High immunoexpression of Ki67, EZH2, and SMYD3 in diagnostic prostate biopsies independently predicts outcome in patients with prostate cancer

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Abstract

Introduction: Overtreatment is a major concern in patients with prostate cancer (PCa). Prognostic biomarkers discriminating indolent from aggressive disease in prostate biopsy are urgently needed. We aimed to evaluate the prognostic value of Ki67, EZH2, LSD1, and SMYD3 immunoexpression in diagnostic biopsies from a cohort of PCa patients with long term follow-up.

Materials and methods: A series of 189 consecutive prostate biopsies diagnosed with PCa (1997–2001) in a cancer center was included in the study, with follow-up last updated in November 2016. Biopsies were reviewed and graded according to 2016 WHO criteria. Immunohistochemistry was performed in the most representative block. Nuclear staining was assessed using digital image analysis. Study outcomes included disease-specific, disease-free, and progression-free survival. Statistical analysis was tabulated using SPSS version 22.0. Survival curves and hazard ratios (HRs) were estimated using Kaplan-Meyer and Cox-regression models, respectively. Statistical significance was set at $P < 0.05$.

Results: The proportion of patients who completed the study was 177/189 (94%). In univariable analysis, high Ki67, EZH2, and SMYD3 immunoexpression associated with significantly worse disease-specific survival (HR = 1.86, 95% CI: 1.05–3.29; HR = 1.87, 95% CI: 1.10–3.27; HR = 2.68, 95% CI: 1.02–7.92). In multivariable analysis, the 3 biomarkers displayed significantly worse DSS adjusted for CAPRA score (HR = 1.78, 95% CI: 1.01–3.16; HR = 1.93, 95% CI: 1.12–3.32; HR = 2.71, 95% CI: 1.04–7.10). Among patients with low/intermediate risk CAPRA score, high Ki67 immunoexpression identified those more prone to experience disease recurrence (HR = 9.20, 95% CI: 1.27–66.44) and progression (HR = 2.97, 95% CI: 1.05–8.43).

Conclusions: High Ki67, EZH2, and SMYD3 immunoexpression, adjusted for standard clinicopathological parameters, independently predicts outcome in patients with PCa, at diagnosis. This might assist in discriminating indolent from aggressive PCa, improving treatment selection.

Keywords: Biopsy; EZH2; Ki67; Prognosis; Prostate cancer; SMYD3

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1. Introduction

Prostate cancer (PCa) is the most prevalent and the second most common malignant neoplasm in men, comprising 15% of cancers diagnosed worldwide in 2012, with 75% occurring in industrialized countries. It constitutes the fifth cause of cancer-related death in males, accounting for 6.6% of the total. More than 1.8 million new cases are expected in 2030, about 758,000 more than in 2012 [1]. In the United States, it is estimated that 12.9% of men will be diagnosed PCa during their lifetime [2,3].

PCa diagnosis generally requires histological examination of prostate biopsy cores, allowing for evaluation of parameters that predict disease aggressiveness, including grading. Gleason score (GS) has been the mainstay of PCa grading for over 4 decades [4], despite subsequent refinements [5] and introduction of grade groups (GG) [6], recently adopted by the newest WHO (World Health Organization) classification [7]. Its ability to prognostically stratify patients with PCa was validated in large cohorts, both in radical prostatectomy (RP) and prostate biopsy specimens, considering relapse-free [8] or disease-specific [9] survival (DSS) as endpoints.

Owing to disseminated serum prostate-specific antigen (PSA) screening, 80% of patients with PCa are diagnosed with organ-confined disease, entailing excellent 5-year survival [3]. Given the 5-fold difference between incidence and mortality, choosing optimal treatment and avoiding overtreatment constitutes a major challenge. Standard clinicopathological parameters, however, cannot accurately differentiate indolent from aggressive disease and, thus, discovery and validation of biomarkers that better predict disease behavior and assist clinicians in treatment tailoring, is imperative. Notwithstanding, most biomarkers reported have been difficult to validate, with most studies focusing on RP specimens and very few analyzing cohorts of patients managed conservatively [10–12]. As diagnostic biopsies represent the whole spectrum of PCa and allow for assessment before therapeutic decision, validation of biomarkers that may accurately distinguish clinically insignificant from curable and from lethal disease is mandatory.

Although molecular pathology has thrived in recent years, immunohistochemistry remains a major ancillary tool in pathology owing to its widespread availability. Immunohistochemistry-based biomarkers offer a significant advantage for universal, fast and reliable transference to clinical routine. Despite the plethora of reported immunohistochemical biomarkers with potential for PCa prognostication, however, none has reached clinical use, mostly owing to lack of reproducibility of evaluation procedures [10–14]. Herein, using a cohort of pretherapeutic PCa biopsies, we sought to evaluate the prognostic value of 4 biomarkers previously shown to have clinical potential, including Ki67 [10–14], and a panel of epigenetic modifiers—EZH2, LSD1, and SMYD3—that we and others have shown to convey prognostic information and allow for discrimination of aggressive PCa among patients submitted to RP [15–21]. To increase the accuracy and consistency of results, evaluation of nuclear biomarkers was performed using a digital image analysis system, and cutoffs were determined based on data distribution.

2. Materials and methods

2.1. Patients and samples

Prostate biopsies from patients consecutively diagnosed with PCa at Portuguese Oncology Institute of Porto (IPO Porto, a tertiary healthcare institution) from 1997 to 2001 were enrolled after informed consent. All slides were reviewed by 2 uropathology dedicated pathologists and graded according to 2016 WHO criteria. Percentage of tumor in biopsy and number of involved cores were also recorded. A representative block (the one representing the highest GS) was selected for immunohistochemistry. When several cores in different blocks depicted the same prognostic GS, the one with greatest amount of tumor was selected for analysis.

Clinical data was retrieved from patients' charts (dates of birth, diagnosis, treatment start, biochemical recurrence (BCR), and death; serum PSA at diagnosis; clinical stage (CS); treatments performed; evidence of BCR or clinical recurrence/progression; last follow-up date; and vital status). Follow-up was updated as of 30th November, 2016. CAPRA score was calculated for each patient as a tool for risk assessment [22]. BCR/progression was considered as previously defined [23]. Patient death was rendered as PCa-related (death from disease) when patients died owing to progression/metastasized disease or in the sequence of treatments performed, or as PCa-unrelated (including either death with disease or death with no evidence of disease).

2.2. Immunohistochemistry and image analysis

Immunohistochemistry was performed using Novolink Max Polymer Detection System (Leica Biosystems, Germany). Sections (3μm-thick) were cut and microwaved for 20 to 30 minutes in EDTA buffer at 800 W for antigen retrieval.

Endogenous peroxidase was blocked through incubation in hydrogen peroxide in 3% methanol for 30 minutes. Primary monoclonal antibodies for Ki67 (MIB-1, DAKO, Denmark), EZH2 (clone 6A10, Novocastra, UK), LSD1 (clone 1b2f2, Novus Biologicals, UK), and SMYD3 (Active Motif, Carlsbad, CA) were used at 1:100, 1:1,000, 1:1,750, and 1:300 dilution in 1% PBS-BSA, respectively, and incubated overnight at 4°C. 3,3′-diaminobenzidine (Sigma-Aldrich, Germany) was used for visualization and hematoxilin for nuclear counterstaining. Appropriate positive controls were used for each antibody and negative control consisted on omission of primary antibodies. Because all cases analyzed contained nonneoplastic prostatic epithelium, this served as (internal) negative control as appropriate. However, when the immunohistochemical protocols were developed, negative external controls were used.

SMYD3 immunoexpression was assessed as previously described [15,16]. Digital image analysis system (GenASIs, Israel) was used for Ki67, EZH2, and LSD1 nuclear immunostaining quantification. Nuclei were considered positive if any staining was present, independently of intensity. Study protocol is outlined in detail in Fig. 1.

2.3. Statistical analysis

For analysis purposes, patients were divided into 3 CS groups (stage I/II, stage III, and stage IV) and 2 major GG subgroups (GG 1–3 and 4–5). Patients were also divided into low/intermediate (<6) and high risk (≥6) CAPRA score. SMYD3 immunoexpression was categorized as weak, moderate, or strong. Cutoffs for Ki67, EZH2, and LSD1 nuclear immunostaining (high vs. low) were set at respective 75th percentile (P75). Diagnostic performance of other Ki67 cutoff values reported [12,24–32] was also assessed. Association between biomarkers' distribution and GG or CS was evaluated using chi-square test. Distribution of continuous variables between groups was compared using nonparametric tests (Mann-Whitney or Kruskall-Wallis, as appropriate). Survival curves were constructed using Kaplan-Meyer nonparametric estimator. Survival between groups was compared using log-rank test. Hazard ratios (HR) and respective 95% CI were estimated using Cox-regression models. Statistical significance was set at $P < 0.05$. Statistical analysis was performed using SPSS Statistics for Windows, version 22.0 (SPSS, Chicago, IL).

3. Results

3.1. Cohort characterization and biomarker immunoexpression

A total of 189 patients were enrolled and their clinicopathological features are depicted in Table 1. Most patients displayed high GS and GG, advanced disease stage, and high-risk CAPRA score.

Illustrative examples of Ki67, EZH2 and LSD1 (nuclear), and SMYD3 (cytoplasmic) immunostaining are shown in Fig. 2. Digital imaging-assisted immunoexpression assessment of nuclear biomarkers took approximately 5 minutes per case. The median number of tumor cells and of 200x fields evaluated per case, as well as respective P75

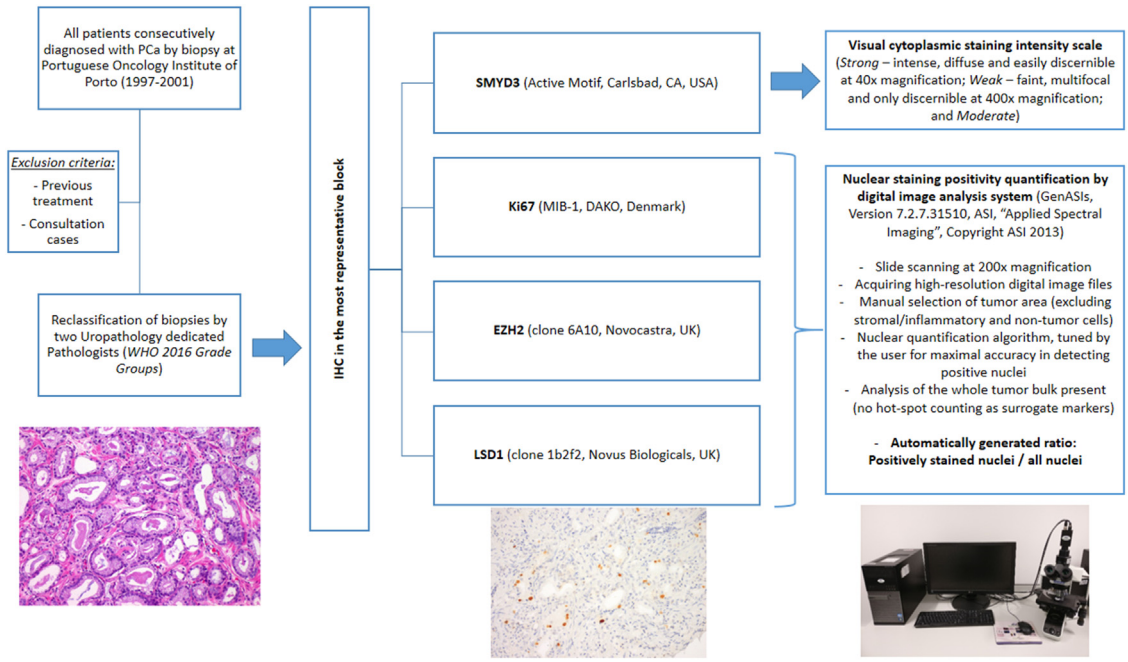

Fig. 1. Study methodology outline. EZH2 = enhancer of zeste homolog 2; IHC = immunohistochemistry; LSD1 = lysine-specific demethylase 1; PCa = prostate cancer; SMYD3 = SET and MYND domain-containing protein 3; WHO = World Health Organization.

Table 1
Clinicopathological features of patients with prostate cancer

Age at diagnosis (y, median [IQR])	72 (67–76)
PSA at diagnosis (ng/ml, median [IQR])	43.0 (15.8–124.0)
Gleason grades, biopsy	
3 + 3	25/189 (13.2%)
3 + 4	33/189 (17.5%)
4 + 3	45/189 (23.8%)
4 + 4	68/189 (36.0%)
4 + 5	14/189 (7.4%)
5 + 4	3/189 (1.6%)
5 + 5	1/189 (0.5%)
Prognostic grade groups, biopsy	
Group 1	25/189 (13.2%)
Group 2	33/189 (17.5%)
Group 3	45/189 (23.8%)
Group 4	68/189 (36.0%)
Group 5	18/189 (9.5%)
Percentage of tumor, biopsy	
<25%	52/189 (27.5%)
<50%	40/189 (21.2%)
<75%	59/189 (31.2%)
≥75%	38/189 (20.1%)
Clinical stage	
Stage I	3/188 (1.6%)
Stage II	58/188 (30.8%)
Stage III	71/188 (37.8%)
Stage IV	56/188 (29.8%)
CAPRA score groups	
<6 (low/intermediate risk)	45/189 (23.8%)
≥6 (high risk)	144/189 (76.2%)
Primary treatment	
RP ± others	16/187 (8.6%)
RT alone or with ADT	45/187 (24.0%)
ADT only	123/187 (65.8%)
WW	3/187 (1.6%)
Ki67	
P75 (cutoff value)	5.33%
Tumor cell count/case (median [IQR])	2,630 (1,198–5,124)
200x fields/case (median [IQR])	8 (4–11)
EZH2	
P75 (cutoff value)	12.40%
Tumor cell count/case (median [IQR])	2,856 (1,195–5,346)
200x fields/case (median [IQR])	9 (5–12)
LSD1	
P75 (cutoff value)	41.40%
Tumor cell count/case (median [IQR])	3,160 (1,204–5,260)
200x fields/case (median [IQR])	8 (5–12)
SMYD3	
Weak	25/178 (14.0%)
Moderate	95/178 (53.4%)
Strong	58/178 (32.6%)

IQR = interquartile range; P75 = 75th percentile for the percentage of positively stained nuclei; RT = radiation therapy; WW = watchful waiting.

value for nuclear biomarkers are provided in Table 1. PCa cases displaying Ki67, EZH2, or LSD1 immunostaining above P75, as well as those with strong SMYD3 cytoplasmic immunoexpression were designated "Ki67-high," "EZH2-high," "LSD1-high," and "SMYD3-high," respectively, for subsequent analyses.

Patients' age associated with GG ($P = 0.003$), and serum PSA at diagnosis with both GG and CS ($P < 0.001$ for both) (Table 2). Significant association between Ki67, EZH2, LSD1, and SMYD3 immunoexpression, and GG, but not CS, was found ($P = 0.001$, $P = 0.003$, $P = 0.005$, and $P = 0.004$, respectively). Serum PSA showed no association with Ki67, EZH2, LSD1, and SMYD3 immunoexpression ($P = 0.750$, $P = 0.184$, $P = 0.837$, and $P = 0.070$).

3.2. Survival analyses

Complete follow-up data was available for 177/189 (94%) patients and median follow-up time was 206 months (range: 0.6–232 mo). A total of 65 (34.4%) patients died from PCa and 133 (70.4%) displayed BCR/progression. Both GG (Fig. 3A) and CS (Fig. 3B) stratified patients in respect to DSS ($P < 0.001$ for both).

3.2.1. Univariable analysis

3.2.1.1. Considering the whole cohort. Patients with SMYD3 strong and moderate immunoexpression experienced significantly worse overall survival (OS) compared to patients with weak expression (strong: HR = 2.19, 95% CI: 1.21–3.97; moderate: HR = 1.91, 95% CI: 1.08–3.39).

Also, Ki67- and EZH2-high patients with PCa experienced significantly worse DSS (Ki67: HR = 1.86, 95% CI: 1.05–3.29; EZH2: HR = 1.87, 95% CI: 1.10–3.27). No significant effect on DSS was found for LSD1 immunoexpression (HR = 0.94, 95% CI: 0.50–1.75). SMYD3-high cases were also more likely to die from PCa compared to weak immunostaining (HR = 2.68, 95% CI: 1.02–7.92). Such association was not found for moderate vs. weak immunostaining (HR = 1.97, 95% CI: 0.76–5.08) (Fig. 4).

Considering patients treated with curative intent (RP or radiation therapy), DSS, and OS analyses are not possible owing to the limited number of events.

3.2.1.2. Considering only androgen deprivation therapy treated patients. Ki67-high patients experienced significantly worse DSS (Ki67: HR = 1.91, 95% CI: 1.05–3.47), but not those with EZH2-high (HR = 1.45, 95% CI: 0.83–2.55), LSD1-high (HR = 0.67, 95% CI: 0.35–1.32), or SMYD3 strong immunoexpression (HR = 1.57, 95% CI: 0.54–4.55).

3.2.2. Nonstratified multivariable analysis

3.2.2.1. Considering the whole cohort. Patients with SMYD3 strong and moderate immunoexpression had significantly worse OS adjusted for CAPRA score (strong: HR = 2.22, 95% CI: 1.22–4.02; moderate: HR = 1.90, 95% CI: 1.07–3.37).

Ki67-high, but not EZH2-high or SMYD3-high, patients had a significantly worse DSS adjusted for patient age, CS and GG (Ki67: HR = 1.91, 95% CI: 1.01–3.57; EZH2: HR = 1.01, 95% CI: 0.56–1.80; SMYD3: HR = 0.92, 95% CI: 0.54–1.57). However, both Ki67 and EZH2-high cases displayed significantly worse DSS adjusted for CAPRA

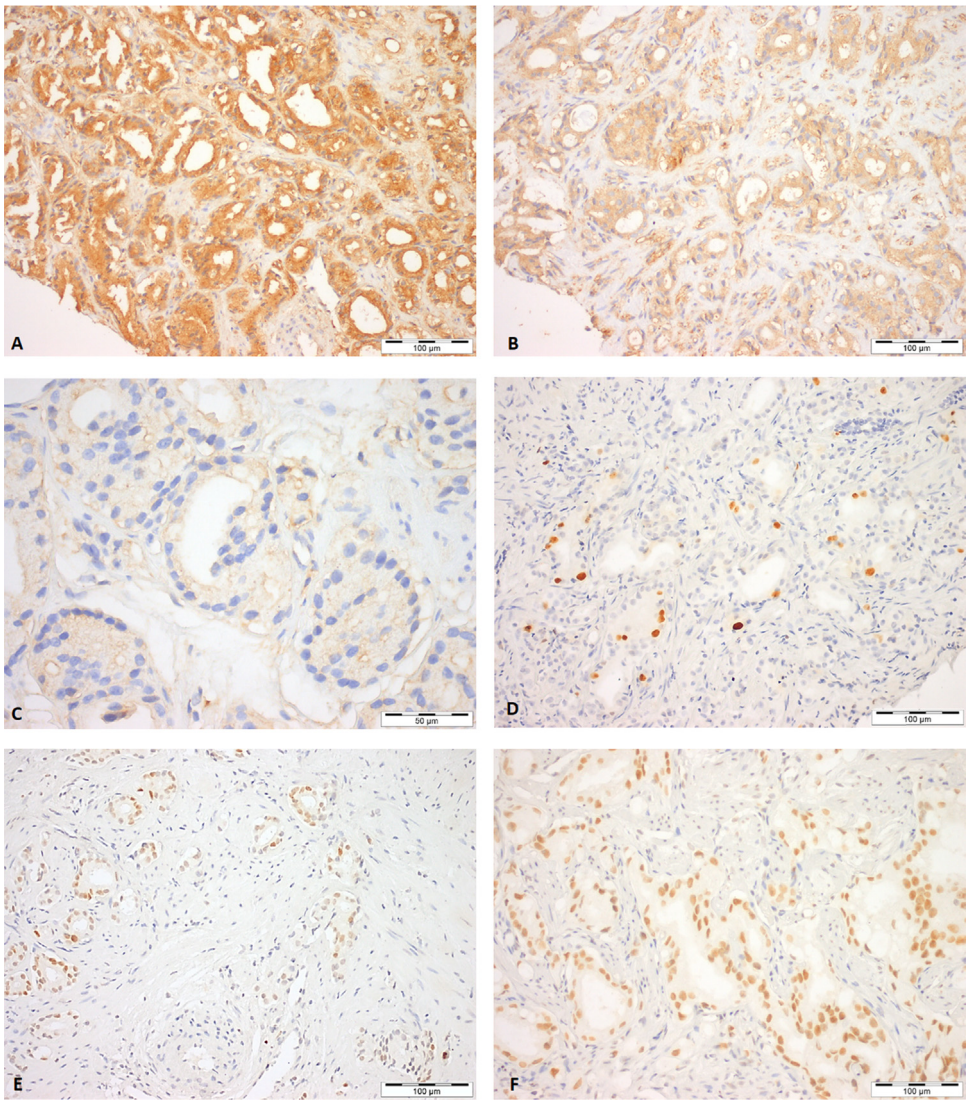

Fig. 2. Immunohistochemical expression of biomarkers in prostate cancer biopsies. (A–C) SMYD3 cytoplasmic staining (A) Strong intensity staining (×200); and (B) moderate intensity staining (×200); and (C) weak intensity staining (×400). (D) Ki67 nuclear staining (×200); (E) EZH2 nuclear staining (×200); (F) LSD1 nuclear staining (×200).

score (Ki67: HR = 1.78, 95% CI: 1.01–3.16; EZH2: HR = 1.93, 95% CI: 1.12–3.32). The same was apparent for SMYD3-high patients compared to weak SMYD3 immunostaining, adjusted for CAPRA score (HR = 2.71, 95% CI: 1.04–7.10).

3.2.2.2. Considering only androgen deprivation therapy treated patients. Ki67-high patients experienced significantly worse DSS when adjusting for patient age, CS and GG (HR = 2.27, 95% CI: 1.17–4.39), which did not occur for the other biomarkers (EZH2: HR = 0.94, 95% CI: 0.52–1.72; SMYD3: HR = 0.82, 95% CI: 0.27–2.52). When adjusting for CAPRA score patients with Ki67-high also showed significantly worse DSS (HR = 1.89, 95% CI: 1.04–3.45); the same was not disclosed for EZH2 or SMYD3-high cases (EZH2: HR = 1.46, 95% CI: 0.83–2.57; SMYD3: HR = 1.59, 0.55–4.60).

3.2.3. Stratified multivariable analysis

3.2.3.1. Considering the whole cohort. Among low/intermediate (but not high) risk CAPRA score, Ki67-high patients were significantly more likely to die from PCa (HR = 4.53, 95% CI: 1.36–15.10 and HR = 1.40, 95% CI: 0.74–2.69, respectively) (Table 3). Moreover, among GG 4 to 5, Ki67-high patients were significantly more likely to die from PCa when adjusted for patients' age and CS (HR = 2.18, 95% CI: 1.09–4.38). Also, in GG 1 to 3 patients, EZH2-high cases displayed significantly worse DSS adjusted for patients' age and CS (HR = 3.66, 95% CI: 1.15–11.60).

3.2.3.2. Concerning only patients treated with curative intent (RP or radiation therapy). Low/intermediate (but not high) risk CAPRA score patients with Ki67-high were significantly more prone to experience disease recurrence

Table 2
Association between clinical data and biomarker immunoexpression

Grade groups				
	Groups 1–3	Groups 4–5		*P* value
Age (y, median [IQR])	71 (66–75)	74 (70–78)		0.003
PSA (ng/ml, median [IQR])	21.7 (11.6–64.5)	93.2 (38.6–253.9)		<0.001
Biomarkers (*n*, %)				
Ki67				0.001
<P75	71/82 (86.6%)	48/76 (63.2%)		
≥P75	11/82 (13.4%)	28/76 (36.8%)		
EZH2				0.003
<P75	74/87 (85.1%)	50/78 (64.1%)		
≥P75	13/87 (14.9%)	28/78 (35.9%)		
LSD1				0.005
<P75	70/82 (85.4%)	50/77 (64.9%)		
≥P75	12/82 (14.6%)	27/77 (35.1%)		
SMYD3				0.004
Weak	20/100 (20.0%)	5/78 (6.4%)		
Moderate	56/100 (56.0%)	39/78 (50.0%)		
Strong	24/100 (24.0%)	34/78 (43.6%)		
Clinical stage groups				
	Stage I/II	Stage III	Stage IV	
Age (y, median [IQR])	71 (66–75)	74 (68–77)	73 (69–78)	0.06
PSA (ng/ml, median [IQR])	14.0 (8.8–23.2)	43 (21.2–89.0)	204.0 (93.5–953.0)	<0.001
Biomarkers (*n*, %)				
Ki67				0.18
<P75	43/51 (84.3%)	43/61 (70.5%)	32/45 (71.1%)	
≥P75	8/51 (15.7%)	18/61 (29.5%)	13/45 (28.9%)	
EZH2				0.12
<P75	45/53 (84.9%)	45/63 (71.4%)	33/48 (68.7%)	
≥P75	8/53 (15.1%)	18/63 (28.6%)	15/48 (31.3%)	
LSD1				0.25
<P75	41/51 (80.4%)	42/61 (68.9%)	37/46 (80.4%)	
≥P75	10/51 (19.6%)	19/61 (31.1%)	9/46 (19.6%)	
SMYD3				0.42
Weak	9/57 (15.8%)	12/69 (17.4%)	4/51 (7.8%)	
Moderate	33/57 (57.9%)	34/69 (49.3%)	27/51 (53.0%)	
Strong	15/57 (26.3%)	23/69 (33.3%)	20/51 (39.2%)	

IQR = interquartile range; P75 = 75th percentile for the percentage of positively stained nuclei.

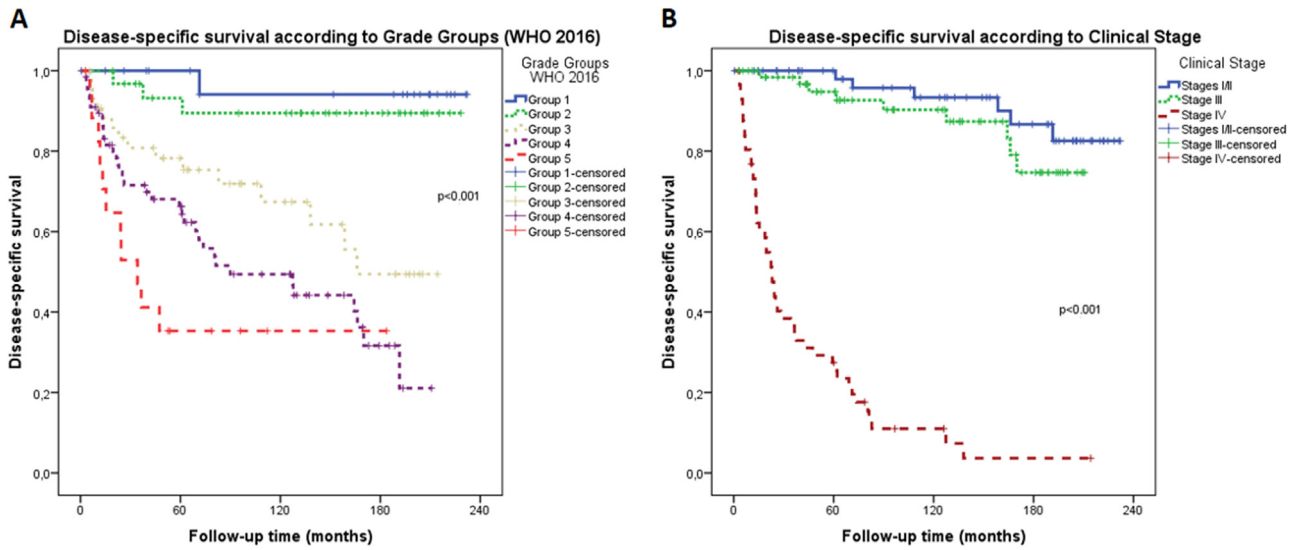

Fig. 3. Cohort stratification according to WHO grade groups and clinical stage, concerning disease-specific survival. (A) Disease-specific survival according to WHO grade groups. (B) Disease-specific survival according to clinical stage. WHO = World Health Organization.

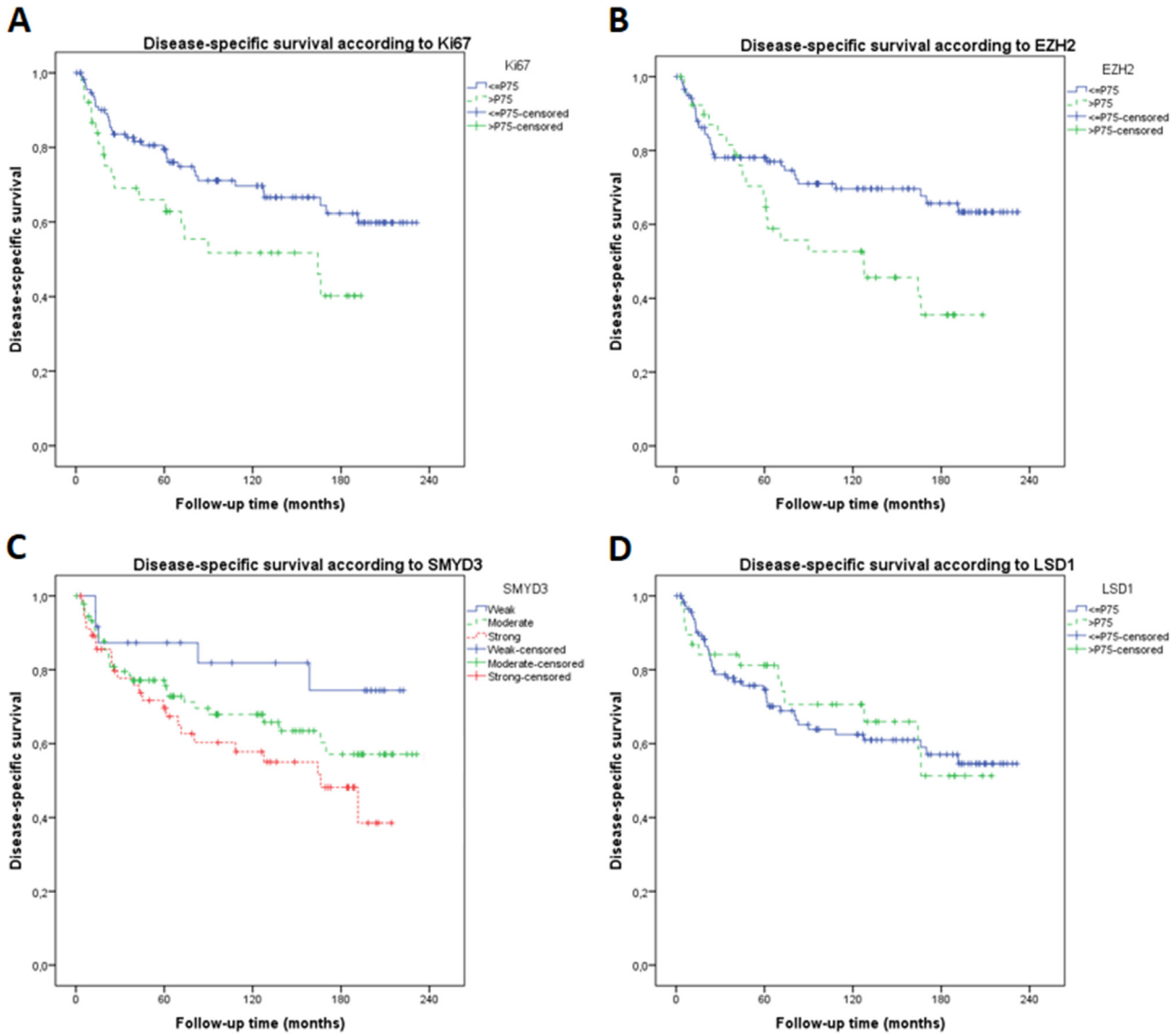

Fig. 4. Disease-specific survival according to biomarker expression levels: (A) Ki67, (B) EZH2, (C) SMYD3, and (D) LSD1.

(HR = 9.20, 95% CI: 1.27–66.44 and HR = 1.39, 95% CI: 0.43–4.52, respectively).

3.2.3.3. Concerning only androgen deprivation therapy treated patients. Low/intermediate (but not high) risk CAPRA score patients with Ki67-high were significantly more prone to experience disease progression (HR = 2.97, 95% CI: 1.05–8.43 and HR = 1.12, 95% CI: 0.64–1.97, respectively).

3.3. Prognostic performance of Ki67 cutoffs reported in literature in this cohort

The prognostic performance of other Ki67 cutoff values previously reported in literature was tested in our cohort (Table 4). Only 10% cutoff revealed a significant association with DSS both in univariable analysis (HR = 2.32, 95% CI: 1.16–4.65) and adjusted for CAPRA score (HR = 2.25, 95% CI: 1.12–4.51), whereas 5% cutoff only associated with DSS in univariable analysis.

4. Discussion

Growing evidence concerning PCa overdiagnosis and overtreatment emphasizes the need for accurate disease aggressiveness stratification. Owing to limitations of standard clinicopathological parameters, there is an unmet need for novel biomarkers that may assist clinicians in deciding therapeutic strategy. Although many studies have reported promising candidate biomarkers, few have stood the test of reproducibility and none has effectively reached clinical routine. Thus, based on our previous experience in epigenetic-based biomarkers and taking into consideration the

Table 3
Multivariable analysis concerning disease-specific survival, disease-free survival, and progression-free survival

	CAPRA risk score		Prognostic grade groups[a]	
	Low CAPRA HR (95% CI)	High CAPRA HR (95% CI)	Groups 1–3 HR (95% CI)	Groups 4–5 HR (95% CI)
DSS (all patients)				
Biomarker				
Ki67				
<P75	1	1	1	1
≥P75	4.53 (1.36–15.10)[b]	1.40 (0.74–2.69)	2.13 (0.44–10.20)	2.18 (1.09–4.38)[b]
EZH2				
<P75	1	1	1	1
≥P75	2.57 (0.82–7.99)	1.76 (0.94–3.29)	3.66 (1.15–11.6)[b]	0.72 (0.37–1.40)
SMYD3				
Weak	1	1	1	1
Moderate	2.19 (0.27–17.86)	1.86 (0.64–5.38)	1.55 (0.32–7.44)	0.50 (0.14–1.79)
Strong	2.27 (0.27–19.51)	2.84 (0.97–8.33)	3.95 (0.76–20.70)	0.40 (0.11–1.45)
DFS (patients treated with curative intent)				
Biomarker				
Ki67				
<P75	1	1	1	1
≥P75	9.20 (1.27–66.44)[b]	1.39 (0.43–4.52)	2.95 (0.75–11.58)	0.24 (0.02–3.60)
EZH2				
<P75	1	1	1	1
≥P75	0.98 (0.11–8.38)	2.62 (0.81–8.47)	1.47 (0.32–6.87)	4.15 (0.52–33.07)
SMYD3				
Weak	1	1	–	–
Moderate	0.99 (0.10–9.63)	0.52 (0.16–1.64)	1[c]	1[c]
Strong	2.44 (0.22–27.15)	0.91 (0.29–2.85)	3.04 (0.96–9.62)	0.18 (0.01–2.65)
PFS (ADT treated patients)				
Biomarker				
Ki67				
<P75	1	1	1	1
≥P75	2.97 (1.05–8.43)[b]	1.12 (0.64–1.97)	1.22 (0.36–4.16)	1.29 (0.71–2.32)
EZH2				
<P75	1	1	1	1
≥P75	1.03 (0.43–2.47)	1.09 (0.64–1.84)	2.24 (0.81–6.17)	0.66 (0.38–1.15)
SMYD3				
Weak	1	1	1	1
Moderate	2.97 (0.37–23.60)	0.95 (0.44–2.05)	1.86 (0.62–5.57)	0.52 (0.19–1.40)
Strong	2.47 (0.31–19.79)	0.85 (0.38–1.92)	1.94 (0.56–6.69)	0.40 (0.15–1.09)

ADT = androgen deprivation therapy; DFS = disease-free survival; DSS = disease-specific survival; PFS = progression-free survival.
[a]Adjusted for patient age and clinical stage.
[b]Values with statistical significance.
[c]Reference category: SMYD3 weak/moderate.

most promising and scrutinized candidates [10–21], we sought to investigate, in a diagnostic prostate biopsy cohort, whether immunoexpression assessment, assisted by digital imaging analysis, might provide prognostically relevant information for patients with PCa, discriminating indolent from aggressive disease, in a pretherapeutic setting.

Our study is based on a series of 189 consecutive diagnostic prostate biopsies from a single tertiary hospital, encompassing the full spectrum of clinically managed PCa, as denoted by the different treatment strategies depicted. Interestingly, only a small proportion of patients were submitted to RP, which has been used as the main setting for validation of PCa biomarkers [10–12]. Thus, a prostate biopsy cohort broadens the biological and clinical features of PCa under evaluation, although at the cost of increased heterogeneity. Nevertheless, CS and GG allowed for statistically significant stratification of patients concerning DSS, validating the series, and notwithstanding the relatively limited size, our study further demonstrates the clinical value of GG, irrespective of treatment strategy, thus complementing previous reports [8,9] as it includes, for the first time, patients submitted to androgen deprivation therapy (ADT).

We found that PCa cases with high Ki67 expression displayed significantly worse DSS, both in univariable and multivariable analysis, in line with some previous findings in RP and prostate biopsy series [10–14]. Remarkably, Ki67 was the only tested biomarker that significantly associated with disease recurrence and progression, both in low/intermediate risk CAPRA score groups. Thus, quantitative Ki67 immunoexpression adds to existing PCa prognostication tools, providing independent information, in a

Table 4
Performance of various cutoffs mentioned in literature for Ki67 percentage nuclear expression in our cohort

Study	*N*	Date of diagnosis	Median follow-up (months)	Ki67 cutoff, %	HR (95% CI)
DSS—univariable analysis					
Stattin et al.[a] [25]	125	1975–1983	71	3	1.27 (0.73–2.21)
Pollack et al.[b] [31]	106	1987–1993	62	3.5	1.33 (0.76–2.34)
Li et al.[a,b] [32]	108	1997–2003	72		
Miyake et al.[f] [30]	193	1997–2003	63	5	1.77 (1.001–3.14)[c]
Berney et al.[a] [29]	693	1990–1996	117		
Gunia et al.[f] [28]	528	1996–2003	46.4		
Rubio et al.[b,f] [27]	91	1997–2001	46.5		
Bubendorf et al.[f] [24]	137	1978–1993	64.8	7.5	1.83 (0.96–3.48)
Zellweger et al.[b] [12]	279	1996–2005	16	10	2.32 (1.16–4.65)[c]
Vis et al.[f] [26]	92	1980–1988	112.8		
Lobo et al.	189	1997–2001	206	5.33	1.86 (1.05–3.29)[c]
DSS—multivariable analysis[d]					
Stattin et al.[a] [25]	125	1975–1983	71	3	1.24 (0.67–2.29)
Pollack et al.[b] [31]	106	1987–1993	62	3.5	1.28 (0.69–2.38)
Li et al.[a,b] [32]	108	1997–2003	72		
Miyake et al.[f] [30]	193	1997–2003	63	5	1.39 (0.74–2.59)
Berney et al.[a] [29]	693	1990–1996	117		
Gunia et al.[f] [28]	528	1996–2003	46.4		
Rubio et al.[b,f] [27]	91	1997–2001	46.5		
Bubendorf et al.[f] [24]	137	1978–1993	64.8	7.5	2.00 (0.997–3.99)
Zellweger et al.[b] [12]	279	1996–2005	16	10	1.88 (0.91–3.89)
Vis et al.[f] [26]	92	1980–1988	112.8		
Lobo et al.	189	1997–2001	206	5.33	1.91 (1.01–3.57)[c]
DSS—multivariable analysis[e]					
Stattin et al.[a] [25]	125	1975–1983	71	3	1.23 (0.70–2.14)
Pollack et al.[b] [31]	106	1987–1993	62	3.5	1.27 (0.72–2.24)
Li et al.[a,b] [32]	108	1997–2003	72		
Miyake et al.[f] [30]	193	1997–2003	63	5	1.70 (0.95–3.01)
Berney et al.[a] [29]	693	1990–1996	117		
Gunia et al.[f] [28]	528	1996–2003	46.4		
Rubio et al.[b,f] [27]	91	1997–2001	46.5		
Bubendorf et al.[f] [24]	137	1978–1993	64.8	7.5	1.77 (0.93–3.38)
Zellweger et al.[b] [12]	279	1996–2005	16	10	2.25 (1.12–4.51)[c]
Vis et al.[f] [26]	92	1980–1988	112.8		
Lobo et al.	189	1997–2001	206	5.33	1.78 (1.01–3.16)[c]

DSS = disease-specific survival.
[a]Biomarker analysis in transurethral resection specimens.
[b]Biomarker analysis in biopsy specimens.
[c]Values with statistical significance.
[d]Adjusted for patient age, clinical stage, and grade groups.
[e]Adjusted for CAPRA score.
[f]Biomarker analysis in radical prostatectomy specimens.

subgroup of patients which are at risk to endure overtreatment. In this setting, Ki67 assessment might assist in discriminating indolent from more aggressive PCa among low-risk patients, eventually identifying those that will mostly benefit from therapeutic intervention. Interestingly, results of immunohistochemical analysis of a single marker of cell proliferation—Ki67—seem to compare well with those of a commercially available cell-cycle progression score (Prolaris), a molecular test that evaluates RNA signature of several cell-cycle progression genes [33,34]. If confirmed in larger and independent series, Ki67 immunoexpression might provide an easier and less expensive method for widespread risk stratification of patients with PCa at diagnosis.

Several previous studies have indicated Ki67 as a promising prognostic biomarker for PCa [12,24–32]. Nevertheless, there is considerable heterogeneity among studies, including different cutoffs (most standing in the 5%–10% interval), cohort types (mostly RP specimens), sample sizes and endpoints, precluding a definitive judgment and translation to the clinics. Hence, we sought to determine the prognostic performance of the various Ki67 cutoffs in our series, using DSS as endpoint. Remarkably, and in addition to our P75 cutoff (Ki67 index of 5.33%), only the 10%

cutoff reported by Zellweger et al. [12] revealed significant association with worse DSS, both in univariable and multivariable analysis. Interestingly, this is one of the few studies based on a prostate biopsy cohort. It should also be emphasized that, in our study, a more accurate and potentially more reproducible method for Ki67 assessment was used (digital image immunoscorer) and the Ki67 cutoff was defined based on the statistical distribution of results (P75) instead of empirically assigned values.

Owing to our previous experience in PCa epigenetic-based biomarkers, immunoexpression of 3 histones' modifying enzymes—EZH2, LSD1, and SMYD3—was also assessed. Whereas LSD1, previously shown to associate with risk of disease recurrence [18], did not confirm its potential, high EZH2 and SMYD3 immunoexpression predicted PCa-related death, both in univariable and multivariable analysis adjusted for CAPRA score. Moreover, in lower GG, high EZH2 immunoexpression predicted worse DSS, adjusted for patients' age and CS. These results are in line with previous reports and significantly extend their reach as they were mostly based on RP cohorts [15,16,20,21,35,36]. Additionally, they are biologically sound, as EZH2 is part of the polycomb complex group 2, regulating genes involved in development and cell cycle progression by interacting both with histone and nonhistone proteins, functioning either as a transcriptional activator or repressor [20,21,35], whereas SMYD3 is a methyltransferase that upregulates the androgen receptor, being involved in transcriptional regulation, either repressing tumor suppressor genes or inducing oncogene expression [15,16,36]. Importantly, both EZH2 and SMYD3 constitute potential therapeutic targets in PCa and, thus, expression assessment might also be predictive of response to targeted therapy.

Major limitations of our study include its retrospective nature, that may potentially bias the results, cohort size, making subgroup analysis for disease-free survival and progression-free survival difficult, treatment heterogeneity and the use of cases dating from 1997 to 2001, implying that sextant biopsy was performed and therapy was different from that currently used. Nevertheless, only long follow-up time enables appropriate assessment of DSS, which is a major endpoint for any biomarker. Moreover, although we have presented data concerning OS, the potential impact of comorbidities was not assessed, as we mostly focused on DSS, disease-free and progression-free survival. Furthermore, despite treatment heterogeneity, this study is based in a series of patients that were consistently managed by the same multidisciplinary clinical team over the years, imparting homogeneity in decision-making strategy. The fact that both CS and GG clearly stratified patients with PCa further supports the validity of our findings. Importantly, this prostate biopsy cohort allowed for improved evaluation of prognostic biomarker performance, which has been seldom achieved, as it represents the entire spectrum of primary PCa. Finally, the use of a digital image analysis system to improve biomarker quantification and reduce evaluation subjectivity, should also be emphasized. We found this to be a practical and easily learned procedure, which took only about 5 minutes per biomarker in each case, and that may be incorporated into routine practice, increasing the consistency and reliability of pathological assessment.

5. Conclusions

High Ki67, EZH2, and SMYD3 immunoexpression independently predicted PCa patient outcome, adjusted for standard clinicopathological parameters, in a cohort of diagnostic biopsies. If incorporated into routine practice, this assessment might assist clinicians in discriminating indolent from aggressive PCa, improving treatment selection. Nevertheless, owing to the limitations of this study [use of sextant biopsies, lack of multiparametric magnetic resonance imaging data, heterogeneity of therapeutic strategies with predominance of advanced-stage PCa treated with ADT], validation of our findings in independent series is required.

Ethics approval

This study was approved by institutional review board of IPO Porto (CES-IPOFG-EPE215/013).

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