN0 vs pN1+2+3)		2.96	1.06, 8.27	<b>0.038</b>
CTLA4/lymphocytic infiltrate	CTLA4 invasion front (cut-off 490 cells/mm <sup>2</sup> )	143	2.17	1.12, 4.17	<b>0.022</b>
	Lymphocytic infiltrate (brisk vs non-brisk+absent)		2.17	1.23, 3.81	<b>0.007</b>

CI, confidence interval.

*p* values for significant prognostic markers are in bold.

prognostic factor of cancer-related death (HR=0.65, *p*=0.3) nor of death from another cause (HR=0.89, *p*=0.7) (Fig. 4B).

### CTLA4

An optimal cut-point of 490 positive cells/mm<sup>2</sup> at the tumour invasion front and 38 positive cells/mm<sup>2</sup> in the tumour centre was used as the cut-off value. The high number of CTLA4+ cells at the tumour invasion front was a significant favourable OS prognostic factor: high CTLA4 (*n*=42, rmean=10.01 years) vs low CTLA4 (*n*=101, rmean=5.73 years), *p*=0.0033 (Fig. 3C). The prognostic effect of a high CTLA4+ cell number remained significant in the multivariate Cox regression if adjusted to the patient's age (HR=0.36, 95% CI=0.19–0.69, *p*=0.002). A low number of CTLA-4 cells at the tumour invasion front was a negative prognostic factor for cancer-related death (HR=3.33, *p*=0.046) (Fig. 4C), but not for death from another cause (HR=1.67, *p*=0.2). In the tumour centre, there was only a trend suggesting a beneficial prognostic effect of a high CTLA4+ cell number: high CTLA4 (*n*=106, rmean=7.69 years) vs low CTLA4 (*n*=37, rmean=5.36 years), *p*=0.16 (Fig. 3D).

### Logistic regression/Pearson chi squared test

The complete results of the analysis are presented in Supplementary Table 4 (Appendix A). High TMB ( $\geq 10$  mut/Mb) was significantly associated with strong (50–100%) PD-L1 expression [odds ratio (OR)=6.11, *p*=0.003] and showed a borderline significant association with p16 negative status (OR=2.83, *p*=0.065). Strong PD-L1 expression was linked to high TMB (see above), p16 negativity (OR=5.36, *p*=0.001), a high number of CTLA4+ cells in the tumour centre (OR=not applicable, *p*=0.004), and brisk lymphocytic infiltrate (OR=3.23, *p*=0.018). There was also a trend suggesting an association between strong PD-L1 expression and a high number of CTLA4+ cells at the tumour invasion front (OR=2.46, *p*=0.1). A high number of CTLA4+ cells was associated with brisk lymphocytic infiltrate in both the tumour centre (OR=2.63, *p*=0.021) and the invasion front (OR=3.45, *p*=0.001); CTLA4+ cell amount in both the centre and the front was mutually linked (OR=6.53, *p*<0.001). A low CTLA4+ cell number in the tumour invasion was associated with histologically verified lymph node metastases (OR=not applicable, *p*=0.029). The associations with advanced pT stage (OR=3.57, *p*=0.051), and high tumour budding (OR=2.08, *p*=0.065) did not reach statistical significance. Histopathological grade 3 was associated with non-

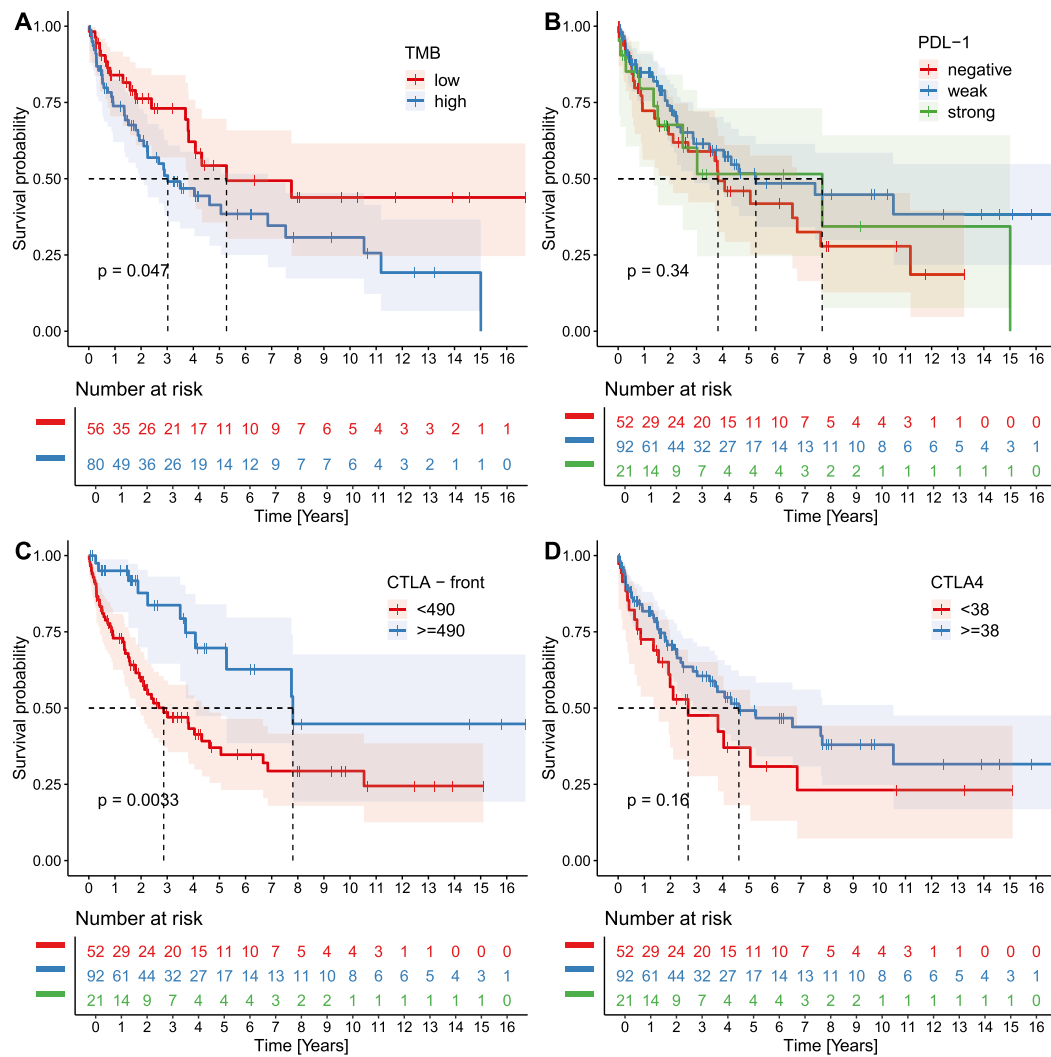
brisk/absent lymphocytic infiltrate (OR=2.49, *p*=0.016), p16 block positivity (OR=3.85, *p*<0.001), lymph node metastases (OR=4.26, *p*=0.021), and high tumour budding (OR=5.47, *p*<0.001). High tumour budding was strongly associated with grade 3 (OR=5.47, *p*<0.001), advanced pT3+4 stage (OR=3.30, *p*=0.008), and lymph node metastases (OR=10.7, *p*<0.001).

### Multivariate Cox regression survival analysis

Multivariate Cox regression was used to evaluate the prognostic significance of variables adjusted to the parameters which displayed a significant association revealed by logistic regression (Table 4). High TMB (using a median cut-off value of 4.3 mut/Mb) retained its negative prognostic significance if adjusted to PD-L1 (HR=1.74, *p*=0.048) and p16 (HR=1.9, *p*=0.027) expression. A low number of CTLA4+ cells at the tumour invasion front (using an optimal cut-off value of 490 cells/mm<sup>2</sup>) was significant when adjusted to the subjectively evaluated lymphocytic infiltrate (HR=2.17, *p*=0.022), but not significant when adjusted to the pN stage (HR=1.16, *p*=0.9). Both non-brisk/absent lymphocytic infiltrate (HR=2.17, *p*=0.007) and regional lymph node metastases (HR=2.96, *p*=0.038) remained negative prognostic markers in this analysis. We do not present a detailed survival analysis in association with these because it is included in our previous publication.<sup>6</sup>

### DISCUSSION

Our retrospective study shows an association of high TMB with strong PD-L1 expression, p16 negativity, and decreased overall survival but not cancer-specific survival. This observation may give an insight to better understanding the aetiology of penile SCC. As in head and neck SCC or vulvar SCC, the spectrum of penile lesions includes either HPV-associated SCC related to undifferentiated squamous intraepithelial lesion (SIL) and expression of oncoprotein p16, or HPV-negative SCC associated with chronic inflammation and differentiated dysplasia (d-PeIN). In the most recent World Health Organization (WHO) classification, pSCC is classified into HPV-associated and HPV-independent tumours.<sup>17</sup> Positive p16 immunohistochemical expression is considered a reliable and robust sign of HPV-associated SCCs.<sup>20</sup> HPV-associated SCCs constitute about 30–80% of penile cancers in various studies.<sup>14,21</sup> In our study, there were 93/161 (57.8%) of pSCC with block p16 positivity (Table 1). HPV-16 and HPV-18 represent the leading subtypes involved in penile cancerogenesis.<sup>22</sup> The molecular mechanisms of



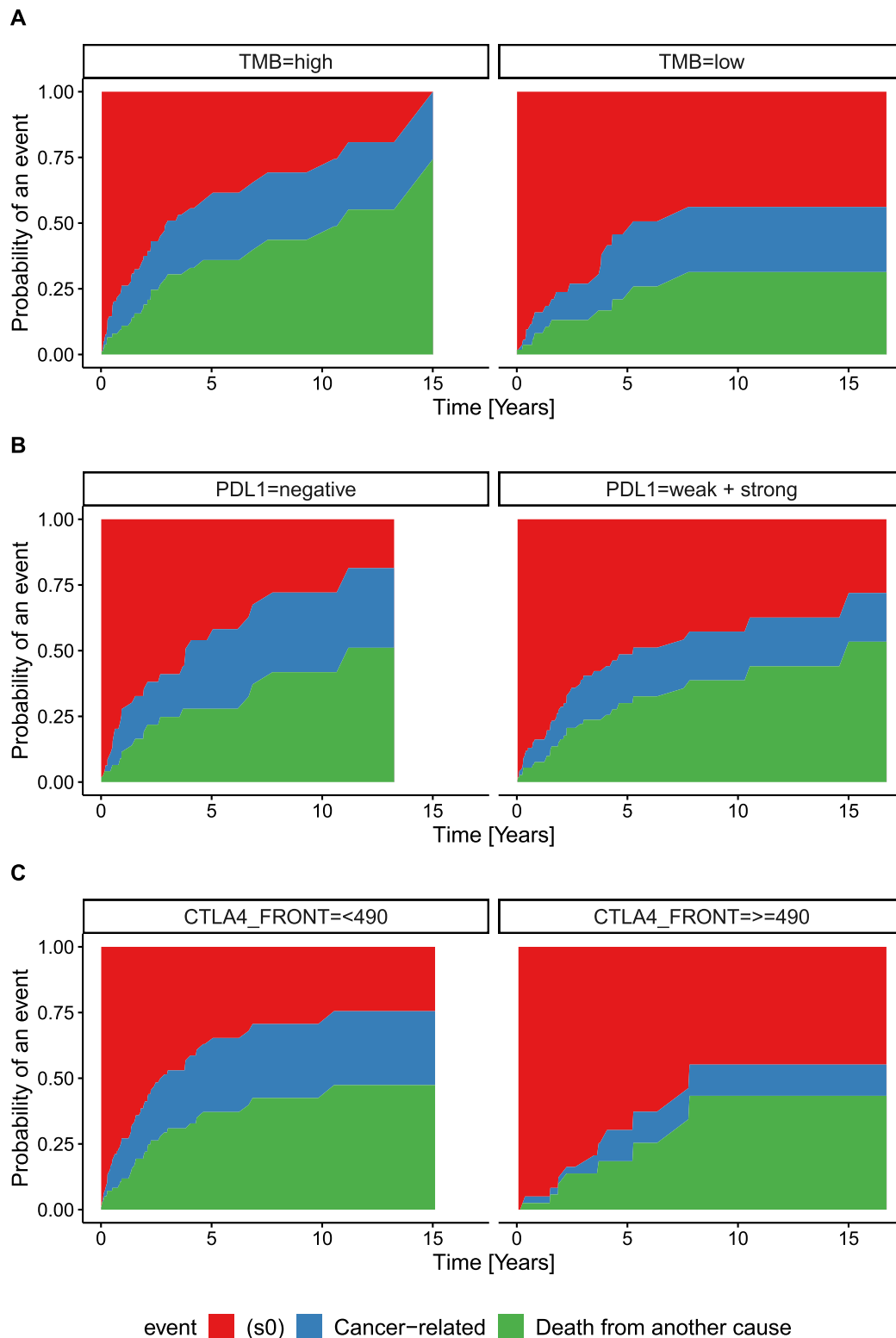
**Fig. 3** Kaplan–Meier curves documenting significantly shorter overall survival (OS) of high tumour mutational burden (TMB) with a cut-off value 4.3 mut/Mb (A) and low CTLA4+ T-cell number in tumour invasion front using optimal cut-point 490 cells/mm<sup>2</sup> (C) in penile squamous cell carcinoma. There was insignificantly shorter OS in those with low CTLA4+ T-cell number in the tumour centre (D) and no prognostic impact of PD-L1 tumour cell expression (B).

HPV-associated tumorigenesis include p53 inactivation by HPV protein E6 interacting with transcription factors (myc), autocrine motility factors which regulate cell adhesion and polarity (paxillin), apoptosis-inducing factors (bcl2), and replication and DNA repair factors (mcm7); and E7 mediated retinoblastoma tumour-suppressor protein inactivation via proteasome-dependent degradation causing p16INK4a over-expression.<sup>20</sup> On the other hand, the non-HPV pSCC risk factors include low socioeconomic status, phimosis, poor genital hygiene, chronic inflammation, lichen sclerosus, and smoking.<sup>22</sup>

The results of our study support these considerations. High-risk HPV infection leads to tumorigenic alterations in several key genes leading to increased proliferation, disordered epithelium architecture, and p16 overexpression, along with lower TMB. Inversely, poor hygiene leads to a chronic inflammatory environment, along with environmental carcinogens (i.e., smoking) eventually leading to an increased amount of acquired mutations via free radicals. Of note, the previously described association of p53 mutated immunoprofile with p16 negativity<sup>6,23,24</sup> supports this theory. These two aetiological pathways may explain the association of

high TMB with HPV/p16-negative status. A high mutation load leads to an increased neoantigen production which may interfere with the host immune system (PD-L1). Neoantigen production may explain the association of high TMB status with strong PD-L1 expression. The association of high TMB with PD-L1 expression has been widely documented in several cancers such as gastric,<sup>25</sup> colorectal, biliary,<sup>26</sup> triple negative breast carcinoma,<sup>27</sup> melanoma, and most robustly in non-small cell lung cancer.<sup>28</sup>

Despite the interesting correlation of our data with pSCC biology, we identified a significant prognostic impact of high TMB using the median cut-off value of 4.3 mut/Mb, but not when using the arbitrary FDA-approved cut-off value of 10 mut/Mb. This may be explained by the overall low TMB in pSCC compared to SCC of other sites, such as lungs, which was also observed by Chahoud *et al.*<sup>29</sup> However, in pSCCs with high TMB only the decreased OS but not CSS showed a statistical significance. In contrast with previously published studies describing decreased survival in PD-L1+ pSCCs,<sup>30–39</sup> we did not confirm any significant prognostic impact. Similarly to our study, Müller *et al.* revealed no significant impact of PD-L1 (in both tumour/combined positivity score



**Fig. 4** Aalen–Johansen plots documenting shorter cancer-specific survival (CSS) in penile squamous cell carcinoma with a low number of CTLA4+ T-lymphocytes in the tumour invasion front (C). Note the blue area difference between both plots. There was no impact of tumour mutational burden (TMB) and PD-L1 tumour cell expression on CSS (A,B).

TPS/CPS) on patients' survival.<sup>30</sup> Ottenhof *et al.*<sup>31</sup> described a favourable prognostic impact of marginal and a detrimental impact of diffuse PD-L1 expression, particularly in HPV-negative pSCCs, which has not been confirmed by our data. This discrepancy may be caused by methodological issues,

e.g., tissue microarray (TMA) vs whole mount section used as well as usage of a different scoring system. In our study, we used the anti-PD-L1 antibody clone SP263 for assessing the tumour proportion score (TPS). TPS is widely used in routine non-penile SCC predictive diagnostics.<sup>40,41</sup> Similarly,

Ottenhof *et al.* also documented more frequent PD-L1 expression in HPV-negative tumours. In contrast, De Bacco *et al.* described association of PD-L1 expression with worse outcome but also with p16 positivity,<sup>32</sup> but the authors used a 10% tumour cell positivity cut-off which differs from the standard recommendations (75%). Feber *et al.* described lower TMB in HPV-positive compared to HPV-negative pSCC in a relatively small cohort comprised of 27 cases but provided no survival analysis.<sup>33</sup> To the best of our knowledge, the association of TMB and PD-L1 has not yet been described in pSCC.

Our results document the crucial prognostic role of tumour immune cell infiltration. In our previous study,<sup>6</sup> brisk lymphocytic infiltrate based on H&E slide examination was a favourable prognostic factor. In this study, the same effect was observed for the high number of CTLA4+ T-cells at the pSCC invasion front. The important role of tumour infiltrating lymphocytes in pSCC corresponding to host anti-tumour immunity has already been documented in several studies. Ottenhof *et al.*<sup>34</sup> described the association of low CD8+ T-cell number in the tumour stroma with nodal metastases. Müller *et al.* reported a high number of all immune cells evaluated in HE slides using a three-tiered grading linked to longer survival and lower tumour stage.<sup>30</sup> Cocks *et al.* found an association of FOXP3 expression in tumoral immune cells with increased tumour thickness,<sup>35</sup> and Hladek *et al.* did not prove any link to survival.<sup>42</sup> However, both studies are limited by the use of the TMA technique, which may easily miss a hot-spot containing a high number of immune cells compared to whole slide analysis. Vassallo *et al.* identified the low number of FOXP3+ T-cells as an unfavourable prognostic factor.<sup>43</sup> Lohneis *et al.* described an increased number of intratumoural T-cells in HPV-positive pSCC,<sup>44</sup> which is not in accordance with our data. In our recent study, we found a negative prognostic significance of low CD3+/CD8+ cell immunoscore according to Galon *et al.*<sup>45</sup> in terms of OS but not CSS, and a favourable prognostic impact of brisk lymphocytic infiltrate compared to cases with non-brisk/absent infiltrate evaluated in H&E slides for both OS and CSS.<sup>6</sup>

Microsatellite instability is currently widely used as an immune-checkpoint therapy predictor in several cancer types. Our results documenting only 2/136 (1.5%) MSI-high cases are in line with previously published studies on pSCC.<sup>46–48</sup> Given its rarity, it remains debatable whether MSI is suitable for routine testing in pSCC. Compared to non-penile skin SCC caused usually by ultraviolet light exposure, both MSI and TMB are low in pSCC;<sup>29,47</sup> clinical trials focused on these predictive markers as well as immune-checkpoint therapy are strongly needed.

There are several limitations and perspectives for further research in our study. Despite the relatively large cohort, a subset of samples ( $n=29$ ) was not suitable for successful high quality DNA extraction. A larger cohort could increase the level of significance, although on the other hand, low quality DNA may limit routine TMB testing mainly in formalin-fixed retrospective samples. Another limitation is that a subset of cases ( $n=22$ ) was not suitable for accurate CTLA4+ quantification, mainly in small biopsies where it was not possible to reliably identify the tumour centre and tumour invasion front. It also needs to be emphasised that despite p16 immunohistochemistry being a WHO-accepted tool for determining the HPV status of pSCC, there are limitations in its agreement

with genetic diagnostic methods. Olesen *et al.* reported a p16 sensitivity of 79.6% and specificity of 81.5% when compared to DNA PCR obtained results in a recent meta-analysis on pSCC.<sup>49</sup> p16 as a surrogate marker of active HPV replication does not reach 100% sensitivity, possibly due to dormant/persistent HPV genome in the tumour cells identifiable only by genetic methods. On the other hand, p16 expression may rarely be seen regardless of the presence of HPV. In our study, we decided to use p16 expression due to the low-quality DNA in a subset of cases, since almost all cases were suitable for p16 immunohistochemistry. The accuracy of various HPV diagnostic methods needs to be further examined in sufficiently large cohorts. The last limitation could be seen in the selection of PD-L1 clones and scoring systems. In our study, we exclusively used the PD-L1 clone SP263 diagnostic kit suitable for TPS assessment. Although the concordance of various PD-L1 clones and PD-L1 scoring systems certainly is a relevant topic for further research, this aspect was outside the scope of the current study.

Our study provides a novel explanation of high TMB in cases without signs of HPV infection. Nevertheless, despite being an important criterion in the WHO classification of penile tumours, according to our recently published study HPV has no prognostic relevance.<sup>6</sup> Clinical trials with immune-checkpoint inhibitors in pSCC monitoring the therapeutic response according to HPV status, PD-L1 expression, and TMB are strongly needed, although there are difficulties given the rarity of this tumour. Also, when comparing the results of our current and previous study, there is prognostic superiority of the pure histological variables such as budding and density of lymphocytic infiltrate over TMB and PD-L1.

**Ethics approval:** This study was approved by the institutional review board and by the University Hospital Královské Vinohrady ethics committee, approval number EK-VP1261012020. The entire research has been performed in accordance with the Declaration of Helsinki to fully respect patients' privacy.

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pathol.2023.10.010>.

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