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Platinum Priority – Prostate Cancer

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Prospective Multicentre Evaluation of *PCA3* and *TMPRSS2-ERG* Gene Fusions as Diagnostic and Prognostic Urinary Biomarkers for Prostate Cancer

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Abstract

Background: Prostate cancer antigen 3 (*PCA3*) and v-ets erythroblastosis virus E26 oncogene homolog (*TMPRSS2-ERG*) gene fusions are promising prostate cancer (PCa) specific biomarkers that can be measured in urine.
Objective: To evaluate the diagnostic and prognostic value of Progensa PCA3 and TMPRSS2-ERG gene fusions (as individual biomarkers and as a panel) for PCa in a prospective multicentre setting.
Design, setting, and participants: At six centres, post–digital rectal examination first-catch urine specimens prior to prostate biopsies were prospectively collected from 497 men. We assessed the predictive value of Progensa PCA3 and TMPRSS2-ERG (quantitative nucleic acid amplification assay to detect TMPRSS2-ERG messenger RNA [mRNA]) for PCa, Gleason score, clinical tumour stage, and PCa significance (individually and as a marker panel). This was compared with serum prostate-specific antigen and the European Randomised Study of Screening for Prostate Cancer (ERSPC) risk calculator. In a subgroup ($n = 61$) we evaluated biomarker association with prostatectomy outcome.
Outcome measurements and statistical analysis: Univariate and multivariate logistic regression analysis and receiver operating curves were used.
Results and limitations: Urine samples of 443 men contained sufficient mRNA for marker analysis. PCa was diagnosed in 196 of 443 men. Both PCA3 and TMPRSS2-ERG had significant additional predictive value to the ERSPC risk calculator parameters in multivariate analysis ($p < 0.001$ and resp. $p = 0.002$). The area under the curve (AUC) increased from 0.799 (ERSPC risk calculator), to 0.833 (ERSPC risk calculator plus PCA3), to 0.842 (ERSPC risk calculator plus PCA3 plus TMPRSS2-ERG) to predict PCa. Sensitivity of PCA3 increased from 68% to 76% when combined with TMPRSS2-ERG. TMPRSS2-ERG added significant predictive value to the ERSPC risk calculator to predict biopsy Gleason score ($p < 0.001$) and clinical tumour stage ($p = 0.023$), whereas PCA3 did not.
Conclusions: TMPRSS2-ERG had independent additional predictive value to PCA3 and the ERSPC risk calculator parameters for predicting PCa. TMPRSS2-ERG had prognostic value, whereas PCA3 did not. Implementing the novel urinary biomarker panel PCA3 and TMPRSS2-ERG into clinical practice would lead to a considerable reduction of the number of prostate biopsies.

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1. Introduction

The gold standard for the diagnosis of prostate cancer (PCa) is based on the histopathologic evaluation of prostate biopsies, an invasive procedure with significant morbidity. Because localised PCa often does not present with symptoms, the selection of men qualifying for prostate biopsies relies on serum prostate-specific antigen (PSA) testing and digital rectal examination (DRE). PSA is currently the only widely used serum biomarker for PCa. However, PSA has a low specificity of 25–40% in the so-called grey area of PSA levels 4.0–10.0 ng/ml, resulting in a high negative biopsy rate [1,2]. Widespread PSA testing also leads to the diagnosis of clinically insignificant tumours, resulting in potential overtreatment, causing morbidity and leading to unnecessary increased health care costs. In the ongoing search for more specific biomarkers for PCa, prostate cancer antigen 3 (PCA3) and v-ets erythroblastosis virus E26 oncogene homolog (ERG, also known as TMPRSS2-ERG) gene fusion transcripts have been identified as promising urinary novel biomarkers [3,4].

The Progensa PCA3 test is approved by the US Food and Drug Administration and commercially available to aid in the decision of taking repeat prostate biopsies. A PCA3 cut-off score of 35 is generally used. However, most recent studies show that a lower cut-off score of 25 might be preferable [5–8]. The results of a possible correlation with established prognostic factors (histologic Gleason grade and tumour stage) are conflicting [5,8–11]. The consensus in most papers is that PCA3 is often negative in patients with indolent cancer, yet in the clinically significant cancers there is no evidence for an association with histopathologic prognostic factors (stage, grade).

In PCa, transmembrane protease, serine 2 (*TMPRSS2*) can be fused to *ERG* (a member of the ETS family of oncogenes) [4]. These *TMPRSS2-ERG* gene fusions are highly specific for PCa and are present in approximately half of white PCa patients [12]. *PCA3* and *TMPRSS2-ERG* are also found occasionally in high-grade prostatic intraepithelial neoplasia, in prostate glands in which PCa is also mostly found [4]. In 2006, the TMPRSS2-ERG gene fusion transcripts were successfully detected in urine samples [13]. This urine test had a sensitivity of 37% and a specificity of 93% for the prediction of PCa on prostate biopsy [14]. The prognostic value of this urine test has not yet been assessed.

Considering the heterogeneous character of the disease, the preferred approach in the diagnostic process of PCa will likely be the use of a panel of biomarkers. In 2007, it was shown that the combined use of PCA3 and TMPRSS2-ERG gene fusion transcripts improved sensitivity significantly [14]. This was recently confirmed by Tomlins et al.; however, that study was not conducted prospectively [15]. The aim of our study was to evaluate the diagnostic and prognostic predictive value of Progensa PCA3 plus TMPRSS2-ERG gene fusions (as individual biomarkers and as a panel) in a prospective multicentre setting, in accordance with the Standards for Reporting of Diagnostic Accuracy criteria [16].

2. Material and methods

2.1. Study design: clinical study

Inclusion criteria were men scheduled for prostate biopsy based on elevated serum PSA levels (≥3 ng/ml), a family history of PCa, or an abnormal DRE. Exclusion criteria were a history of PCa, medical therapy known to affect serum PSA levels, symptoms of urinary tract infection, prostate biopsy within 3 mo prior to enrolment, or invasive treatment for benign prostatic hyperplasia within 6 mo prior to enrolment. Subjects were recruited at six urology centres in the Netherlands (Radboud University Nijmegen Medical Centre, Nijmegen; Academic Medical Centre, Amsterdam; ZGT Hospital, Hengelo; Canisius Wilhelmina Hospital, Nijmegen; Scheper Hospital, Emmen; and St. Elisabeth Hospital, Tilburg). Prostate biopsies were performed and evaluated per each hospital's standard procedure (8–12 biopsy cores). One experienced genitourinary pathologist reviewed all the biopsy Gleason scores independently and was blinded for the biomarker scores. Three biopsy samples were not available for Gleason review; in these case, the local Gleason scores were used. The respective independent ethics committees approved the study protocol, and written informed consent was obtained.

Specimen processing is described in the Appendix.

2.2. Data collection

The following data were extracted from the records: age, serum PSA, DRE and transrectal ultrasound (TRUS) results, prostate volume, biopsy results (current and history), radiologic results, clinical TNM stage (if diagnosed with PCa), and radical prostatectomy results (if applicable). These data and the coded assay results were entered in a secured preset Web-based database with audit trail (in compliance with the International Conference on Harmonisation-Good Clinical Practices guidelines). The assay results and revised Gleason scores were not provided to the clinical sites for patient care.

2.3. Statistical analyses

PCA3 was assessed both as a continuous and a dichotomous variable with cut-off scores of 25 and 35. TMPRSS2-ERG was assessed as a dichotomous variable; all samples with measurable TMPRSS2-ERG (≥10 copies TMPRSS2-ERG messenger RNA [mRNA]) were marked positive. The following end points were used: PCa, biopsy Gleason score, clinical tumour stage, PCa significance, and in a subgroup, prostatectomy histologic outcome. Clinical significance of the tumour was assessed according to the Epstein criteria: clinical stage ≥T2, Gleason score ≥7, prostate-specific antigen density >0.15, and >33% positive cores. The Fisher exact test (for dichotomous variables) and the nonparametric Mann-Whitney test (for continuous variables) were used to test differences in levels of serum PSA, PCA3, and TMPRSS2-ERG for statistical significance between groups of patients. Univariate logistic regression analysis was used to study the influence of the biomarkers on each end point separately. Multivariate logistic regression analysis with selection procedures was used to test whether PCA3 and TMPRSS2-ERG had independently additional predictive value to the ERSPC risk calculator parameters on the three outcomes. The ERSPC risk calculator parameters are serum PSA level (nanograms per millilitre), DRE normal/abnormal, TRUS normal/abnormal, and prostate volume (millilitre). In addition, we tested which combination of the biomarker panel PCA3 plus TMPRSS2-ERG had the best additional discriminative value for the ERSPC risk calculator parameters. The odds ratios (ORs) and corresponding 95% confidence intervals (CIs) of the final model of each outcome are presented. The area under the curve (AUC) of the receiver operating

Table 1 – Patient characteristics

	All evaluable men (n = 443) Median (range) or n (%)
Age, yr	65 (44–86)
Serum PSA, ng/ml	7.4 (0.4–756)
PSAD, ng/ml/ml	0.15 (0.01–22.6)
Abnormal DRE	126 (28)
Abnormal TRUS	86 (19)
Prostate volume, ml	48 (15–200)
Family history PCa	82 (19)
Initial biopsy	348 (79)
Diagnosis PCa	196 (44)
Gleason score ≥7	115 (59)
Gleason score 7	60 (31)
Gleason score 8–10	55 (28)
Clinically significant PCa (Epstein)*	177 (40)
Radical prostatectomy	61 (14)

DRE = digital rectal examination; PCa = prostate cancer; PSA = prostate-specific antigen; PSAD = PSA density (serum PSA/prostate volume); TRUS = transrectal ultrasound.
* Clinically significant PCa according to the Epstein criteria: clinical stage ≥T2, Gleason score ≥7, PSAD >0.15, and >33% positive cores on biopsy.

characteristic curves was used to measure the additional predictive discrimination of PCA3 and TMPRSS2-ERG for the ERSPC risk calculator parameters. Two-sided p values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS v.18.0 software.

3. Results

Urine samples of 497 men were collected between September 2009 and July 2011, of which 54 samples were excluded for containing too much (in)organic precipitate or inadequate amounts of prostate cells (<1000 copies PSA mRNA). Thus, in total, the urine samples of 443 men were analysed successfully (89%). Table 1 shows the patient characteristics. Median serum PSA was 7.4 ng/ml, of which 274 men (62%) had a serum PSA level 4.0–10.0 ng/ml and 140 men (32%) had a serum PSA level ≥10.0 ng/ml. Of the 95 patients who had previous biopsies, one, two, or three or more previous biopsies had been performed in 70, 17, and 8 patients, respectively.

3.1. Total biopsy cohort

Table 2 shows the biomarker characteristics per biopsy outcome: PCa, Gleason score, clinical tumour stage, and PCa significance. Serum PSA, PCA3, and TMPRSS2-ERG were all significantly higher in patients with PCa compared with patients without PCa. Serum PSA and TMPRSS2-ERG were significantly higher in patients with Gleason scores ≥7 (7.8 vs 10.7 ng/ml and 7% vs 35% TMPRSS2-ERG positive resp.) and clinical tumour stage T3–T4 (8.0 vs 12.3 ng/ml and 16% vs 36% TMPRSS2-ERG positive resp.). PCA3 scores did not differ significantly between these groups. When evaluating a subcohort of patients with PSA levels <10 ng/ml (n = 303; data not shown), only TMPRSS2-ERG correlated significantly (p = 0.016) with Gleason score. In this subcohort of

Table 2 – Biomarker characteristics by prostate cancer, Gleason score, and clinical tumour stage

	PCa			Gleason score			Clinical tumour stage			PCa significance*		
	No (n = 247) Median (range) or n (%)	Yes (n = 196) Median (range) or n (%)	p value	≤6 (n = 81) Median (range) or n (%)	≥7 (n = 115) Median (range) or n (%)	p value	T1–T2 (n = 124) Median (range) or n (%)	T3–T4 (n = 72) Median (range) or n (%)	p value	No (n = 266) Median (range) or n (%)	Yes (n = 177) Median (range) or n (%)	p value
Age, yr	63 (46–80)	66 (44–86)	0.001[a]	64 (52–85)	67 (44–86)	0.002[a]	65 (44–85)	66 (54–86)	0.396[a]	64 (46–80)	66 (44–86)	0.002[a]
Serum PSA, ng/ml	6.8 (0.4–48)	9.2 (0.6–756)	<0.001[a]	7.8 (0.6–38)	10.7 (2.3–756)	<0.001[a]	8.0 (0.6–67.9)	12.3 (5.3–756)	<0.001[a]	6.7 (0.4–48)	9.6 (2.3–756)	<0.001[a]
PCA3 score	24 (0–219)	60 (0–522)	<0.001[a]	59 (2–308)	60 (0–522)	0.552[a]	60 (2–308)	54 (0–522)	0.582[a]	24 (0–308)	60 (0–522)	<0.001[a]
PCA3 score ≥25	118 (48)	159 (81)	<0.001[b]	64 (79)	95 (83)	0.580[b]	101 (82)	58 (81)	1.000[b]	131 (49)	146 (83)	<0.001[b]
PCA3 score ≥35	98 (40)	134 (68)	<0.001[b]	55 (68)	79 (69)	1.000[b]	88 (71)	46 (64)	0.341[b]	111 (42)	121 (68)	<0.001[b]
TMPRSS2-ERG ≥10	15 (6)	46 (24)	<0.001[b]	6 (7)	40 (35)	<0.001[b]	20 (16)	26 (36)	0.003[b]	18 (7)	43 (24)	<0.001[b]

mRNA = messenger RNA; PCa = prostate cancer; PCA3 = prostate cancer antigen 3; PSA = prostate-specific antigen; TMPRSS2-ERG = v-ets erythroblastosis virus E26 oncogene homolog.
* Clinically significant PCa according to Epstein criteria: clinical stage ≥T2, Gleason score ≥7, PSA density >0.15, and >33% positive cores.
No PCa significance = no PCa plus clinically insignificant PCa.
PCA3 score = [copies PCA3 mRNA] / [copies PSA mRNA] × 1000; TMPRSS2-ERG positive = ≥10 copies TMPRSS2-ERG mRNA.
[a] Mann-Whitney test.
[b] Fisher exact test.

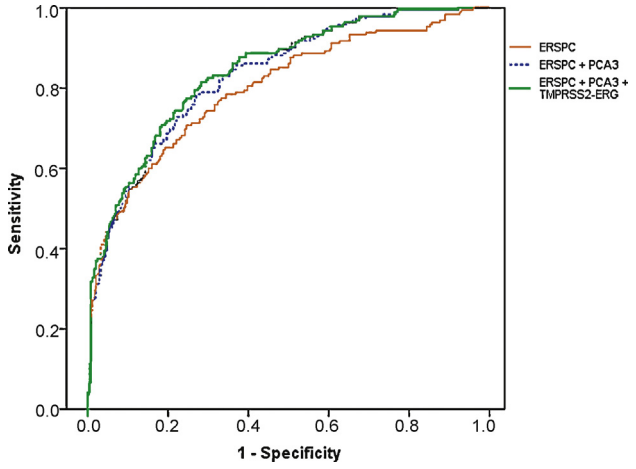

Fig. 1 – The receiver operating characteristic curves for the three models for the prediction of prostate cancer on biopsy: (1) European Randomised Study of Screening for Prostate Cancer (ERSPC) risk calculator parameters only (orange solid line; area under the curve [AUC]: 0.799); (2) ERSPC risk calculator parameters and PCA3 (blue dashed line; AUC: 0.833); and (3) ERSPC risk calculator parameters, PCA3, and TMPRSS2-ERG (green solid line; AUC: 0.842).
PCA3 = prostate cancer antigen 3; TMPRSS2-ERG = v-ets erythroblastosis virus E26 oncogene homolog.

patients, only serum PSA correlated significantly with clinical tumour stage ($p = 0.002$).

Table 3 shows the crude OR (95% CI) of the biomarkers predicting PCa, Gleason score, clinical tumour stage, and PCa significance, respectively, using univariate logistic regression analysis. Regarding PCa, all variables were significant predictors ($p < 0.001$). PCA3 demonstrated the highest accuracy in predicting PCa (AUC: 0.720). Both PCA3 (OR: 3.64; 95% CI, 2.19–6.05; $p < 0.001$) and TMPRSS2-ERG (OR: 3.28; 95% CI, 1.57–6.85; $p = 0.002$) had independent additional predictive value for the ERSPC risk calculator parameters for predicting PCa, using multivariate models with a selection procedure (Table 4). The ERSPC risk calculator parameters had a predictive accuracy for PCa diagnosis of 0.799, compared with 0.833 when PCA3 was included and 0.842 when both PCA3 and TMPRSS2-ERG were included (Fig. 1).

Regarding biopsy Gleason score and clinical tumour stage, serum PSA ($p = 0.001$ and $p < 0.001$ resp.) and TMPRSS2-ERG ($p < 0.001$ and $p = 0.002$ resp.) were significant predictors, whereas PCA3 was not, using univariate logistic regression analysis (Table 3). Only TMPRSS2-ERG was an independent predictor of biopsy Gleason score (OR: 7.16; 95% CI, 2.54–20.15; $p < 0.001$) and clinical tumour stage (OR: 2.60; 95% CI, 1.14–5.90; $p = 0.023$), in addition to the ERSPC risk calculator parameters, using multivariate logistic regression analysis with the selection procedure (Table 4).

The urinary biomarker panel was defined as negative if a negative TMPRSS2-ERG (<10 copies mRNA) and PCA3 <25, and positive otherwise. It had the best predictive value for PCa compared with all the other panels defined by these markers, using multivariate logistic regression models with a selection procedure. Therefore, sensitivity and specificity were calculated for this panel and for PCA3 and TMPRSS2-ERG individually. TMPRSS2-ERG was a highly specific biomarker for clinically significant PCa, with a sensitivity of 24.3% and a specificity of 93.2%. PCA3 (cut-off score: 35) had a sensitivity of 68.4% and a specificity of 58.3%, compared with 82.5% and 50.8%, respectively, when using a PCA3 cut-off score of 25. The biomarker panel PCA3 (cut-off: 25) plus TMPRSS2-ERG showed an increased sensitivity of 88.1% without compromising the specificity of PCA3 (49.6%). Sensitivity and specificity were not significantly different for the subgroup of patients without previous biopsies (data not shown).

3.2. *Clinical implications*

Based on serum PSA, all men in this study cohort underwent prostate biopsies. Table 5 shows the number of prostate biopsies that would be avoided and PCa that would be missed if the urinary biomarker panel was used to select men for prostate biopsies. The biomarker panel PCA3 plus TMPRSS2-ERG was indicated as positive if TMPRSS2-ERG and/or PCA3 were positive. The biomarker panel PCA3 (cut-off: 25) plus TMPRSS2-ERG would avoid 35% of prostate biopsies; 13% of prostate tumours would be missed. By combining PCA3 with TMPRSS2-ERG, an additional 13 of 443 men would be biopsied, of which 11 would be diagnosed with PCa including 9 men with Gleason score ≥ 7.

3.3. *Prostatectomy subcohort*

We also evaluated the subgroup of men ($n = 61$) who underwent a radical prostatectomy (data not shown). Gleason score on biopsy was upgraded in the prostatectomy specimen in 21% of men. TMPRSS2-ERG was an independent predictor of extracapsular extension (ECE) of the tumour on radical prostatectomy (OR: 4.98; 95% CI, 1.13–21.98; $p = 0.034$); serum PSA and PCA3 were not. None of the biomarkers correlated significantly with prostatectomy Gleason score or seminal vesicle invasion ($n = 8$). The biomarker panel PCA3 plus TMPRSS2-ERG did not correlate with any of the previously mentioned prognostic parameters on prostatectomy.

4. Discussion

In our study, PCA3 was a highly accurate biomarker for predicting PCa, exceeding the performance of the widely used serum PSA. The optimal PCA3 cut-off score is still subject to debate. Several studies suggested lowering the cut-off score from 35 to 25 [5–8]. In this study, the sensitivity for diagnostically clinically significant PCa increased remarkably when lowering the cut-off score to 25 (68% vs 83%). Concurrently, the specificity decreased from 58% to 51%. Defining the so-called optimal cut-off score will always be a compromise between sensitivity and specificity, depending on what risk of missing PCa is clinically acceptable. Based on the intended use, the optimal cut-off point can be determined based on the need for a high negative predictive value (NPV) or positive predictive value (PPV) in a particular setting (eg, excluding cancer in a

Table 3 – The crude odds ratios with 95% confidence intervals of the biomarkers for the probability of prostate cancer, Gleason score, and clinical tumour stage, respectively, using univariate logistic regression

	PCa			Gleason score ≥7			Clinical tumour stage T3–T4			Clinically significant PCa*		
	OR (95% CI)	*p* value	AUC (95% CI)	OR (95% CI)	*p* value	AUC (95% CI)	OR (95% CI)	*p* value	AUC (95% CI)	OR (95% CI)	*p* value	AUC (95% CI)
Serum PSA, ng/ml	1.08	<0.001	0.67	1.07	0.001	0.68	1.05	<0.001	0.71	1.10	<0.001	0.72
	(1.05–1.12)		(0.62–0.72)	(1.03–1.12)		(0.61–0.75)	(1.02–1.08)		(0.64–0.79)	(1.06–1.14)		(0.67–0.78)
PCA3 score	1.01	<0.001	0.72	1.00	0.224	0.53	1.00	0.436	0.48	1.01	<0.001	0.68
	(1.01–1.02)		(0.67–0.77)	(1.00–1.01)		(0.44–0.61)	(1.00–1.01)		(0.39–0.56)	(1.01–1.02)		(0.62–0.73)
PCA3 score												
<25	1.00	<0.001		1.00	0.527		1.00	0.877		1.00	<0.001	
	(reference)			(reference)			(reference)			(reference)		
≥25	4.70		0.67	1.26		0.52	0.94		0.50	4.85		0.64
	(3.04–7.27)		(0.62–0.72)	(0.61–2.59)		(0.44–0.60)	(0.45–1.98)		(0.41–0.58)	(3.08–7.66)		(0.58–0.69)
PCA3 score												
<35	1.00	<0.001		1.00	0.906		1.00	0.305		1.00	<0.001	
	(reference)			(reference)			(reference)			(reference)		
≥35	3.29		0.64	1.04		0.50	0.72		0.47	3.02		0.61
	(2.22–4.88)		(0.59–0.70)	(0.56–1.91)		(0.42–0.59)	(0.39–1.34)		(0.38–0.55)	(2.02–4.50)		(0.55–0.67)
TMPRSS2-ERG												
<10	1.00	<0.001		1.00	<0.001		1.00	0.002		1.00	<0.001	
	(reference)			(reference)			(reference)			(reference)		
≥10	4.74		0.59	6.67		0.64	2.94		0.60	4.42		0.64
	(2.56–8.80)		(0.53–0.64)	(2.67–16.66)		(0.56–0.71)	(1.49–5.79)		(0.52–0.68)	(2.45–7.97)		(0.58–0.71)

AUC = area under the curve; CI = confidence interval; mRNA = messenger RNA; OR = odds ratio; PCa = prostate cancer; PCA3 = prostate cancer antigen 3; PSA = prostate-specific antigen; TMPRSS2-ERG = v-ets erythroblastosis virus E26 oncogene homolog.

* Clinically significant PCa according to Epstein criteria: clinical stage ≥T2, Gleason score ≥7, PSA density >0.15, and >33% positive cores.

PCA3 score = [copies PCA3 mRNA] / [copies PSA mRNA] × 1000; TMPRSS2-ERG positive = ≥10 copies TMPRSS2-ERG mRNA. Gleason score = Gleason score ≤6 versus ≥7; clinical tumour stage = T1–T2 versus T3–T4; clinically significant PCa = clinically significant PCa according to Epstein criteria versus the rest of the patients (no PCa plus insignificant PCa).

Table 4 – The adjusted odds ratios with 95% confidence intervals of the biomarker for the probability of prostate cancer, Gleason score, and clinical tumour stage, respectively, using multivariate logistic regression

	OR (95% CI)	*p* value	AUC (95% CI)
Prostate cancer:			
ERSPC parameters			0.799 (0.756–0.841)
Serum PSA	1.08 (1.04–1.12)	<0.001	
Volume	0.98 (0.97–0.99)	<0.001	
DRE abnormal*	2.90 (1.60–5.24)	<0.001	
TRUS abnormal†	2.10 (1.06–4.18)	0.035	
PCA3 score ≥25‡	3.64 (2.19–6.05)	<0.001	0.833 (0.795–0.870)
TMPRSS2-ERG ≥10§	3.28 (1.57–6.85)	0.002	0.842 (0.806–0.878)
Gleason score ≥7:			
ERSPC parameters			0.801 (0.738–0.865)
Serum PSA	1.06 (1.02–1.11)	0.003	
Volume	0.99 (0.97–1.01)	0.205	
DRE abnormal*	7.06 (3.17–15.73)	<0.001	
TRUS abnormal†	1.26 (0.52–3.03)	0.613	
TMPRSS2-ERG ≥10§	7.16 (2.54–20.15)	<0.001	0.840 (0.785–0.895)
Clinical tumour stage T3–T4:			
ERSPC parameters			0.824 (0.764–0.884)
Serum PSA	1.05 (1.02–1.08)	0.002	
Volume	0.98 (0.96–1.00)	0.093	
DRE abnormal*	3.98 (1.84–8.62)	<0.001	
TRUS abnormal†	2.91 (1.35–6.27)	0.006	
TMPRSS2-ERG ≥10§	2.60 (1.14–5.90)	0.023	0.834 (0.776–0.892)

AUC = area under the curve; CI = confidence interval; DRE = digital rectal examination; ERSPC = European Organisation for Research and Treatment of Cancer; OR = odds ratio; PCA3 = prostate cancer antigen 3; PSA = prostate-specific antigen; TMPRSS2-ERG = v-ets erythroblastosis virus E26 oncogene homolog; TRUS = transrectal ultrasound.
* DRE normal.
† TRUS normal.
‡ PCA3 score <25.
§ TMPRSS2-ERG <10 copies.

Table 5 – Clinical implications of the urinary biomarkers

	Prostate biopsies avoided (*n* = 443) *n* (%)	Prostate cancers missed (*n* = 196) *n* (%)	Prostate cancers Gleason ≥7 missed (*n* = 115) *n* (%)
PCA3 score ≥25	166 (37)	37 (19)	20 (17)
PCA3 score ≥35	211 (48)	62 (32)	36 (31)
TMPRSS2-ERG ≥10	382 (86)	150 (77)	75 (65)
PCA3-25 plus TMPRSS2-ERG	153 (35)	26 (13)	11 (10)
PCA3-35 plus TMPRSS2-ERG	195 (44)	48 (24)	24 (21)

MRNA = messenger RNA; PCA3 = prostate cancer antigen 3; PCA3-25 = PCA3 test with a cut-off of 25; PSA = prostate-specific antigen; TMPRSS2-ERG = v-ets erythroblastosis virus E26 oncogene homolog.
PCA3 score = [copies PCA3 mRNA] / [copies PSA mRNA] × 1000.
TMPRSS2-ERG positive = ≥10 copies TMPRSS2-ERG mRNA.
PCA3-25 plus TMPRSS2-ERG = TMPRSS2-ERG positive and/or PCA3 ≥25.
PCA3-35 plus TMPRSS2-ERG = TMPRSS2-ERG positive and/or PCA3 ≥35.

clinical cohort [high-sensitivity/NPV]) or for a population-based screening (high specificity/PPV). Based on our results, a PCA3 cut-off score of 25 might be optimal; also when it is combined with TMPRSS2-ERG.

TMPRSS2-ERG was highly specific (93.2%) for predicting clinically significant PCa on biopsy. Therefore, combining PCA3 with TMPRSS2-ERG will not compromise the specificity of PCA3. This makes TMPRSS2-ERG a valuable marker to combine in a panel with PCA3. Because of its specificity, reaching nearly 100% in our opinion, TMPRSS2-ERG–positive patients without PCa on biopsy would need immediate rebiopsy or magnetic resonance imaging. However, TMPRSS2-ERG is not yet validated as a PCa biomarker to indicate the need for rebiopsy. Thus we cannot yet use this test for this indication in daily practice.

Both PCA3 and TMPRSS2-ERG had independent additional predictive value for the ERSPC risk calculator parameters for predicting PCa. If this biomarker panel PCA3 plus TMPRSS2-ERG would have been used in this study cohort to select men for prostate biopsies, a substantial number of (unnecessary) prostate biopsies (35%) would have been avoided, missing only 10% of the men with PCa with a Gleason score ≥7. Our results confirm and validate the results of Hessels et al. on this novel biomarker panel for PCa [14]. Recently, Tomlins et al. demonstrated that TMPRSS2-ERG in combination with PCA3 improved the performance of the multivariate Prostate

Cancer Prevention Trial risk calculator [15]. The limitation of their study was that it was not prospectively conducted and that the various centres did not use the same assays thresholds.

Knowing that Gleason score has high interpathologist variability, we asked one experienced genitourinary pathologist to review all the biopsy Gleason scores independently. This resulted in a 4% downgrading of the Gleason score; the Gleason score was upgraded in 28% of reviewed biopsies. However, the pathology review did not change the significance of our results (data not shown). When evaluating its potential prognostic value, PCA3 was not correlated with biopsy Gleason score and clinical tumour stage. When evaluating a subgroup of men who underwent a radical prostatectomy, PCA3 again had no correlation with established risk factors. Our results are comparable with several large studies that have been published recently showing that PCA3 had no additional predictive value for Gleason score and tumour stage [5,8–10,17]. Although these studies showed PCA3 to be correlated with insignificant PCa and tumour volume, the additional prognostic value of PCA3 is most likely limited.

This study demonstrates that the TMPRSS2-ERG urine assay adds significant value in the prediction of biopsy Gleason score, clinical tumour stage, and ECE of the tumour in the radical prostatectomy specimen. This may indicate that the TMPRSS2-ERG urine test could be used to select men with clinically significant PCa. However, TMPRSS2-ERG was not correlated with Gleason score in radical prostatectomy specimens. Thus the prognostic value of TMPRSS2-ERG in this subgroup of patients may be limited and needs further evaluation. The prognostic value of TMPRSS2-ERG when measured in tissue has been studied extensively, and the results are conflicting [18–24]. The hypothesis that TMPRSS2-ERG has prognostic value when measured in urine could be explained by the fact that aggressive PCa cells are more invasive and invade surrounding structures including the prostatic ductal system. Therefore, aggressive PCas are considered to shed their cells more easily into the prostatic ductal system after DRE, resulting in a urine specimen with more TMPRSS2-ERG mRNA.

Our cohort consisted of a relatively small proportion of patients with clinically insignificant PCa. This raises the question whether our study consists of a representative cohort. However, we have to emphasise that our study is a multicentre prospective trial, and we analysed an intent-to-use cohort; thus per definition the selection bias was minimal. Our results might not be applicable to a screening cohort. Although when analysing the subcohort of patients with a normal DRE, sensitivity and specificity for PCA3, TMPRSS2-ERG, and the panel did not change significantly (data not shown). Of the 126 patients with an abnormal DRE, 24% had no PCa on biopsies. This shows that DRE is very subjective; therefore, we do not think it is appropriate to preselect a population on the basis of this criterion. Our intention was to use objective criteria to diagnose patients. Biomarkers particularly fit this purpose.

5. Conclusions

In this prospective multicentre study we evaluated two novel urinary biomarkers for PCa; Progensa PCA3 and TMPRSS2-ERG had independent additional predictive value for PCA3 and ERSPC risk calculator parameters for predicting PCa. In addition, TMPRSS2-ERG had prognostic value. Implementing the novel biomarker panel PCA3 and TMPRSS2-ERG into clinical practice would lead to a considerable reduction of prostate biopsies.

Author contributions: Jack A. Schalken had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data: Leyten, Hessels, Jannink, Smit, de Jong, de Reijke, Cornel, Vergunst, Knipscheer, Kil, van Oort, Hulsbergen-van de Kaa.
Analysis and interpretation of data: Leyten, Hessels, Schalken, Mulders.
Drafting of the manuscript: Leyten, Hessels, Schalken.
Critical revision of the manuscript for important intellectual content: Mulders, van Oort, de Reijke, Cornel, Vergunst, Knipscheer, Kil, van Oort, Hulsbergen-van de Kaa.
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Obtaining funding: Schalken, Mulders.
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Supervision: Schalken, Mulders.
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Appendix A – Specimen processing

First-catch urine specimens after DRE were processed using a validated standard operating procedure based on the integration of procedures described by Groskopf et al. [25]; whole urine and urinary sediments were prepared as described by Hessels et al. [26]. In brief, first voided urine after DRE was collected in a coded container with 4 ml 0.5 M ethylenediamine tetra-acetic acid (EDTA). Then 2.5 ml of the urine was immediately transferred to a PCA3 urine sample collection tube containing sample transport medium (Gen-Probe Inc.). The urine samples were immediately cooled to 4 °C and mailed with cold packs to NovioGendix Research BV (Nijmegen, The Netherlands). Urine samples were processed within 48 h after collection to guarantee good sample quality. The urine, EDTA stabilised, was centrifuged at 4 °C and 1.800 × g for 10 min. The obtained

Table 6 – Primer sequences and TaqMan probes

Gene	GenBank	Primer	Position	Sequence
TMPRSS2-F	NM_005656	Forward	1–17	5′- CGC GAG CTA AGC AGG AG-3′
ERG-R	NM_004449	Reverse	315–334	5′-GTC CAT AGT CGC TGG AGG AG-3′
ERG-probe	NM_004449		80–109	5′-FAM-TGG TCC TCA CTC ACA ACT GAT AAG GCT TCC-BBQ-3′
PSA-F	NM_001648	Forward	74–91	5′-CGT GAC GTG GAT TGG TGC-3′
PSA-R	NM_001648	Reverse	179–194	5′-GCC GCA GAC TGC CCT G-3′
PSA-probe	NM_001648		97–122	5′-610-CCC TCA TCC TGT CTC GGA TTG TGG GA-BBQ-3′

GenBank = Database accession number.

urinary sediments were washed twice with ice-cold buffered sodium chloride solution. On centrifugation at 4 °C and 1.000 × g for 10 min, the sediments were snap frozen in liquid nitrogen and stored at −70 °C. The urine in the PCA3 urine sample collection tube was used for the Progensa PCA3 test at NovioGendix as described by Groskopf et al. [25]. The PCA3 score was calculated as [PCA3 mRNA] / [PSA mRNA] × 1000.

Quantitative real-time polymerase chain reaction (PCR) for PCA3 and TMPRSS2-ERG RNA was extracted from the urinary sediments using a modified TriPure reagent (Roche, Cat. no. 11 667 165 001) protocol. After the chloroform extraction, GlycoBlue (Ambion, Cat. no. AM 9515) was added to the aqueous phase to precipitate the RNA using isopropanol. The RNA samples that were obtained were DNase treated prior to the amplification protocol using DNase I enzyme (Invitrogen, Cat. no. 18068-015). The RNA samples were purified using GlycoBlue and sodium acetate (Ambion, Cat no. AM 9740). Total RNA from the sediments was used to generate amplified sense-strand cDNA (complementary DNA) using the Whole Transcriptome (WT) Expression Kit (Ambion, Cat no. 4411974) according to the manufacturer's protocol. Fluorescence-based real-time PCR assays were designed and optimised specifically for the TMPRSS2-ERG gene fusion transcripts and PSA. The TMPRSS2-ERG assay detects the three prime gene fusions identified in PCa [14], detecting 85% of all ETS gene fusions. The primers were located in exon 1 of TMPRSS2 and exon 4 of ERG. The probe was located in exon 4 of ERG and labelled with two fluorochromes; a 5′ end reporter fluorescent dye, and a quencher dye at the 3′ end. The primers and probe for the PSA assay are located in exon 1 and 2 of the PSA gene (Table 6). All primers and probes were designed by and manufactured by TIB Molbiol Berlin. Blunt-ended PCR products were cloned in the PCR-Blunt cloning vector (Invitrogen). Calibration curves with a wide linear dynamic range (10–1 000 000 copies) were generated using serial dilutions of the plasmids. The amplification efficiency of the primer pair was determined using the calibration curve and was >1.85. Control samples with known template concentrations were used as a reference. Two microlitres of each cDNA sample were amplified in a 20-μl PCR reaction containing 10 pmol of each primer, 2 pmol of TaqMan probe, and 1 × Probe Master mix (Roche). The following amplification conditions were used: 95 °C for 10 min followed by 50 cycles at 95 °C for 10 s, 60 °C for 30 s, and cooling at 40 °C for 55 s (LightCycler LC480, Roche). The crossing point (Cp) values were determined using the LightCycler 480 SW v.1.5 software (Roche). The Cp values of the samples were converted to concentrations by extrapolation in the generated calibration curve. For TMPRSS2-ERG, real-time PCR experiments were performed twice for each sample and classified as positive for TMPRSS2-ERG whenever they contained at least ≥10 copies in a single experiment. A fluorescent signal <1 (465–510) was classified as background signal. The assay performance of the real-time PCR experiments was evaluated during in-study validation. The reference control samples had an inter- and intra-assay variation <30%. A total of 97–100% of all samples was informative for PCA3 and TMPRSS2-ERG.

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