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Low GATA3 predicts worse survival in penile cancer

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Penile squamous cell carcinoma (pSCC) is a rare genitourinary tumor associated with notable psychosexual distress and poor prognosis. Traditional prognostic factors for pSCC include TNM stage and histological grade, with lymph node metastases being a critical indicator of poor prognosis. This study aimed to evaluate the prognostic impact of the following immunohistochemistry markers routinely used in histopathology practice: GATA3, IMP3, HIF-1-α, CK7, CA-IX, HER2, and TTF-1. A retrospective cohort of 145 patients with pSCC was analyzed using tissue microarray and immunohistochemical techniques. Overall survival (OS) was correlated statistically with detected marker expression. Key findings include that low GATA3 expression is associated with significantly worse OS in univariate Cox regression truncated at 3 years of follow-up. Low GATA3 retained prognostic impact when adjusted for major clinicopathological variables: Age, pT and pN stage, grade, lymphatic, venous, and perineural invasion, lymphocytic infiltrate, and expression of p16, p53, and PD-L1. Low GATA3 expression was associated with shorter cancer-specific survival (CSS) at 10 years follow-up. IMP3, CK7, and CA-IX showed statistically insignificant trends towards poorer prognosis. CK7 and CA-IX were more frequently expressed in high grade pSCC and in p16/HPV-positive tumors. IMP3 and CA-IX were associated with regional lymph node metastases. All cases were negative in TTF-1 and HER2. This study suggests GATA3 as a potential prognostic marker in pSCC.

Keywords Penile cancer, Squamous cell carcinoma, GATA3, IMP3, HIF-1-α, Cytokeratin 7, Carbonic anhydrase IX

Penile squamous cell carcinoma (pSCC) is a relatively rare genitourinary tumor whose prognosis improvement has remained elusive, especially in advanced stages, over the past several decades[1]. Some studies on Western populations describe a slowly increasing incidence, particularly among young men, which is likely related to sexually transmitted human papillomavirus (HPV) infection[2,3]. In pSCC, two distinct types of lesions may arise: HPV-associated SCC, characterized by undifferentiated squamous intraepithelial lesions (SIL) and the expression of the oncoprotein p16, and HPV-independent SCC, associated with chronic inflammation and differentiated dysplasia/differentiated penile intraepithelial neoplasia (d-PeIN). d-PeIN is usually associated with older age, relatively preserved morphology of squamous epithelium, and a high risk of progression into an invasive pSCC. The traditional prognostic factors include TNM stage[4], histological grade[5], and histological subtype[6]. Lymph node metastases represent a late indicator of a poor prognosis[7]. There are controversies concerning the pT1b stage as it displays worse prognosis compared to pT2-4[8]. Novel prognostic markers such as immune cell infiltration, tumor budding[9], high tumor mutational burden (TMB)[10], and several genomic alterations[11] have been described, but more efficient markers are needed. Recently, there has been a global surge in scientific research concentrating on prognostic factors pertinent to pSCC.

Given the increasing recognition of certain markers as surrogate prognostic indicators or therapeutic targets in squamous and epithelial cancers, we aimed to explore the prognostic utility of a routinely available IHC panel, which could be related to modern anticancer therapy or used as specific markers for other tumors: GATA

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binding protein 3 (GATA3), Insulin-like growth factor II-messenger RNA-binding protein 3 (IMP3), Hypoxia-inducible factor 1-α (HIF-1-α), Cytokeratin 7 (CK7), carbonic anhydrase IX (CA-IX), Human epidermal growth factor receptor-2 (HER2), and Thyroid transcription factor-1 (TTF-1). The most important clinical-pathological variables (stage, histological subtype, grade, lymphatic/vascular/perineural invasion) and pivotal prognostic immunohistochemistry markers (i.e. p16, p53, PD-L1) have been studied in our previous works[9,10]. This study aims to analyze the prognostic impact of other important previously unexplored markers in pSCC.

Materials and methods

Cohort acquisition

A retrospective cohort of 156 patients with surgically removed or biopsied and histologically verified invasive pSCC with sufficient amount of representative formalin-fixed paraffin-embedded (FFPE) archival tumor tissue was analyzed. Invasive carcinomas of all stages were included, without regard to adjuvant therapy. No patient in the cohort received neoadjuvant therapy. Follow-up data were obtained from medical records or exported from the Czech National Oncological Registry. Overall survival (OS) was calculated from the date of surgery to the date of recorded death. The stage of the tumors was recorded based on medical records. Stage I-IV was assigned in accordance with TNM Classification[4] and Union for International Cancer Control (UICC). All available haematoxylin and eosin (H&E) 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[5]. The presence or absence of lymphatic, vascular, and perineural invasion were recorded. Lymphocytic infiltrate in H&E slides was evaluated as brisk/non-brisk + absent according to Clark´s scheme used in assessment of intratumoral lymphocytes in malignant melanoma[12], as described in detail elsewhere[9].

Immunohistochemistry

For immunohistochemistry, the tissue microarray (TMA) technique was used, using two cylindrical cores each measuring 2 mm in diameter from each case. Tissue sections of 4 µm thickness were stained using the Ventana BenchMark ULTRA autostainer (Ventana Medical Systems, USA). Monoclonal antibodies used included GATA3 (L50-823, CellMarque, ready to use/RTU), IMP3 (EP286, BioSB, 1:20), HIF-1-α (EP118, BioSB, 1:800), CK7 (OV-TL 12/30, BioSB, 1:500), CA-IX (EP161, BioSB, 1:100), HER2 (4B5, Roche, RTU), TTF-1 (SP141, Roche, RTU). The reactions were visualized using the Ultraview Detection System (Ventana Medical Systems) and counterstained with haematoxylin. The stained slides were then dehydrated and mounted in a xylene-based medium.

Immunohistochemistry evaluation

The immunohistochemistry slides were evaluated independently by two experienced surgical pathologists (JH and ZP) without knowledge of other variables and clinical outcome. Cytoplasmic (CA-IX, IMP3, HIF-1-α, CK7), nuclear (GATA3, TTF-1), and membranous (HER2) staining were noted. In each case, the percentage of positively stained cells was scored (0 = none, 1–9% = 1, 10–49% = 2, 50–79% = 3, 80–100% = 4), and staining intensity was semi-quantitatively classified into the following four categories: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong positivity. In each core, an overall immunoreactivity score was calculated as proportion (0–4) × intensity (0–3), range 0–12 (Fig. 1). Each case rendered four scores: 2 cores from 2 pathologists. For the statistical analysis, the average value from individual cores was used. The immunoreactivity score was recorded for each core only if ≥ 100 invasive pSCC cells were found. If three or four cores were insufficient, the case was excluded from the study. In total, 11 cases were excluded from the study, and the final cohort size was $n = 145$.

Immunohistochemistry profiles of p53 and p16 antibodies were analyzed in whole sections and classified into mutated/wild type and block-positive/non-block-positive + negative, as described in detail elsewhere[9]. Programmed death ligand 1 (PD-L1) tumor proportion score was evaluated as described in detail elsewhere[10], classified into negative (< 1%)/positive (1–100%).

Statistics

For the overall survival (OS) analysis, Kaplan–Meier analysis was conducted using the log-rank test, and confidence intervals were calculated using the log-log method. This was followed by a restricted mean survival time (RMST) analysis with a 95% confidence interval (CI). Univariate Cox regressions with 95% CIs were performed to calculate the hazard ratio (HR) for each parameter. The OS analyses were truncated at 5 years of follow-up, and in case of significant p value (< 0.05), separate analyses truncating at 3 years and 10 years of follow-up were performed. The significant results were subsequently analyzed by multivariate Cox regression adjusting the marker on the patient's age and other clinical-pathological variables (see below). Multivariable analyses were conducted as pairwise models across key covariates due to limited events per variable. The markers with an impact on OS were further subjected to analysis of cancer-specific survival (CSS) using Fine Gray regression (a proportional hazards model for the subdistribution of a competing risk) distinguishing between penile cancer-related deaths and other causes of death, based on information from clinical documentation and Czech National Oncological Registry. The cohort was binarized according to immunoreactivity score for each marker using the surv_cut-point function (survminer) determining the optimal cut-point using the maximally selected rank statistics from the `maxstat` R package. According to the optimal cut-point, the cohort was classified into high and low for each marker. Interobserver agreement between the two pathologists was evaluated by calculating Cohen's kappa. To assess association between immunohistochemistry findings and clinical-pathological variables, logistic regression/Pearson's chi-squared test was performed. The clinical-pathological variables were grouped as follows: pT1 + pT2/pT3 + pT4; pNX + pN0/pN1 + pN2 + pN3; grade 1 + 2/grade 3; lymphatic invasion yes/no; vascular invasion yes/no; perineural invasion yes/no; lymphocytic infiltrate brisk/non-brisk + absent. The patients who underwent lymphadenectomy with negative lymph node histology were clustered

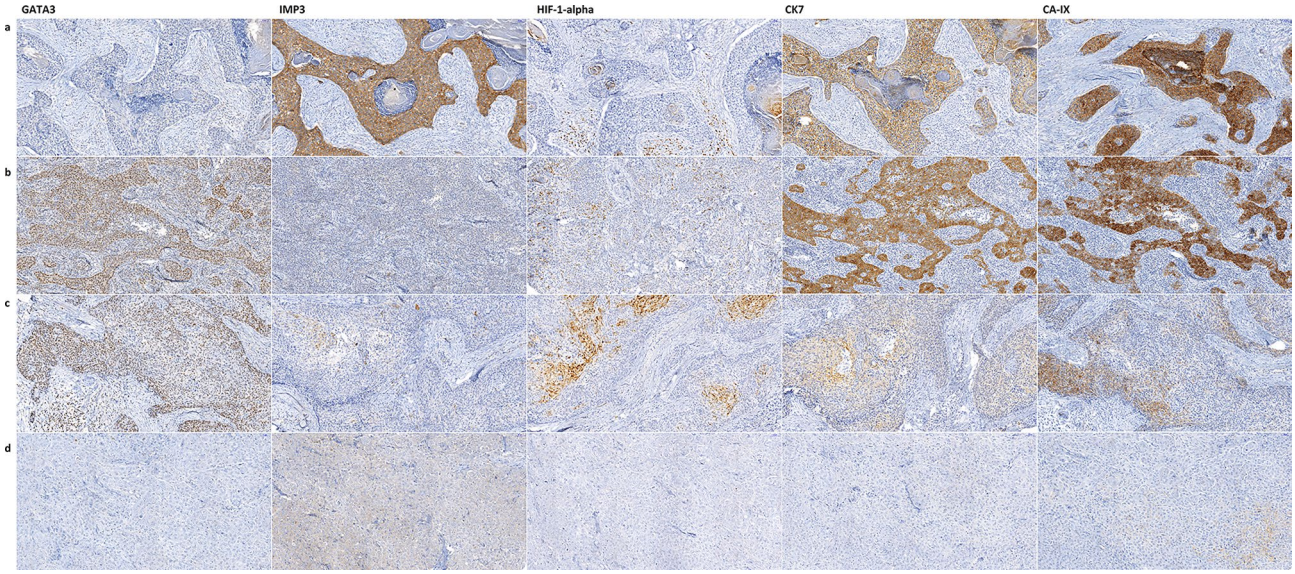

Fig. 1. immunohistochemistry profiles of penile squamous cell carcinoma (pSCC), 200x.a: pSCC evaluated as GATA3-low (negative) by both pathologists, IMP3-high (intensity 3 in 85% and 100%), HIF-1-α negative, CK7-high (intensity 3 in 100% and 95%), CA-IX-high (2 in 60%, 3 in 60%).b: pSCC evaluated as GATA3-high by both pathologists (intensity 3 in 90% and 2 in 80%), IMP3 negative, HIF-1-α negative, CK7-high (3 in 100% and 95%), CA-IX-high (3 in 80% and 90%).c: pSCC evaluated as GATA3-high (intensity 2 in 60% by both pathologists), IMP3-low (zero and 1 in 10%), HIF-1-α-high (intensity 3 in 30% and 2 in 15%), CK7-low (2 in 5% and 1 in 30%), CA-IX-low (2 in 10% by both pathologists).d: pSCC evaluated as negative/low in all markers by both pathologists. The microphotographs from four selected cases illustrate the highly variable immunoreactivity with studied markers in pSCC.

together with patients without lymphadenectomy (pNX/cN0). The immunohistochemistry marker profiles have been compared with mutational profiles examined by next generation sequencing (NGS), whose results and methods have been described by our group elsewhere[11]. Genes which have been altered in ≥ 10 cases have been included in the analysis.

All analyses were performed using R version 4.2.2 (2022-10-31), with survival analyses carried out using the survival package version 3.4–0.17; p values < 0.05 were considered statistically significant.

Ethics

The study was approved by the institutional review board and by the University Hospital Královské Vinohrady ethics committee, approval number EK-VP1261012020. Informed consent was waived due to the retrospective nature of the study. The entire research was performed in accordance with the Declaration of Helsinki.

Results

Optimal cut-points

The cohort description is summarized in Table 1. All research data are listed in Supplementary Table 1 (Table S1). Samples from all 145 cases were HER2- and TTF-1-negative. The remaining six markers showed varying intensity and percentage of staining. In the HIF-1-α, only cytoplasmic staining without any nuclear staining was observed. The optimal cut-points using the survminer function were set at immunoreactivity score 0.5 in GATA3, 0.75 in IMP3, 3.0 in HIF-1-α, 3.25 in CK7, and 8.75 in CA-IX.

The cohort ($n = 145$) was classified according to the optimal cut-points as low/n (%) as follows: GATA3 = 60/145 (42%), IMP3 = 70/145 (48%), HIF-1-α = 122/144 (85%), CK7 = 129/145 (87%), CA-IX = 126/143 (88%). Detailed description of the mutual associations is in Supplementary Table 2 (Table S2). Cohen's kappa reached for binarized markers: GATA3 K = 0.78, IMP3 K = 0.84, HIF-1-α K = 0.65, CK7 K = 0.92, CA-IX K = 0.72. This calculation showed almost perfect interobserver agreement in CK7 and IMP3, and substantial agreement in GATA3, HIF-1-α and CA-IX.

OS analysis

The results of Kaplan-Meier analysis, RMST, and univariate Cox regression are summarized in Table 2, the results of Kaplan-Meier analysis in Fig. 2. The results of multivariate Cox regression are summarized in Table 3.

Patients with GATA3-low tumors displayed significantly worse OS in the univariate (Hazard ratio/HR = 1.69, 95%CI = 1.02–2.78, $p = 0.042$), but not in the multivariate age-adjusted (HR = 1.41, 95%CI = 0.85–2.33, $p = 0.2$) Cox regression. The Cox regression truncated at 3 years of follow-up displayed significant negative prognostic impact of GATA3-low status (univariate HR = 2.08, 95%CI = 1.22–3.57, $p = 0.007$; age-adjusted HR = 1.75, 95%CI = 1.03–3.03, $p = 0.04$). GATA3-low status was a significant negative prognostic marker also in 10 years

Variable	Value	*N*	%
Death	Yes	71	49.0
	No	74	51.0
pT stage	pT1a	62	42.8
	pT1b	21	14.5
	pT2	35	24.1
	pT3	24	16.6
	pT4	2	1.4
	pTX	1	0.7
Lymphadenectomy	Yes	47	32.4
	No	98	67.6
Surgery type	Excision	22	15.2
	Biopsy	10	6.9
	Circumcision	21	14.5
	Partial penectomy	69	47.6
	Total penectomy	19	13.1
	Other/unknown	4	2.8
pN stage	pN0	27	18.6
	pN1	12	8.3
	pN2	7	4.8
	pN3	5	3.5
	pNX	94	64.8
Lymphatic invasion	Yes	47	32.4
	No	98	67.6
Vascular invasion	Yes	26	17.9
	No	119	82.1
Perineural invasion	Yes	15	10.4
	No	130	89.6
Grade	1	33	22.8
	2	66	45.6
	3	46	31.6
Histological subtype	Usual SCC	101	69.7
	Verrucous	17	11.7
	Basaloid	13	9.0
	Papillary	5	3.5
	Warty-basaloid	3	2.1
	Sarcomatoid	3	2.1
	Warty	2	1.4
	Pseudoglandular	1	0.7
Resection margin	R0	108	74.9
	R1	32	22.1
	R2	4	2.8
	Unknown	1	0.7
Lymphocytic infiltrate	Brisk	59	40.7
	Non-brisk + Absent	86	59.3
p16	Block	84	57.9
	Non-block + Negative	61	42.1
p53	Wildtype	117	80.7
	Mutated	28	19.3
PD-L1	Negative (0%)	32	22.1
	Positive (1–100%)	113	77.9
GATA3	Low (< 0.5)	60	41.4
	High (≥ 0.5)	85	58.6
IMP3	Low (< 0.75)	74	51.0
	High (≥ 0.75)	71	49.0
HIF-1-α	Low (< 2.75)	116	80.0
	High (≥ 2.75)	29	20.0

Continued

Variable	Value	N	%
CK7	Low (< 3.25)	129	89.0
	High (≥ 3.25)	16	11.0
CA-IX	Low (< 8.75)	126	88.1
	High (≥ 8.75)	17	11.9

Table 1. Description of the cohort ($n = 145$): clinical-pathological variables, studied immunohistochemistry results, profiles of p16, p53, PD-L1.

Marker	Status	N	deaths	rmean	Median	p (Kaplan Meier)	HR univariate Cox regression (95% CI)	p
GATA3 3-year follow-up	Low (< 0.5)	60	27	2.72	2.25	**0.006**	2.08 (1.22, 3.57)	**0.007**
	High (≥ 0.5)	85	31	3.61	NA			
GATA3 5-year follow-up	Low (< 0.5)	60	30	2.81	2.40	**0.04**	1.69 (1.02, 2.78)	**0.042**
	High (≥ 0.5)	85	31	3.63	NA			
GATA3 10-year follow-up	Low (< 0.5)	60	30	2.81	2.40	**0.014**	1.82, (1.12, 2.94)	**0.016**
	High (≥ 0.5)	85	31	3.63	NA			
IMP3 5-year follow-up	Low (< 0.75)	74	28	3.58	NA	0.13	1.48 (0.89, 2.44)	0.13
	High (≥ 0.75)	71	33	3.06	4.04			
HIF-1-α 5-year follow-up	Low (< 2.75)	116	50	3.23	4.33	0.38	0.75 (0.39, 1.44)	0.4
	High (≥ 2.75)	29	11	3.68	NA			
CK7 5-year follow-up	Low (< 3.25)	129	54	3.38	4.70	0.31	1.46 (0.69, 3.07)	0.3
	High (≥ 3.25)	16	8	2.78	3.82			
CA-IX 5-year follow-up	Low (< 8.75)	126	52	3.38	NA	0.26	1.53 (0.73, 3.22)	0.3
	High (≥ 8.75)	17	8	2.73	2.40			
	High (> 0)	65	30	3.18	4.08			

Table 2. Summary of overall survival analysis - restricted mean (rmean) survival time, Kaplan-Meier analysis, univariate Cox regression. Significant p values (< 0.05) in bold.

of follow-up in the univariate (HR = 1.82, 95%CI = 1.12–2.94, $p = 0.016$) but not in the age adjusted (HR = 1.49, 95%CI = 0.92–2.44, $p = 0.1$) analysis. GATA3-low status remained significantly associated with shorter 3 years OS if adjusted on pT stage, pN stage, grade, lymphatic, venous, and perineural invasion, lymphocytic infiltrate, p16 status, p53 profile, and PD-L1 expression (Table 2). In the multivariate Cox regression, the following variables were negative prognostic factors independently from GATA3 status: pN stage, lymphatic, venous, and perineural invasion, lymphocytic infiltrate, and p53 profile (Table 2).

IMP3-high tumors displayed an insignificant trend towards worse OS in the univariate 5 years Cox regression (HR = 1.48, 95%CI = 0.89–2.44, $p = 0.13$). HIF-1-α-high tumors tended insignificantly to show better OS (HR = 0.75, 95%CI = 0.39–1.44, $p = 0.4$). CK7-high expression was associated with an insignificant trend towards shorter OS (HR = 1.46, 95%CI = 0.69–3.07, $p = 0.3$). CA-IX-high status showed an insignificant negative prognostic impact (HR = 1.53, 95%CI = 0.73–3.22, $p = 0.3$).

CSS analysis of GATA3

In Fine Gray regression, GATA3-low status showed insignificant trends towards worse survival in 3 years (HR = 1.85, 95%CI = 0.88–4.88, $p = 0.1$) and 5 years (HR = 1.82, 95%CI = 0.9–3.7, $p = 0.094$) follow-up, but significantly worse CSS in 10 years follow-up (HR = 2.04, 95%CI = 1.03–4.17, $p = 0.039$).

Logistic regression/Pearson's Chi squared test – immunohistochemistry and clinical pathological variables

Complete list of associations between analyzed variables is presented in Supplementary 2 (Table S2).

GATA3-low tumors (negative prognostic feature) were significantly associated with locally advanced (pT3 + 4) tumor stage (Odds ratio/OR = 4.17, 95%CI = 1.54–12.5, $p = 0.002$), lymphatic invasion (OR = 2.17, 95%CI = 2.15–4.76, $p = 0.031$), non-brisk lymphocytic infiltrate (OR = 2.5, 95%CI = 2.5–5.56, $p = 0.01$), HIF-1-α-low status (OR = 3.42, 95%CI = 1.23–11.1, $p = 0.01$), and borderline insignificantly also with p53 mutated profile (OR = 2.17, 95%CI = 0.87–5.54, $p = 0.087$).

IMP3-high status was significantly more frequent in patients with regional lymph node metastases (OR = 4.16, 95%CI = 1.47–13.7, $p = 0.004$), borderline insignificantly more in G3 tumors (OR = 2.05, 95%CI = 0.96–4.50, $p = 0.051$), PD-L1 + tumors (OR = 2.11, 95%CI = 0.88–5.32, $p = 0.075$), and tumor with mutated p53-profiles (OR = 2.17, 95%CI = 0.88–5.88, $p = 0.092$).

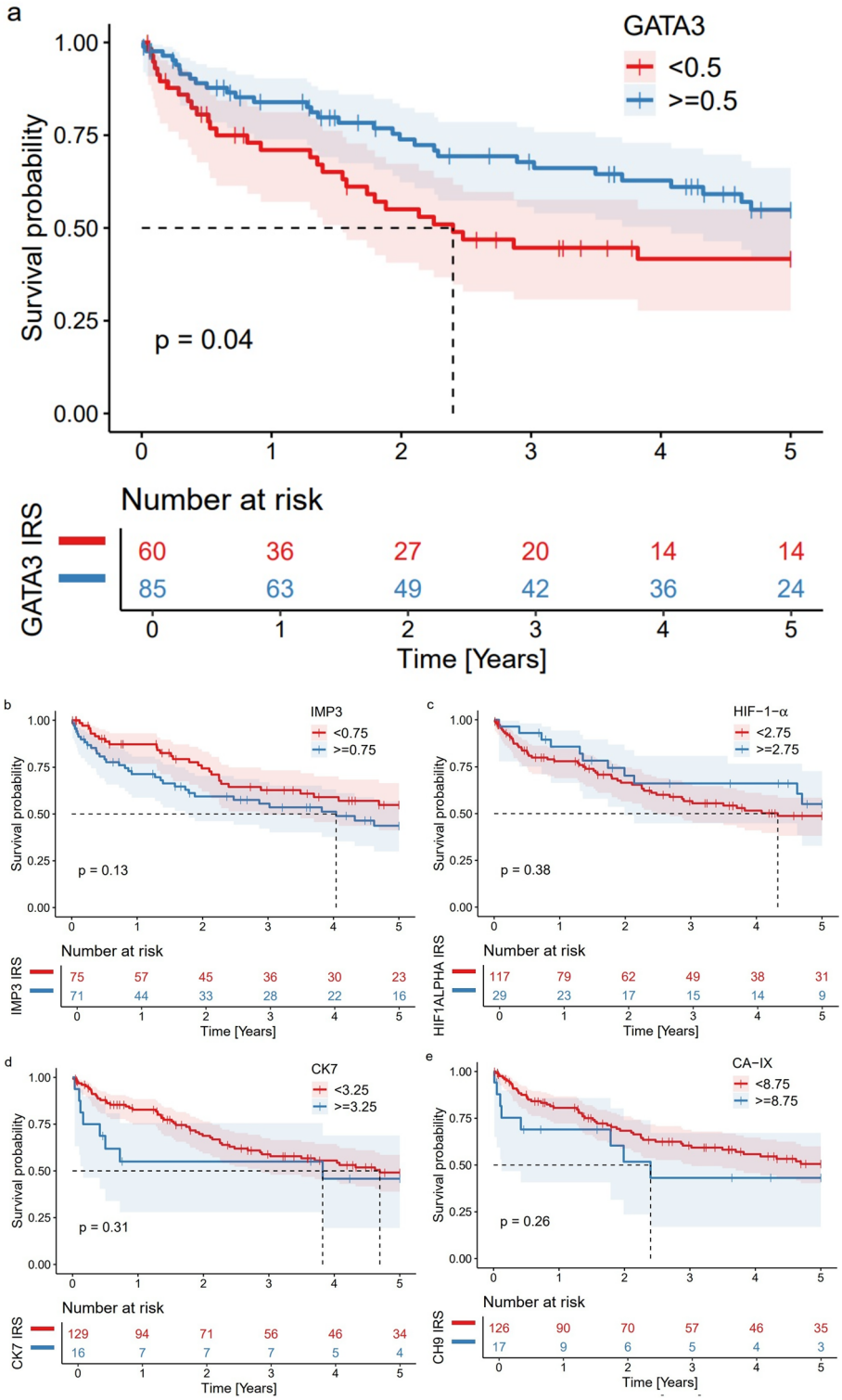

Fig. 2. Kaplan-Meier curves demonstrating the prognostic effects of six immunohistochemistry markers (GATA3, IMP3, HIF-1-α, CK7, CA-IX) on 5 year overall survival (OS) in penile squamous cell carcinoma (pSCC). Note the significant negative impact of GATA3-low status (a), insignificant trends towards worse prognosis in IMP3-high (b), HIF-1-α-low (c), CK7-high (d), and CA-IX-high (e) tumors.

HIF-1-α-high profile was significantly associated with beneficial prognostic parameters - low stage pT1 + 2 (OR = 0.14, 95%CI = 0–0.95.95, $p = 0.028$), low grade G1 + 2 (OR = 0.13, 95%CI = 0.01–0.56, $p = 0.001$), and borderline insignificantly with absent lymphatic (OR = 0.40, 95%CI = 0.11–1.19, $p = 0.11$) and absent venous (OR = 0.30 95%CI = 0.03–1.33, $p = 0.11$) invasion.

Adjusted variables		status	*n*	HR multivariate Cox (95%CI)	*p*
GATA3 + pT stage	GATA3	Low (< 0.5)	60	1.79 (1.01, 3.13)	**0.046**
		High (≥ 0.5)	85		
	pT	pT1 + 2	118	1.80 (0.97, 3.33)	0.063
		pT3 + 4	26		
GATA3 + pN stage	GATA3	Low (< 0.5)	60	2.13 (1.25, 3.70)	**0.006**
		High (≥ 0.5)	85		
	pN	pN0 + pNX	120	2.48 (1.34, 4.59)	**0.004**
		pN1 + pN2 + pN3	25		
GATA3 + grade	GATA3	Low (< 0.5)	60	1.96 (1.14, 3.33)	**0.015**
		High (≥ 0.5)	85		
	Grade	G1 + 2	99	1.70 (0.98, 2.94)	0.06
		G3	46		
GATA3 + lymphatic invasion	GATA3	Low (< 0.5)	60	1.85 (1.06, 3.23)	**0.029**
		High (≥ 0.5)	85		
	Lymphatic invasion	No	99	1.77 (1.01, 3.09)	**0.046**
		Yes	46		
GATA3 + venous invasion	GATA3	Low (< 0.5)	60	1.82 (1.05, 3.13)	**0.032**
		High (≥ 0.5)	85		
	Venous invasion	No	119	2.66 (1.48, 4.79)	**0.001**
		Yes	26		
GATA3 + perineural invasion	GATA3	Low (< 0.5)	60	1.92 (1.12, 3.33)	**0.017**
		High (≥ 0.5)	85		
	Perineural invasion	No	130	2.06 (1.00, 4.24)	**0.049**
		Yes	15		
GATA3 + Lymphocytic infiltrate	GATA3	Low (< 0.5)	60	1.79 (1.02, 3.13)	**0.041**
		High (≥ 0.5)	85		
	Lymphocytic infiltrate	Brisk	59	2.65 (1.37, 5.12)	**0.004**
		Non-brisk + Absent	86		
GATA3 + p16	GATA3	Low (< 0.5)	60	2.0 (1.18, 3.45)	**0.011**
		High (≥ 0.5)	85		
	p16	Block	84	1.42 (0.83, 2.45)	0.2
		Non-block + Negative	61		
GATA3 + p53	GATA3	Low (< 0.5)	60	1.96 (1.12, 3.33)	**0.017**
		High (≥ 0.5)	85		
	p53	Wildtype	117	3.33 (1.89, 5.88)	**< 0.001**
		Mutated	28		
GATA3 + PD-L1	GATA3	Low (< 0.5)	60	2.13 (1.25, 3.70)	**0.006**
		High (≥ 0.5)	85		
	PD-L1	Negative	32	1.52 (0.82, 2.78)	0.2
		1–100%	113		
GATA3 adjusted on patient`s age				1.75 (1.03, 3.03)	**0.04**
Patient`s age adjusted on GATA3				1.05 (1.02, 1.07)	**< 0.001**

Table 3. Multivariate/bimodal overall survival Cox regression adjusting GATA3 on several clinical-pathological variables, truncated at 3 years of follow-up. Significant p values (< 0.05) in bold.

CK7-high status was significantly associated with G3 (OR = 6.06, 95%CI = 1.78–23.9, $p = 0.001$), p16 positivity (OR = 12.5, 95%CI = 1.88-∞, $p = 0.002$), CA-IX-high status (OR = 6.09, 95%CI = 1.52–23.2, $p = 0.005$), and p53 wild type status (HR = ∞, 95%CI = 0-∞, $p = 0.042$).

CA-IX-high status was significantly associated with G3 (OR = 4.9, 95%CI = 1.52–17.5, $p = 0.004$), lymphatic invasion (OR = 4.07, 95%CI = 1.24–14.7, $p = 0.01$), with regional lymph node metastases (OR = 4.14, 95%CI = 1.18–14.0.18.0, $p = 0.013$) and borderline insignificantly with perineural invasion (OR = 3.10, 95%CI = 0.63–12.6, $p = 0.087$), non-brisk lymphocytic infiltrate (OR = 2.58, 95%CI = 0.74–11.5, $p = 0.12$), and p16 positivity (OR = 2.7, 95%CI = 0.77–12.5, $p = 0.12$).

No further associations with $p < 0.15$ were found.

Logistic regression/Pearson's Chi squared test – immunohistochemistry and NGS variables

Complete list of associations between analyzed variables is presented in Supplementary Tablet 2 (Table S2).

GATA3-low status was associated with mutations in *NOTCH1* (OR = 0.45, 95%CI = 0.19–1.06, $p = 0.048$) and *ATM* (OR = 0.12, 95%CI = 0.01–0.60, $p = 0.003$). IMP3-low status has been linked to *HRAS* mutation (OR = 0.09, 95%CI = 0.00–0.66.00.66, $p = 0.008$). CK7-high status was associated with *PIK3CA* mutation (OR = 4.92, 95%CI = 1.40–18.6, $p = 0.005$) but with absent *CDKN2A* mutation (OR = 0, 95%CI = 0–0.87.87, $p = 0.021$). There were no significant associations between CA-IX, HIF-1-α, and genomic variables.

Discussion

This study is a retrospective analysis of several immunohistochemical markers on a relatively large cohort of patients with pSCC ($n = 145$), whose prognostic significance has not yet been described in this context. Although some markers showed borderline trends towards a worse prognosis, only the GATA3-low status reached a p-value below 5% in our survival analysis.

GATA3

GATA binding protein 3 (GATA3) is a zinc-finger transcription factor playing a key role in cell differentiation during development. In the epidermis, GATA3 suppresses cell proliferation and induces expression of differentiation markers[13].

Over the past ten years, GATA3 immunohistochemistry has been widely used by pathologists to identify breast or urothelial origin of cancer[14]. Moreover, GATA3 is expressed in cutaneous adnexal tumors (including basal cell carcinoma), trophoblastic and endodermal sinus tumors[13,15]. Miettinen et al. performed an extensive study on 2500 various tumors in terms of nuclear GATA3 expression, regarding at least 5% of staining tumor cell nuclei positive. In SCC, there was a relatively frequent GATA3 expression: 81% of cutaneous SCC, with a median of 50% tumor cells positive (range, 5–100%), 33% cervical carcinomas (33% of cases, nearly half of them showing positivity in > 50% of tumor cells), 16% laryngeal and 12% pulmonary SCCs. Half of the GATA3-positive examples of the pulmonary tumors and 2 of 8 of the laryngeal ones showed positivity in > 50% of tumor cells[15]. Reportedly, the expression of GATA3 in SCCs varies by site, being frequent in skin tumors[16] and less common in respiratory SCCs[13,17]. Loss of GATA3 has been described as a part of HPV-independent carcinogenesis towards SCC, as a helpful hallmark of differentiated (HPV-negative) vulvar intraepithelial lesion (dVIN) distinguishing this from HPV-associated dysplasia[18], and as a correlate of sun induced skin dysplasia and cancer[19]. Prognostic significance of GATA3 expression varies among various tumors. GATA3 expression has been linked to poorer survival in lung adenocarcinoma[20], pancreatic adenocarcinoma[21], high grade serous ovarian carcinoma[22], and soft tissue sarcomas[23].

In our study on penile cancer, we used immunoreactivity score cut-off value 0.5 (mean from two cores and two pathologists), which counts almost any staining as positive, and there were 85/145 (58%) positive/high cases. The prognostic implications of GATA3 expression in SCC are equivocal. There was a negative prognostic impact of GATA3 expression in esophageal cancer[24]. On the other hand, knockdown of GATA3 stimulated proliferation and invasion in oral SCC cell cultures, whereas anticancer function of *MEG3* was GATA3-dependent[25]. Vaziri Fard et al. described GATA3 loss in 40% of 86 vulvar SCC[26], which is etiologically, morphologically, and prognostically similar to pSCC. In our data, there were 42% of GATA-low (negative) cases. In vulvar SCC, associations between GATA3 loss, HPV-independency, p53 abnormal profile and shorter OS were found. In line with this, our study showed negative prognostic implications of GATA3 loss in terms of shorter survival and association with advanced stage and lymphatic invasion. In vulvar pathology, GATA3 loss seems to be associated with dVIN-associated HPV-independent cancerogenesis, based on the findings of Vaziri Fard et al.[26]. As vulvar SCC is etiologically, morphologically, and prognostically similar to pSCC, analogous association of GATA3 expression with HPV/p16 positivity could be expected. Nevertheless, no association of GATA3 status with p16/HPV profile was found in our study.

IMP3

IMP3 is a member of the RNA-binding protein family, involved in RNA trafficking and stabilization, as well as cell growth and migration during the early stages of embryonic development[27]. Numerous studies have demonstrated that IMP3 is specifically expressed in malignant tumors and functions as an important cancer-specific gene involved in various aggressive and advanced cancers[28]. There is growing evidence that upregulated IMP3 expression in tumor tissues is correlated with poor clinical outcome and can serve as a prognostic factor. Chen et al. performed a robust meta-analysis comprising 53 studies on 8937 patients with a broad spectrum of malignant tumors. High IMP3 expression was significantly associated with shorter OS (HR = 2.08, 95%CI = 1.80–2.42, $p < 0.001$); the prognostic impact was found in the majority of tumor types except for ovarian cancer[29].

Among SCC, there is an association of IMP3 positivity with epithelial dysplasia and carcinoma, together with negative impact on prognosis SCC of the uterine cervix, where the vast majority of dysplasias and carcinomas are HPV-associated[30,31]. Similarly, IMP3 expression signifies poor prognosis in esophageal carcinoma, which is typically HPV-independent[32]. Head and neck SCC (HNSCC) may arise in both HPV-associated and HPV-independent pathways, and IMP3 has been described as negative prognostic marker in several studies, together with IMP3-upregulation in squamous epithelium dysplasia[33–38].

In the first study focusing on IMP3 in pSCC, IMP3-high status showed a borderline insignificant trend towards worse survival in a relatively large cohort. Although IMP3 did not reach statistical significance in the survival analysis, it showed associations with several adverse prognostic features such as lymph node metastases, high grade, and p53-mutated profile, whose negative prognostic role in pSCC has been well documented[9,39–44], together with the role of p53 in epithelial-mesenchymal transition[45] and its association with tumor budding[46], both p53 and tumor budding being strong negative prognostic markers[9]. These findings suggest that IMP3

expression may reflect broader tumor aggressiveness but does not independently predict survival. Its potential role as a surrogate marker of aggressive tumor biology warrants further study.

HIF-1-α

Hypoxia-inducible factors (HIFs) are key regulators of oxygen homeostasis vital for each cell in the human body. Cancer cells utilize HIFs to drive malignant progression. In relation to HIF-1-α prognostic impact, abundant literature is available. Negative prognostic implications of HIF-1-α results were described in meta-analyses on SCC of the esophagus[47,48], head and neck[49,50], and uterine cervix[51].

Notably, findings in our study on HIF-1-α are strikingly discordant with the cited literature. We observed an insignificant tendency to better OS in HIF-1-α-high tumors, and associations with low risk clinical-pathological variables (stage, grade, lymphovascular invasion). The plausible explanation could be that many cited studies lack information regarding HIF-1-α compartmentalization, making it unclear whether the antibody reaction is occurring in the cytoplasm or the nucleus. In uterine cervix SCC, which is etiologically comparable to pSCC, only nuclear HIF-1-α positivity has been associated with a worse prognosis[51]. This has not been observed in our samples. The role of cytoplasmic staining in SCC requires further investigation, but its prognostic value appears to be quite limited in pSCC.

CK7

CK7 is found in various ductal and glandular epithelia; most adenocarcinomas express CK7, except for those originating from the colon, prostate, kidney, thymus, carcinoid tumors, and Merkel cell tumors of the skin[52]. The anomalous expression of CK7 is linked to tumor progression and metastasis, whereas the mechanism is not yet clearly understood[53]. In several cancer types, CK7 (over)expression plays a prognostic role. Approximately 70% of urothelial carcinomas express CK7[54], the overexpression has been linked to advanced stage, poor OS, and shorter disease-free survival[55]. In adenocarcinomas, the negative prognostic implications of abnormal expression have been described in tubo-ovarian high-grade serous carcinoma[56], pancreatic adenocarcinoma[57], and colorectal carcinoma[58,59].

Compared to adenocarcinomas, CK7 expression is less frequent in SCCs. In HNSCC, CK7 positivity has been found in 29%[60], the authors described that CK7 expression is linked to HPV positive status, like we did in this study, but no prognostic impact of CK7 has been found in HNSCC[61,62]. In uterine cervix tumors, which are mostly HPV-dependent, CK7 expression has been described in 57–66% of invasive carcinomas and in 42–100% of dysplasias[63,64], and the CK7 expression has been linked to low grade non-progressive dysplasia[65] and favorable clinical course[64]. Hashiguchi et al. report potential negative prognostic impact of decreased CK7 expression in cervical SCC[66]. On the other hand, Lee et al. suggest CK7 functions as a protector of HPV-induced E7 transcript, thereby, CK7 might be essential for production of proteins for viral replication, and oncogenic transformation. The authors propose CK7 staining as a marker for predicting the physical status of HR HPV and E7 level in SCC and related dysplasias[63].

We present the first description of CK7 prognostic implications in penile cancer, identifying only insignificant trends. In line with studies on cervical SCC, we identified tight associations of CK7 expression with HPV/p16 positivity, CA-IX overexpression, and high grade, whereas most HPV + SCCs with basaloid morphology are evaluated as grade 3. In line with this, there was a negative association between CK7-high status and p53 mutated profile, as the overwhelming majority of HPV/p16 + cases are p53 wild type[9,11]. Products of *TP53* and *CDKN2A* are usually silenced by viral oncoproteins E6 and E7 in HPV-associated pSCCs, and these genes are usually wild type at DNA level[67]. Our result of logistic regression of CK7 and p53 with HR = ∞ shows the absence of CK7 + p53 mutated cases. Although in line with the known negative association of HPV and p53 and novel association of CK7 + with HPV/p16 positivity, the true effect size cannot be reliably determined and further studies in larger cohorts are warranted.

CA-IX

Carbonic anhydrases are metallo-enzymes involved in various pathophysiological processes that require tissue pH regulation. CA-IX, a tumor-associated isoform, is induced by hypoxia and aids tumor cells in adapting to acidosis. Multiple tumor-driving pathways can induce CA-IX expression, which is linked to cancer cell invasion, metastatic properties, stem-like characteristics, and drug resistance. High CA-IX expression has been described as a marker of poor prognosis in breast, lung, ovarian, and bladder carcinomas, but a low expression is a negative prognostic marker in clear cell renal cell carcinoma[68]. A meta-analysis including 147 studies with 24,523 patients suffering from various cancers reported that high CA-IX expression was associated with a worse OS (HR = 1.76, 95%CI = 1.58–1.98, $p < 0.0001$)[69]. Focusing on SCC, upregulated CA-IX has been linked to postoperative recurrence and poorer outcome in oral cavity[70–72], tongue[73], and sinonasal SCC[74]. To the best of our knowledge, only one study aiming to explore the prognostic role of CA-IX specifically in pSCC has been published prior to this article. Zhu et al. analyzed approximately half the number of cases ($n = 73$) compared to our study using an arbitrary cutoff of 10% of positively stained tumor cells. The authors found high expression in 42% cases. The expression was not associated with lymph node metastases, T stage, grade, lymphovascular invasion, Ki-67 expression, and cancer-specific survival, but was associated with p53 expression[75]. Unlike Zhu et al., our study found a lower number of CA-IX-high tumors (11.8%) due to the use of optimal cut-point value. CA-IX-high status was significantly associated with high grade and lymphatic invasion, and borderline significantly with p16/HPV positivity, whereas p16/HPV positivity, CK7-high and CA-IX high profiles were mutually associated. Nevertheless, like Zhu et al., our analysis failed to prove a significant impact on survival rendering just an insignificant trend towards worse OS.

Associations of immunohistochemistry profiles with NGS data

Comparing the immunohistochemistry marker results with previously published NGS data[11], several associations have been found: GATA3-low status was associated with mutations in *NOTCH1* and *ATM*. IMP3-low status was inversely associated with *HRAS* mutation. CK7-high status was positively associated with *PIK3CA* mutation but inversely associated with *CDKN2A* mutation (OR = 0, 95% CI = 0–0.87.87, p = 0.021), indicating absence of CDKN2A mutation in CK7-high cases.

Some of these associations are in line with other results of this study. As already mentioned, CK7 is almost exclusively expressed in HPV/p16 + SCCs, which are characterized by low prevalence of *TP53* and *CDKN2A* mutations (canonical mutations in HPV-independent pSCC cancer pathway)[11,67]. CK7 is a hallmark marker of many adenocarcinomas including breast cancer, with 98% of breast carcinomas showing CK7 positivity. *PIK3CA* mutations are common in estrogen receptor-positive breast cancer (ca. 35%)[76], particularly in luminal subtypes. Also, urothelial carcinoma, which is constitutively positive for CK7[77], displays relatively frequently *PIK3CA* mutation (8–25%)[78], and *CDKN2A* mutation (10–12%)[79]. However, CK7-high status is not specifically correlated with *PIK3CA* and *CDKN2A* mutations in breast cancer and urothelial cancer studies, as CK7 is nearly universally expressed. Also, association of underexpressed IMP3 (correlate of aggressive phenotype in many cancers) and mutated *HRAS* (without prognostic significance in pSCC)[11] has not been reported in the literature. *ATM* and *NOTCH1* mutation occur in ca. 1–7% and 2% of breast cancers, respectively[80–82], both alterations are enriched in triple negative breast cancer[83,84], which is characterized by decreased or absent GATA3 expression[85]. Nevertheless, it is questionable to apply these findings on pSCC, and further studies are warranted.

Limitations

Although it is a cost-effective method, the use of TMAs may represent a potential limiting factor of our study, as the expression of immunohistochemistry markers may vary within the tumor and the area of expression may be potentially missed. However, the same issue concerns small diagnostic biopsy samples in routine practice. For statistical analysis, pNX (clinically cN0) cases were grouped with pN0, acknowledging this as a limitation. In view of the limited number of events and to minimize overfitting, we employed pairwise multivariable models including GATA3 and a single covariate; this approach supports robustness across key factors but does not replace a fully specified joint model adjusting all variables mutually. Larger cohorts are required to confirm GATA3 as a strictly independent prognostic factor.

Conclusion

This study evaluated the prognostic and clinicopathologic relevance of eight immunohistochemical markers in the largest penile cancer cohort to date. Among these, only GATA3 loss demonstrated a statistically significant and independent association with worse overall and cancer-specific survival, as well as correlations with adverse pathological features such as advanced tumor stage and lymphatic invasion. The other markers, including IMP3, CK7, and CA-IX, did not show significant prognostic impact but were associated with relevant tumor characteristics such as high grade, HPV/p16 status, and lymph node metastases, suggesting potential roles in tumor biology. While these findings contribute to the growing understanding of pSCC heterogeneity, they warrant further validation in independent cohorts and mechanistic studies before clinical application.

Data availability

Research data are available in the Supplementary tables, full NGS data are available per email request from the corresponding author.

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Author contributions

JHr contributed to the study design, funding management, cohort acquisition, immunohistochemistry and histopathological analysis, result interpretation, and manuscript writing. ZP was responsible for immunohistochemistry and histopathological analysis. RM was involved in study design, funding management, cohort acquisition, histopathological analysis, result interpretation, and manuscript writing. JHo contributed to the study design, funding management, NGS studies, and interpretation of results. MKB contributed to cohort acquisition and follow-up data acquisition. DČ and NZ were responsible for follow-up data acquisition. PW conducted statistical analysis and contributed to result interpretation. All authors read and approved the final version of the manuscript.

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Declarations

Conflict of interest

The authors state that there are no conflicts of interest to disclose.

Additional information

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