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ORIGINAL RESEARCH ARTICLE

Prospective validation study of a combined urine and plasma test for predicting high-grade prostate cancer in biopsy naïve men

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ABSTRACT

Objective: Early and accurate diagnosis of prostate cancer (PC) is crucial for effective treatment. Diagnosing clinically insignificant cancers can lead to overdiagnosis and overtreatment, highlighting the importance of accurately selecting patients for further evaluation based on improved risk prediction tools. Novel biomarkers offer promise for enhancing this diagnostic process. In this study, we aimed to externally validate a previously developed urine and plasma biomarker test in a biopsy-naïve population.

Materials and methods: Urine and blood samples were prospectively collected from 362 biopsy-naïve men with suspected PC before they underwent transrectal prostate biopsies. The expression levels of a 10-gene mRNA panel were quantified using reverse transcription/quantitative polymerase chain reaction of both urine and plasma. These gene expression levels, combined with clinical features and plasma prostate-specific antigen (PSA) levels, were used to predict the presence of International Society of Urological Pathology grade group ≥ 2 PC.

Results: Complete data were available for 314 patients. The sensitivity and specificity of the biomarker test were 87% (95% CI: 79–93%) and 42% (95% CI: 36–49%), respectively. The area under the curve was 0.76 (95% CI: 0.7–0.82) for the biomarker test probability and 0.65 (95% CI: 0.59–0.72) for PSA ($p = 0.02$). The test's negative predictive value was 89% (CI: 81–94%).

Conclusion: This study did not replicate the previously reported high accuracy of the biomarker test, highlighting the need for further refinement and robust external validation to ensure reliable performance across diverse patient populations.

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Introduction

Accurate differentiation between clinically significant and insignificant prostate cancer (PC) is essential for effective patient management. Clinically significant PC is typically defined as International Society of Urological Pathology (ISUP) grade ≥ 2 [1]. Low-grade PC (ISUP grade 1) grows slowly with minimal risk of progression, and aggressive treatment of such cancers may lead to overtreatment and unnecessary psychological and physical distress [2, 3]. For ISUP grades 2–3, outcomes vary, with some behaving indolently and others progressing more aggressively. Therefore, stratifying risk within this group is critical to identify those who may benefit from curative treatment [4]. Enhanced stratification reduces unnecessary biopsies and overtreatment of low-grade cancers, especially as PC incidence is expected to rise [5].

Current guidelines recommend initial multiparametric Magnetic resonance imaging (mpMRI) for suspected PC prior to biopsy, as mpMRI improves clinically significant cancer detection whilst reducing unnecessary biopsies [6, 7]. Although mpMRI with targeted biopsy is now standard in many countries, its interpretation requires expertise, and access remains limited. Furthermore, mpMRI alone cannot fully exclude PC, underscoring the need for complementary diagnostic tools [8, 9]. Prostate-specific antigen (PSA) testing remains the primary screening method, though its lack of specificity may lead to unnecessary biopsies [10, 11].

Additional blood tests, such as Prostate Health Index (PHI) and 4K score, measure PSA variants to aid in reducing unnecessary biopsies in men with PSA levels between 2 and 10 ng/mL [12, 13]. IsoPSA, based on PSA structure, further distinguishes between high- and low-grade cancers [14]. The Stockholm3 test combines clinical data, biomarkers and genetics to potentially reduce MRI requirements [15], whilst Proclarix estimates cancer risk in ambiguous MRI cases [16]. Urine

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biomarkers like PCA3 and SelectMDX assess cancer presence and aggressiveness, often alongside MRI, to improve risk stratification [17, 18]. Despite their potential, these biomarkers have not yet achieved widespread clinical use.

Combining urine and plasma biomarkers with clinical data offers another promising diagnostic approach. Albitar et al. showed that a panel of these biomarkers could predict biopsy outcomes with high predictive accuracy, even identifying low-grade cancers (ISUP grade ≥ 2) [19, 20]. This study aims to externally validate these findings in a biopsy-naïve population, assessing the biomarker test's potential to reduce unnecessary biopsies and improve patient selection for mpMRI and biopsy.

Materials and methods

Study design and patients

Men scheduled for transrectal ultrasound-guided prostate biopsy on suspicion of PC were invited to participate in this prospective study. A written-informed consent was obtained from all participants prior to inclusion. This study was approved by The Regional Committees on Health Research Ethics for Southern Denmark (#S-20170043). Eligibility criteria excluded patients with PSA values above 20 μg/L, a history of previous prostate biopsy, or previous/current therapy for benign prostatic hyperplasia (BPH), including surgery, medical therapy (except alpha-adrenergic antagonists) or catheterisation (intermittent or indwelling). Patients meeting these criteria were considered biopsy-naïve and eligible for inclusion. We used the identical choice of analysis equipment and reagents as in the previous studies [19, 20].

Urine and plasma processing

Approximately 60 ml of first-void urine and 12 mL (two 6 mL blood Ethylenediaminetetraacetic acid (EDTA) vials) of peripheral whole blood were collected from each participant without prior digital rectal examination. Urine collection was conducted in the morning under non-fasting conditions. Urine samples were immediately placed on ice and processed within 48 hours. The samples were centrifuged using Amicon Ultra-15 Centrifugal Filter Units with a 3 kDa molecular weight cut-off membrane (Millipore, Billerica, MA, USA) in a swinging bucket rotor at 3220xg (approximately 4,000 rpm) until 1 mL of concentrated urine remained. All urine processing was performed on ice to preserve RNA integrity, following previously published protocols [21, 22]. Plasma was separated from the peripheral blood samples by centrifugation at 805 g for 10 minutes.

Nucleic acid isolation for RNA and DNA was carried out using the NUCLISENS® easyMAG® Automated DNA/RNA extraction system (bioMérieux, Marcy-l'Étoile, France). Following purification, nucleic acids were either analysed immediately or stored at −80°C for later analysis. RNA quality was ensured by adhering to protocols designed to mitigate degradation, particularly during storage and transport, which were critical for maintaining sample integrity.

Quantitative reverse transcription-polymerase chain reaction

The biomarker test in this study was based on the expression levels of 10 genes in both urine and plasma (NeoLAB Prostate©, Florida, USA). The gene panel included UAP1, PDLIM5, IMPDH2, HSPD1, PCA3, PSA, TMPRSS2, ERG, GAPDH, B2M, AR and PTEN. GAPDH and B2M were used as housekeeping genes for normalisation. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed as previously described [21, 22], using RNA Ultrasense One-Step Quantitative RT-PCR on a ViiA™ 7 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Equal amounts of RNA were extracted from both plasma and urine, dissolved in water and used in each qRT-PCR assay. All assays followed standardised protocols to ensure consistent gene quantification.

Biopsy procedure

A standard 12-core biopsy protocol was followed, targeting the peripheral zone of the prostate. Patients received antibiotic prophylaxis according to local guidelines. Ultrasound guidance for the biopsy was provided by the BK3000 Ultrasound System (BK Medical A/S, Herlev, Denmark) equipped with an E14C4t (9018) Prostate Triplane Endocavity Transducer.

Biomarker test calculation

The biomarker test used to estimate the probability of ISUP grade ≥ 2 PC has been previously described in detail [21, 23]. In addition to biomarker levels, the test incorporates the following clinical parameters as covariates: age, prostate size, previous biopsy history and PSA levels. These covariates are integrated into the test using a logistic regression model, where they serve as independent variables to adjust and refine the probability estimates for ISUP grade ≥ 2 PC. By accounting for these clinical parameters alongside biomarker levels, the logistic regression framework allows the model to provide individualised risk predictions that are more precise and clinically relevant. The biomarker test generates predictions for three sensitivity cut-off points (high, medium and low); for this study, the high sensitivity cut-off was used to predict ISUP grade group ≥ 2 PC.

Statistical analyses

Statistical analyses were performed using R version 4.4.1 (2024-06-14) [24]. The Prostate Biopsy Collaborative Group Risk Calculator (PBCG-RC) was employed to estimate the risk of high-grade PC (Gleason score ≥ 7 (3 + 4)) [25]. Covariates included in the PBCG-RC are age, prostate size, digital rectal examination findings, family history of PC, use of 5-alpha reductase inhibitors, self-identified race, history of prior biopsy and PSA levels. These variables represent key clinical and demographic factors that are known to influence PC risk. The PBCG-RC integrates these covariates into a logistic regression framework to calculate individualised risk probabilities.

For consistency, information on digital rectal examination findings was categorised as missing in the PBCG-RC calculations, as this parameter was not incorporated into the biomarker test being evaluated. Binomial confidence limits were calculated for sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The area under the curve (AUC) for receiver operating characteristic (ROC) curves was compared using DeLong's nonparametric approach to assess the discriminatory performance of the biomarker test versus PSA. Decision curve analysis was performed to evaluate the clinical utility of the biomarker test probability compared to PSA for clinically relevant biopsy thresholds. Calibration of the biomarker test was assessed through visual inspection of calibration plots. Statistical significance was set at $p < 0.05$.

Results

Patient characteristics

A total of 362 men were included between November 2018 and September 2021. Complete biopsy and biomarker data were available for 314 men. Missing data were attributed to patients declining biopsy after inclusion, inadequate urine volume, technical malfunctions of analysis equipment and withdrawal of consent. The characteristics of the study population are summarised in Table 1. The overall prevalence of ISUP grade group ≥ 2 cancer was 29.6% ($n = 98$).

Table 1. Baseline characteristics of the study population, including demographic and clinical variables.

Variable	($n = 314$)
Age (years)	
Median (IQR)	68 (62, 73)
Range	44–82
Serum PSA (μg/L)	
Median (IQR)	6.5 (5.0, 9.2)
Range	0.32–20
TRUS Volume[a] (cm^3)	
Median (IQR)	50 (37, 72)
Range	14–160
PSA Density (μg/L/cm^3)	
Median (IQR)	0.12 (0.08, 0.20)
Range	0.0076–0.64
Clinical T-Stage	
cTx	2 (1%)
cT1c	234 (75%)
cT2a	31 (10%)
cT2b	15 (5%)
cT2c	13 (4%)
cT3a	16 (5%)
cT3b	3 (1%)
ISUP* grade	
Negative biopsy	145 (46%)
1	75 (24%)
2	59 (19%)
3	17 (5%)
4	8 (3%)
5	10 (3%)

The data were collected between the years 2018 and 2021. IQR: Interquartile range; PSA: prostate-specific antigen. [a]Volume measured by transrectal ultrasound (TRUS). *International Society of Urological Pathology.

Prediction of ISUP grade group ≥ 2

Figure 1 shows the ROC curves for PSA, PBCG-RC and the biomarker test (Figure 1A). The corresponding decision curves are displayed in the right panel (Figure 1B). Overall, the PBCG-RC model demonstrated superior discrimination compared to the biomarker test (AUC: 0.81 (95% CI: 0.76–0.86) vs 0.76 (95% CI: 0.7–0.82), p value = 0.03). Using a 10% risk threshold for biopsy, the biomarker test yielded a lower net benefit than PSA, PBCG-RC and the reference strategy (0.216, 0.222, 0.226 and 0.222). For details on the other sensitivity cut-off points, refer to Table 2.

A visual inspection of the biomarker test's calibration plot suggests fair calibration at low-risk levels, with an underestimation of probability at low overall risk and an overestimation of risk at less clinically relevant higher risk (Figure 1C). The PBCG model, in contrast, overestimated risk across the full probability range (Figure 1D). At a 10% risk threshold, the PBCG-RC model demonstrated a sensitivity of 99% (95% CI: 94–100%), a specificity of 6% (95% CI: 4–10%) and a NPV of 94% (CI: 70–100%). The corresponding sensitivity and specificity of our test biomarker test were 87% (95% CI: 79–93%) and 42% (95% CI: 36–49%), respectively, with a NPV of 89% (CI: 81–94%).

Discussion

We previously evaluated a biomarker test that integrated a panel of urine and plasma biomarkers with clinical data to predict clinically significant PC in a cohort of 33 patients, yielding promising initial results. However, in this external validation study involving 314 biopsy-naïve men, we were unable to replicate those findings. The biomarker test demonstrated a NPV of 89% (CI: 81–94%), with a sensitivity of 87% and specificity of only 42%. By comparison, the PBCG-RC, which does not incorporate biomarkers, exhibited superior discrimination between significant PC and benign conditions. This suggests that clinical factors – such as prostate volume, age and biopsy history – were more informative than the biomarker panel within our study population.

Various urine- and blood-based biomarkers have been proposed to improve early detection and risk stratification of PC, often guiding the decision to perform a biopsy. Amongst the most established biomarker tests are the *Progensa* Prostate Cancer Antigen 3 (PCA3) assay and the PHI, both of which are United States Food and Drug Administration approved [26, 27]. The *Progensa* PCA3 assay, which measures PCA3 RNA and PSA mRNA in urine, is typically used to guide repeat biopsy decisions following an initial negative biopsy. In external validation studies, it has demonstrated an AUC of 0.73–0.75. The PHI test, which combines total PSA, free PSA and proPSA (p2PSA), has consistently outperformed traditional PSA testing, with an AUC of 0.703 in men with PSA levels between 2 and 10 μg/L [27].

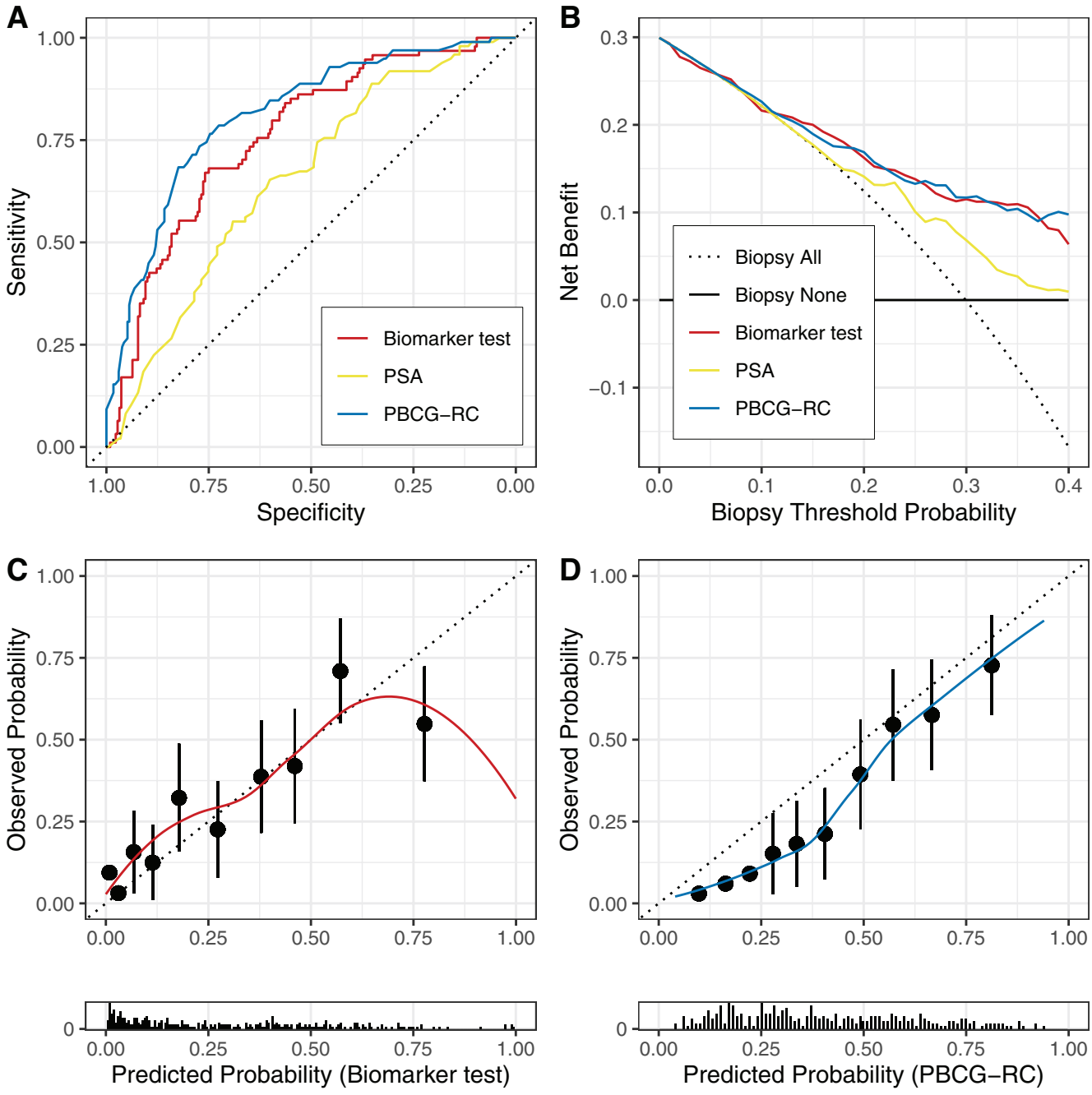

Figure 1. Performance evaluation of the biomarker test, Prostate Biopsy Collaborative Group (PBCG) Risk Calculator (RC) and PSA levels. (A) receiver operating characteristic (ROC) curves for the biomarker test, PBCG-RC and prostate-specific antigen (PSA), showing their respective areas under the curve (AUC): biomarker test: 0.76 (95% CI: 0.7–0.82). PSA: 0.65 (95% CI: 0.59–0.72); PBCG-RC: 0.81 (95% CI: 0.76–0.86). (B) Decision curves illustrating net benefit across various thresholds. The reference strategy assumes biopsying all men. (C) Calibration plot for the biomarker test, with a logistic calibration intercept of −0.301 and slope of 0.47. The dotted line represents perfect calibration, and black dots show observed probabilities with error bars for deciles of predicted probabilities. A distribution plot of predicted probabilities is displayed below. (D) Calibration plot for the PBCG-RC, with similar features as described in panel C.

Other biomarker-based models, such as the four-kallikrein score (4Kscore) and the Stockholm-3 model (S3M), combine plasma protein biomarkers, genetic polymorphisms and clinical features to predict significant PC. However, their contribution to predictive performance can be limited [28]. For instance, S3M achieved an AUC of 0.74 in patients with PSA levels below 10 μg/L, although clinical factors remained the most critical elements for risk stratification [29]. Similarly, the 4Kscore reported an AUC of 0.84 in a cohort of screen-positive men (PSA ≥ 3.0 μg/L) [30]. These studies underscore that, in many cases, clinical parameters dominate biomarker contributions, a pattern we observed in our study.

In our initial study from 2016, PSA values ranged from 0.5 to 283.2 μg/L, a broader range than those typically used in other

Table 2. Performance metrics (sensitivity, specificity, PPV and NPV) of the biomarker test at three different cut-off points for predicting patients with ISUP grade ≥ 2 prostate cancer.

Cutoff	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Low	74	60	44	85
Medium	82	56	44	88
High	87	42	39	89
PBCG-RC[†]	99	6	31	94

Data were derived from validation population. For reference, performance metrics are compared with the Prostate Biopsy Collaborative Group Risk Calculator at a 10% risk threshold (†). PPV: Positive predictive value. NPV: Negative predictive value; PBCG-RC, Prostate Biopsy Collaborative Group Risk Calculator; ISUP: International Society of Urological Pathology.

validation studies [21]. This wide PSA range likely influenced the biomarker test's performance. In the earlier external validation attempts we performed, in a cohort of prostatectomised men, the results were inherently biased due to the high prevalence of clinically significant PC in that group, with only 15% of patients having an ISUP grade group < 2 [20]. The shift in cohort characteristics and the change in reference standards (from biopsy to prostatectomy specimens) likely contributed to the biomarker test's lower specificity in this study. A subsequent validation in a mixed cohort of BPH and PC patients also reported high sensitivity but was limited by a small sample size and cohort heterogeneity [22].

Another critical factor is the reproducibility of the assay across different laboratories. In our study, the calibration plot for the biomarker test indicated an overestimation of cancer risk, particularly at higher probability ranges. This discrepancy may be partly explained by differences in laboratory conditions and technologist expertise, which likely influenced the biomarker test's sensitivity in the prostatectomy cohort. Furthermore, our cohort consisted entirely of biopsy-naïve men, meaning that clinical data points such as prior biopsy history and key elements in the biomarker test did not contribute to risk stratification.

In summary, this study highlights the challenges associated with external validation of biomarker-based tests. The biomarker test examined in this cohort did not perform as expected, particularly in terms of specificity, when compared to clinical tools like the PBCG-RC. Whilst biomarkers hold potential for improving risk stratification, our findings emphasise the need for robust validation across diverse patient populations before clinical implementation. Additionally, the observed variability in test performance across different study settings underscores the importance of standardising assay protocols and addressing factors that may influence reproducibility, such as sample handling and laboratory conditions.

Conclusions

This prospective cohort study was unable to replicate the previously reported high accuracy of the urine and plasma biomarker test for predicting clinically significant PC in biopsy-naïve men. These results underscore the importance of conducting robust external validation across diverse populations before clinical implementation of any biomarker-based diagnostic tests or algorithms.

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