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The performance and limitations of PCA3, TMPRSS2:ERG, HOXC6 and DLX1 urinary markers combined in the improvement of prostate cancer diagnostics

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ABSTRACT

Background: Prostate cancer (PCa) is the second most commonly diagnosed cancer in men. To date, the role of the combined application of long non-coding RNAs (PCA3, DLX1, HOXC6, TMPRSS2:ERG) for obtaining the most accurate method of detection of PCa has not yet been comprehensively investigated.
Methods: In total 240 persons were included in the retrospective study. Among them were 150 patients with confirmed PCa, 30 patients with benign prostatic hyperplasia, 30 patients with active chronic prostatitis and 30 healthy volunteers. In all patients, the urine samples were collected prior to biopsy or treatment. Polymerase chain reaction with reverse transcription was performed to detect the expression level of PCA3, HOXC6, DLX1 and the presence of the TMPRSS2:ERG transcript.
Results: PCA3 was detected in urine samples in all cases. Using a PCA3 score of 56 allowed the differentiation between PCa and all other cases with a sensitivity of 61% and specificity of 96% ($p < 0.001$) while a PCA3 score threshold value of 50 resulted in a differentiation between clinically significant PCa (ISUP grades 2–5) and all other cases with a sensitivity of 93% and specificity of 93% ($p < 0.001$). The TMPRSS2:ERG expression in urine was detected exclusively in the group of patients with PCa and only in 16% of all cases.
Conclusions: PCA3 score detected in urine demonstrated moderate sensitivity and good specificity in differentiation between PCa and non-PCa and high sensitivity and specificity in differentiation between clinically significant PCa and non-PCa.

1. Introduction

Prostate cancer (PCa) is the second most commonly diagnosed cancer in men, with an estimated 1.4 million newly diagnosed cases worldwide [1]. Currently, the only marker widely used for early detection, diagnostics and treatment efficiency assessment is prostate-specific antigen (PSA), a member of the 15-gene family of kallikrein-related peptidases 3 (KLK3) [2]. As an independent predictor of prostate cancer, PSA is better than either digital rectal examination (DRE) or transrectal ultrasound (TRUS) [3]. The main drawback of PSA application for PCa detection is that this marker is organ- but not cancer-specific: it may be elevated due to non-malignant conditions including benign prostatic

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hypertrophy (BPH), acute or chronic prostatitis (CP), or after TRUS, catheterization of the urinary bladder, or DRE, hindering differential diagnosis of cancer [4,5]. The sensitivity of the PSA test in detecting clinically significant cancer is suboptimal and is about 51%. Prostate cancer is detected in almost every 4th man with a normal PSA level [6]. The use of PSA for screening of PCa led to hyper-diagnosis and increased detection of non-threatening forms of PCa (which do not require active treatment in most cases) [7], the result of which is mainly the performance of highly traumatizing surgical interventions with potentially severe complications and unjustified significant economic losses on the part of the state and the patient. The situation in the context of PCa diagnosis is further aggravated by the high level of false-negative results of puncture biopsy of the prostate, which reaches 46% [8] and does not allow diagnosing the existing malignancy in almost every second patient. However it is necessary to recognize the recent improvement of these indicators since the introduction of magnetic resonance imaging (MRI)-targeted biopsy and its wide use instead of a systematic one [9,10].

In the last decade a spectrum of serum and urine markers has been actively investigated and proposed for clinical usage to reduce the number of unnecessary prostate biopsies in men with abnormal PSA results but doubtful imaging or/and clinical findings or after negative prostate biopsies associated with rising suspicion of PCa [11–13]. Among urinary markers, the most studied and promising are long non-coding RNAs such as prostate cancer antigen 3 (PCA3) score, a fusion of the transmembrane protease serine 2 (TMPRSS2) and the ERG gene (TMPRSS2-ERG), Homeobox C6 Protein (HOXC6) and Distal-Less Homeobox 1 (DLX1) [14]. According to recent data diagnostic performance of PCA3 score measured in urine is superior compared to PSA for the detection of PCa. The main indication is a triage to determine whether a repeat prostate biopsy is required after an initially negative result, nonetheless, its clinical effectiveness for this purpose is still unclear. Also, there is conflicting data about whether the PCA3 score could independently predict the International Society of Urological Pathology (ISUP) prostate cancer grade [15–17]. When the urine expression of TMPRSS2-ERG was added to the PCA3 score the PCa prediction improved, allowing to avoid 27% of unnecessary prostate biopsies, however, this tumour marker is still under investigation [13,18]. The detection of HOXC6 and DLX1 mRNA expression in urine is used to assess the risk of both presence of PCa on biopsy as well as the prediction of high-grade malignancy. A combination of HOXC6/DLX1 urine expression measurement and prostate MRI in men with elevated PSA had a negative predictive value of 93% [19]. However, this combination is not widely used and its clinical feasibility requires further investigations.

In the light of the above data, it could be concluded that diagnostic markers for PCa are insufficiently effective and do not meet the modern challenges of oncologic urology. The search for a non-invasive, highly specific, sensitive and economically affordable method of early diagnosis of this disease is one of the priority tasks of modern medicine. To date, the role of combined application of markers determined in urine such as long non-coding RNAs (PCA3, DLX1, HOXC6, TMPRSS2:ERG) for obtaining the most accurate method of detection of PCa, which was the goal of this project, has not yet been comprehensively investigated.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Ethical Committee of Danylo Halytsky Lviv National Medical University, Ukraine, and was executed according to ethical standards formulated in the Declaration of Helsinki 1975. This research was performed at the Urology Department of Lviv National Medical University and the General and Molecular Pathophysiology Department of Bogomoletz Institute of Physiology of the National Academy of Sciences of Ukraine during the years of 2020–2022.

2.2. General data

In total 240 persons were included in the retrospective study. The mean age was 62.99 ± 6.67 years. Among them there were 150 patients (62.5%) with confirmed PCa utilizing standard 12 core TRUS-Bx: there were 39 cases (16.25%) of clinically insignificant prostate cancer (ciPCa) which is grade 1 according to the ISUP and 111 cases (46.25%) of clinically significant cancer (scPCa) which is ISUP grade > 1. The inclusion criteria were: suspicious for PCa clinical/laboratory/imaging findings such as an increased serum PSA level in accordance with the age of the patient, positive DRE, features of PCa based on multiparametric prostate MRI or/and ultrasonography (USG) data. Additionally, 30 patients with BPH (12.5%) and 30 patients with active CP (12.5%) were enrolled into the study. All cases of BPH were histologically confirmed using transurethral resection of the prostate according to clinical indications. Patients with CP and with positive DRE or suspicions for PCa features according to imaging data (multiparametric prostate MRI/USG) were excluded from the study. The additional exclusion criteria for the subgroup with CP were an abnormal level of serum PSA (>3 ng/ml) 3 months after the appropriate antimicrobial/anti-inflammation treatment and clinical improvement according to laboratory findings (expressed prostatic secretion microscopy and bacteriological culturing). For reference, 30 healthy volunteers were included in the study (inclusion criteria were: no clinical/laboratory suspicion or history of prostate disease, serum PSA < 2.5 ng/ml, prostate volume ≤ 30 ml). PSA data was collected retrospectively from the histories of patients. The method used for PSA detection was immunochemical with electrochemiluminescent detection (ELICA). Clinical characteristics of groups and subgroups of the patients are presented in Table 1.

2.3. Sample collection and processing

In all cases, 50 ml of first-catch morning urine was collected into a sterile container. In all patients, the urine samples were collected prior to biopsy or treatment. The sampling was carried out after massaging the prostate gland to exfoliate the cells in the prostatic part of the urethra, according to standard methodology (3 S per lobe). Urine samples were stored for no>2 h at a temperature of +4 °C. Centrifugation was then performed to obtain urine sediment with subsequent stabilization of nucleic acids using RNAlater (Thermo Fisher Scientific, USA) for further storage of clinical material for 7 days at a temperature of +4 °C.

Table 1
The mean age and mean serum PSA level in groups and subgroups of patients.

Group/subgroup	N	Mean age ± SD, years	Mean PSA serum level ± SD, ng/ml
PCa all grades	150	63.0 ± 6.59*	11.97 ± 23.19‡
PCa ISUP grade 1	39	60.98 ± 6.52*	7.04 ± 6.03†
PCa ISUP grade > 1	111	63.71 ± 6.49*	13.57 ± 26.56**, ***,†
BPH	30	61.15 ± 6.69*	2.06 ± 0.90**,†, ‡
Chronic prostatitis	30	62.56 ± 7.22*	7.36 ± 3.58†
Healthy	30	65.19 ± 6.16*	1.24 ± 0.64***, †, ‡

PCa = prostate cancer, BPH = benign prostatic hyperplasia, ISUP = International Society of Urological Pathology prostate cancer grade, SD = standard deviation.
*p > 0,05.
**p = 0.022.
*** p = 0.011.
‡<0,05.

2.4. Messenger RNA isolation from urine and qPCR data

RNA from fixed samples was isolated using phenol–chloroform extraction. Polymerase chain reaction with reverse transcription (RT-PCR) was performed to detect the expression level of PCA3, HOXC6, DLX1 and the presence of the TMPRSS2:ERG transcript. Reverse transcription was performed using random hexamer primer and reverse transcriptase M-mulv (Thermo Fisher Scientific, USA). Quantitative determination of messenger RNA (mRNA) was carried out using two-step PCR using TaqMan® probes (Thermo Fisher Scientific, USA): Hs01371938_m1, Hs03063375_ft and Hs02758991_g1 (GAPDH mRNA determination). The latter sample was used as internal control with control samples in a volume of 10 μl containing 5 μl TaqMan® Gene Expression Master Mix, 0.5 μl TaqMan® probe and 3.5 μl cDNA. This stage was carried out by one-time heating to a temperature of 50 °C for 120 s, then to 95 °C for 120 s, followed by 45 cycles of amplification with temperature changes: 95 °C – 3c, 60 °C – 30c. Transcript expression in the studied sample was considered positive if amplification was observed in duplicates; provided there is amplification in the positive control sample and no amplification in the negative control sample. Urinary PCA3 mRNA levels were normalized to the amount of prostate-derived RNA to calculate a PCA3 score using the standard methodology [20]. The HOXC6, DLX1 and TMPRSS2:ERG expression levels were presented as relative units (RU).

2.5. Statistical analysis

Microsoft Excel 2019 and SPSS v.22 software packages were used for the statistical data processing. The data distribution normality was evaluated using Shapiro–Wilk test. The ANOVA method was used to assess the difference in expression levels in patients' subgroups. The results were considered statistically significant when the p-value was < 0.05. The correlation was measured by means of the Pearson method. The diagnostic performance of expression levels for diagnostics of PCa was evaluated using receiver operating characteristics (ROC) analysis.

3. Results

3.1. PCA3 analysis

The mean PSA in all groups was 8.81 ± 18.87 ng/ml. The PCA3 was detected in urine samples in all cases. There was a significant difference in mean PCA3 score between the group with PCa and groups with BPH, CP, and healthy persons: 69.33 ± 31.58 vs 30.89 ± 20.23, 18.0 ± 16.36 RU, 8.20 ± 2.51 accordingly ($p < 0.001$). However, there was no such difference between groups with BPH and CP ($p = 0.244$), and between the group with CP and healthy persons ($p = 0.488$). We observed a significantly lower PCA3 score in the subgroup with ciPCa (ISUP grade 1) compared to csPCa (ISUP grade > 1) which was 42.97 ± 8.09 vs 78.58 ± 31.55 ($p < 0.001$), Fig. 1. There was a statistically significant difference in the mean PCA3 score between the subgroup with clinically significant PCa and all other subgroups ($p < 0.001$), nevertheless there was no such difference between subgroups with clinically insignificant PCa and BPH ($p = 0.223$).

Analysis of mean PCA3 score inside the subgroups with PCa of different ISUP grades revealed a difference between the subgroup of ISUP grade 1 and subgroups with ISUP grades 2–5 ($p < 0.01$). Albeit, there was no difference in mean PCA3 score between subgroups with PCa ISUP grade 2 and ISUP grade 3 ($p = 0.457$) and also between subgroups with PCa ISUP grade 3 and ISUP grade 4 ($p = 0.590$). The difference in mean PCA3 score between subgroups with PCa ISUP grade 5 and ISUP grades 1–4 was substantial ($p < 0.001$). The detailed statistical characteristics of PCA3 scores in groups and subgroups of patients are presented in Table 2.

Moreover, the PCA3 score demonstrated higher values in high-grade prostate cancer compared to low grade, Fig. 2. As a result of correlation analysis between the PCA3 score and prostate cancer ISUP grade, we re-

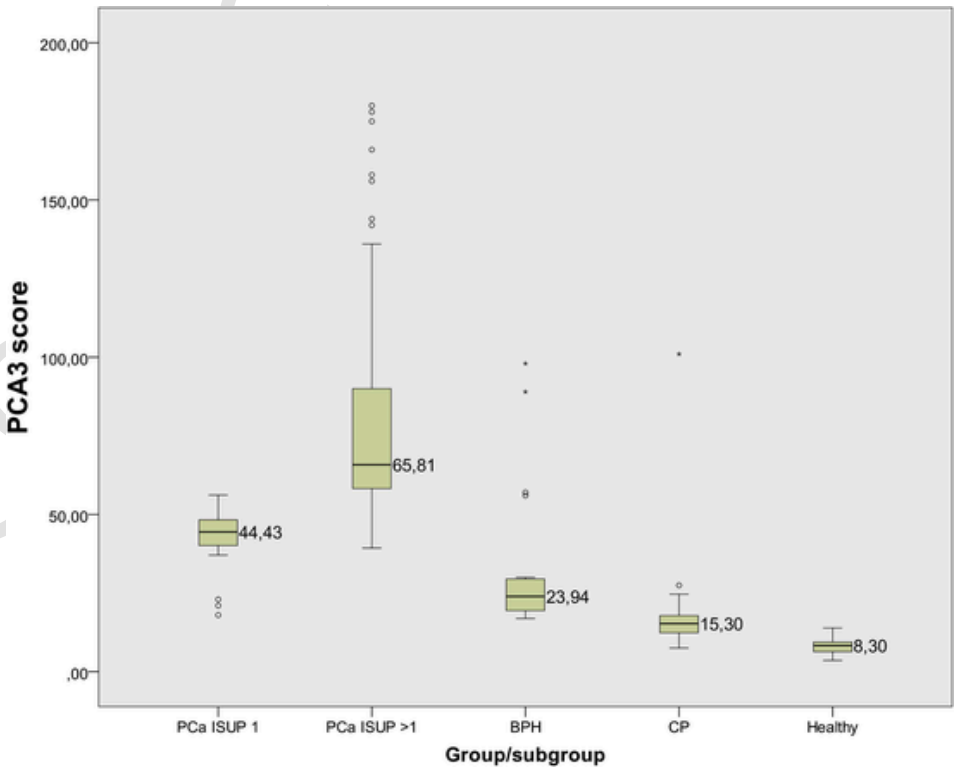

Fig. 1. Box plot of PCA3 score in groups of patients.

Table 2
The detailed statistical characteristics of PCA3 scores in groups and subgroups of patients.

	N	Mean	SD	95% Confidence Interval for Mean		Min.	Max.
				Lower Bound	Upper Bound		
PCa all grades	150	69.33*	31.58	64.23	74.42	18.0	180.0
PCa ISUP grade 1	39	42.97**,***,≠	8.09	40.35	45.60	18.0	56.17
PCa ISUP grade > 1	111	78.58**,‡	31.55	72.65	84.52	39.30	180.0
PCa ISUP grade 2	36	62.16≠	9.43	58.97	65.35	39.30	89.0
PCa ISUP grade 3	30	71.57≠	20.13	64.05	79.09	41.37	120.0
PCa ISUP grade 4	23	80.95≠	28.43	68.65	93.24	44.60	136.0
PCa ISUP grade 5	22	112.56≠	43.71	93.18	131.94	46.39	180.0
BPH	30	30.89*,†,‡,***	20.23	23.34	38.45	16.92	98.0
CP	30	18.0*,†,‡	16.36	11.89	24.11	7.52	101.0
Healthy	30	8.20*,‡	2.51	7.27	9.14	3.59	13.91

PCa = prostate cancer, BPH = benign prostatic hyperplasia, CP = chronic prostatitis, ISUP = International Society of Urological Pathology prostate cancer grade, SD = standard deviation.
* $p < 0.001$.
** $p < 0.001$.
*** $p < 0.001$.
†$p = 0.244$.
‡ $p < 0.001$.
≠ $p < 0.001$.

ceived a strong positive correlation: the Pearson correlation coefficient amounted 0.685 ($p < 0.001$).

According to ROC-analysis, a PCA3 score of 56 allowed the differentiation between PCa and all other cases with a sensitivity of 61% and specificity of 96% (AUC = 0.995, 95% CI = 0.920–0.989, $p < 0.001$). Using a PCA3 score threshold value of 50 resulted in a differentiation between clinically significant csPCa (ISUP grades 2–5) and all other cases with a sensitivity of 93% and specificity of 93% (AUC = 0.966, 95% CI = 0.943–0.989, $p < 0.001$), Fig. 3.

3.2. HOXC6 and DLX1 analysis

Surprisingly, in our study, HOXC6 and DLX1 expression in urine after the prostate massage was detected only in 43 (18%) and in 9 (3,75%) of 240 cases respectively. Due to the small sample size, the latter was excluded from the currently presented analysis. Moreover, there was no detectable HOXC6 expression in urine in the group of patients with BPH or in healthy persons. The mean HOXC6 expression in the group with PCa (n = 26; 11%) was lower compared to the group with active CP (n = 17; 7%) but did not meet statistical significance: 36.31 ± 12.54 RU vs 48.18 ± 26.79 RU ($p = 0.057$). Of note, that in 100% of PCa cases, urine expression of HOXC6 was detected in a clinically significant variant of disease: ISUP grade 2 (n = 4), ISUP grade 3 (n = 8), ISUP grade 4 (n = 6), ISUP grade 5 (n = 8). However, there was no difference in mean HOXC6 expression between subgroups of csPCA ($p > 0.05$).

3.3. TMPRSS2:ERG analysis

Similarly to HOXC6 and DLX1, TMPRSS2:ERG expression in urine after prostate massage was detected only in a small number of cases, particularly exclusively in the group of patients with PCa (n = 39; 16%), namely: ISUP grade 1 (n = 6), ISUP grade 2 (n = 5), ISUP grade 3 (n = 11), ISUP grade 4 (n = 11), ISUP grade 5 (n = 6). No urine expression of TMPRSS2:ERG was detected in groups of patients with BPH, CP and in healthy persons. In contrast to HOXC6 and DLX1, there was a significant difference in mean TMPRSS2:ERG expression in urine between subgroups of csPCa and ciPCa: 41.14 ± 10.21 RU vs 19.67 ± 5.16 RU ($p < 0.001$). In addition, there was a significant dif-

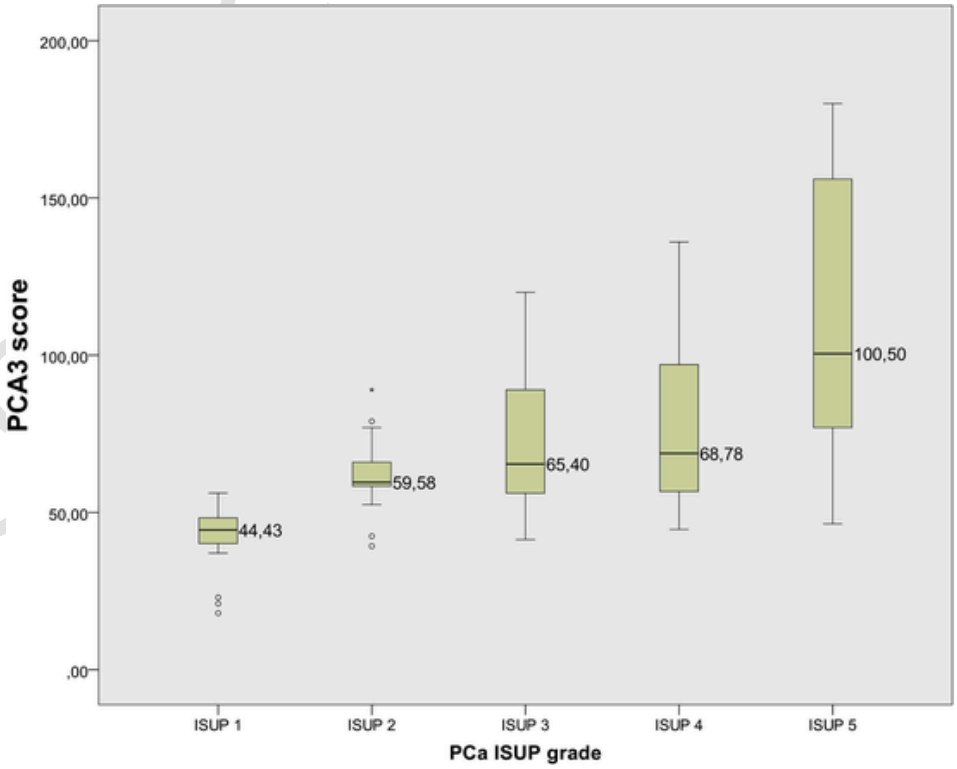

Fig. 2. Box plot of PCA3 score in subgroups of patients with PCa of different ISUP grades.

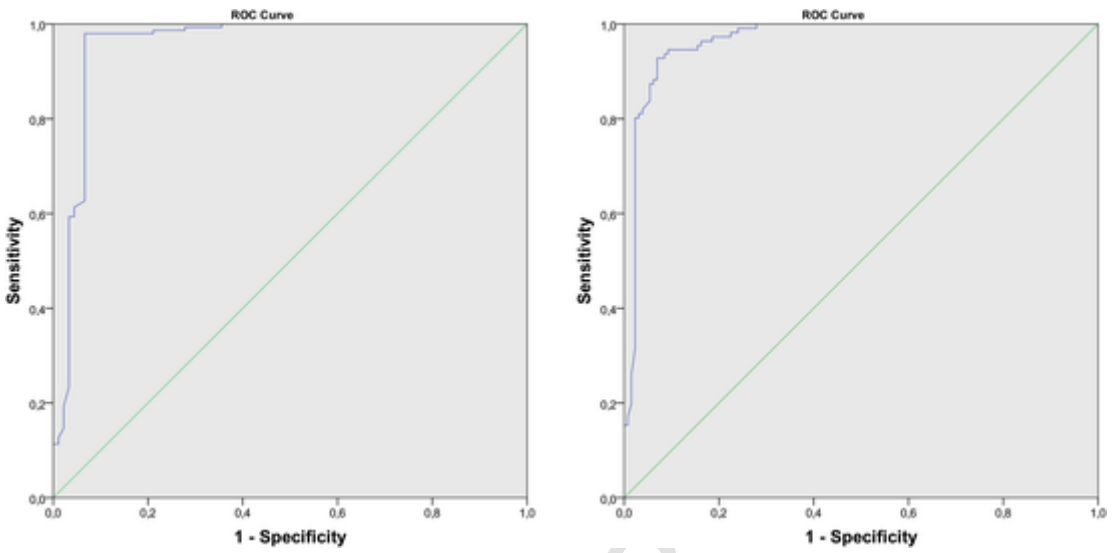

Fig. 3. ROC-analysis of PCA3 score in differentiation between PCa and all other cases (left graph) and differentiation between clinically significant PCa (ISUP grades 2–5) and all other cases (right graph).

ference in mean TMPRSS2:ERG expression values between subgroups of patients with PCa of ISUP grade 3 and ISUP grade 5 ($p = 0.032$), Fig. 4.

In correlation analysis, the TMPRSS2:ERG expression was associated with prostate cancer ISUP grade: a strong positive correlation was discovered, and the Pearson correlation coefficient amounted to 0.683 ($p < 0.001$). The detailed statistical characteristics of TMPRSS2:ERG expression in urine in subgroups of patients with PCa are presented in Table 3.

The ROC-analysis allowed differentiation between clinically significant and clinically insignificant PCa with a sensitivity of 94% and specificity of 100 % using the threshold of TMPRSS2:ERG expression in the urine of 29 RU (AUC = 0.980, 95% CI = 0.941–0.100, $p < 0.001$), Fig. 5.

4. Discussion

In the last decade, the progressive development of genomics and the introduction of genetic assays led to significant improvement in the detection of malignant diseases in oncologic urology. A wide spectrum of scientific data demonstrates a prominent potential of RNAs, mRNAs and microRNAs (miRNAs) measured in blood serum, urine and tissues as diagnostic tumour markers [21–25]. Howsoever, currently, the only tumour marker which is widely used for PCa detection is PSA although

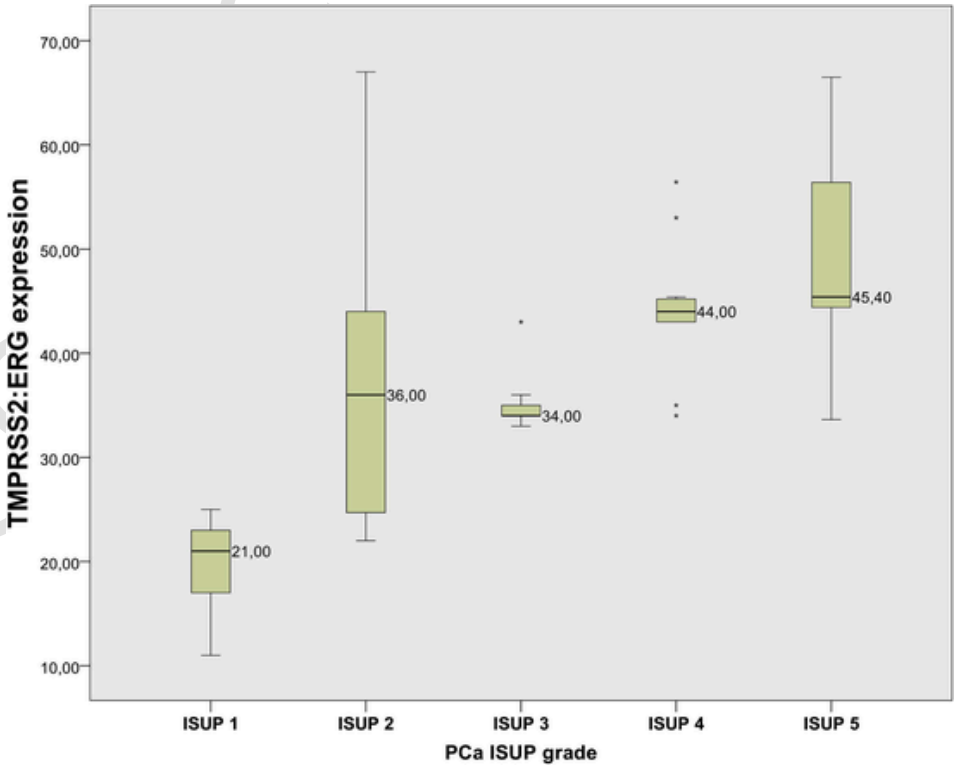

Fig. 4. Box plot of TMPRSS2:ERG expression in urine in subgroups of patients with PCa.

Table 3
The detailed statistical characteristics of TMPRSS2:ERG expression in urine in subgroups of patients with PCa.

	N	Mean	SD	95% Confidence Interval for Mean		Min.	Max.
				Lower Bound	Upper Bound		
PCa ISUP grade 1	6	19.67*	5.16	14.25	25.09	11.0	25.0
PCa ISUP grade 2	5	38.74*	18.10	16.26	61.22	22.0	67.0
PCa ISUP grade 3	11	35.04*, †	2.76	33.18	36.90	33.0	43.0
PCa ISUP grade 4	11	44.26*	6.50	39.89	48.63	34.0	56.43
PCa ISUP grade 5	6	48.62*, †	11.34	36.71	60.53	33.63	66.50
Total	39	37.84	12.36	33.83	41.85	11.0	67.0

PCa = prostate cancer, ISUP = International Society of Urological Pathology prostate cancer grade, SD = standard deviation.
* $p < 0.001$ when PCa ISUP grade 1 compared to ISUP grades 2–5.
†$p = 0.244$ when PCa ISUP grade 3 compared to ISUP grade 5.

it is not cancer-specific. In our study, we investigated the performance and limitations of a combination of long non-coding RNAs (PCA3, TMPRSS2:ERG, HOXC6 and DLX1) as urinary markers for improvement of PCa diagnostics.

PCA3 is the most prostate cancer-specific gene described to date. According to recent data, in real clinical conditions, the main indication for PCA3 score measurement in urine is a determination of whether a repeat prostate biopsy is necessary after a previously negative biopsy. According to Hessels et al., in a study of PCA3 measurement using quantitative RT-PCR assay in urine sediments after prostate massage for the detection of PCa, the test had a sensitivity of 67% and a negative predictive value of 90% [26]. Later Wei et al. demonstrated a 42% sensitivity with a specificity of 91% at a threshold PCA3 score of 60 in primary biopsy setting and a positive predictive value of 80% suggesting that this assay may be used in the primary diagnostics [27]. In our study, PCA3 urine expression was detected in all cases. ROC-analysis using a threshold PCA3 score of 56 allowed the differentiation between PCa of all grades and non-PCa with a sensitivity of 61% and specificity of 96% (AUC = 0.995, 95% CI = 0.920–0.989, $p < 0.001$) while a PCA3 score threshold of 50 resulted in a differentiation between clinically significant PCa and non-PCa with a sensitivity of 93% and specificity of 93% (AUC = 0.966, 95% CI = 0.943–0.989, $p < 0.001$). The hypothesis that higher PCA3 scores are associated with high-grade PCa is based on the suggestion that with decreasing differentiation, tumour cells become more invasive and could therefore more easily be spread into the ductal system of the prostatic gland after DRE. Although several studies failed to confirm this hypothesis: in most studies PCA3 independently predicted low-volume tumours and clinically insignificant PCa but was not associated with locally advanced disease and demonstrated only limited ability to predict high-grade cancer [28,29]. On the contrary, in our study we observed a strong positive correlation between PCA3 score and PCa ISUP grade, the Pearson correlation coefficient was 0.685 ($p < 0.001$).

According to literature another two most promising biomarkers for PCa are HOXC6 and DLX1. In a large study (n = 519 – main cohort; n = 386 – validation cohort) Van Neste et al. used reverse transcrip-

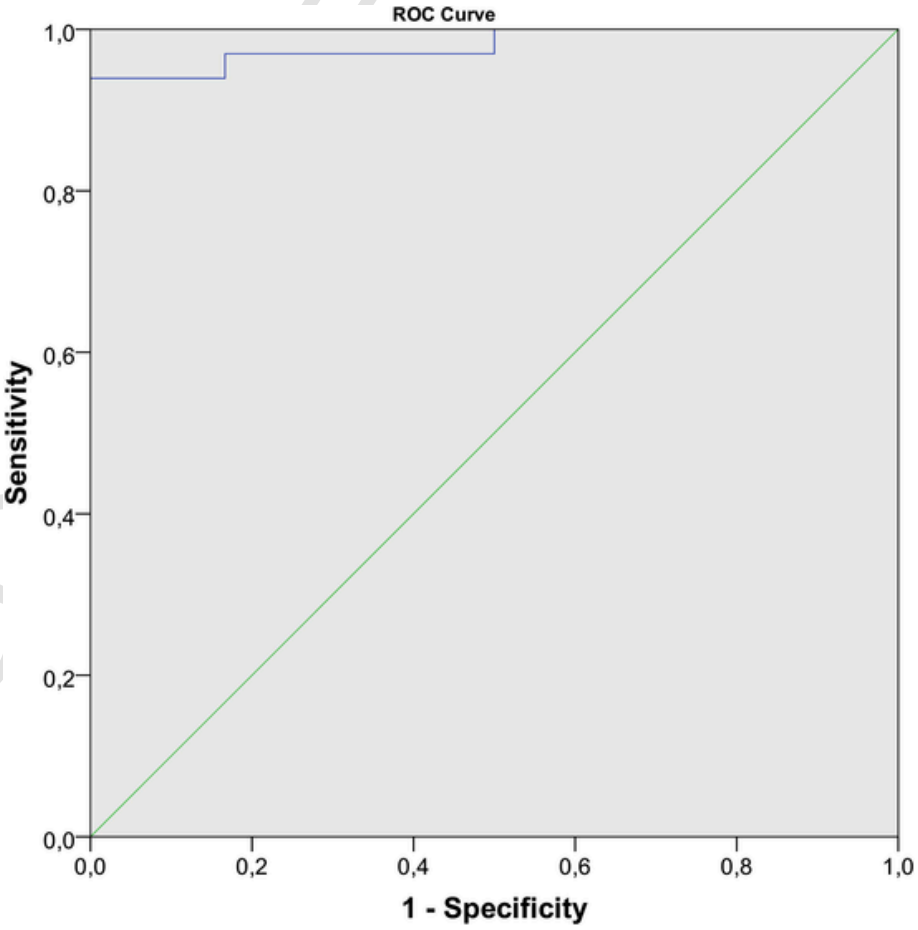

Fig. 5. ROC-analysis of TMPRSS2:ERG urine expression in differentiation between clinically significant and clinically insignificant PCa.

tion-quantitative polymerase chain reaction to measure HOXC6 and DLX1 mRNA urine levels: both markers in combination with clinical parameters such as PSA, PSA density, DRE, family history of PCa and age demonstrated good prediction ability for the detection of high-grade PCa with AUC of 0.90 in the validation cohort and with AUC of 0.86 in the training cohort [15]. Unlike PCA3, in our study, HOXC6 and DLX1 urine expression after the prostate massage was detected only in 43 (18%) and in 9 (3,75%) of 240 cases respectively. HOXC6 was detected only in patients with PCa and with CP, also there was no significant difference in mean marker expression values between both groups ($p = 0.057$). Taking into consideration, that in all PCa cases, urine expression of HOXC6 was detected only in a clinically significant variant of the disease, this marker could be potentially used only as an additive to PSA or PCA3 assays to decide the need for prostate biopsy. High expression of this marker in both groups with PCa and active CP can be explained by its potential role in either inflammatory and tumour-associated signalling pathways, however, this suggestion requires further investigations. The extremely low detection rate of DLX1 urine expression in our study was an unexpected finding and hindered the application of this marker for diagnostic purposes.

Earlier it was reported that ERG is the most commonly overexpressed oncogene in PCa. In Tomlins et al. study TMPRSS2-ERG fusion was detected in 50% of PCas whereas overexpression of these markers was never observed across BPH tissue samples [30]. In a large cohort ($n = 1225$), diagnostic models incorporating TMPRSS2-ERG had a significantly greater AUC compared to PSA in predicting PCa of all ISUP grades (0.693 vs 0.585, $p < 0.001$) and high-grade (ISUP grade > 1) PCa on biopsy (0.729 vs 0.651, $p < 0.001$) [13]. In our study TMPRSS2:ERG expression in urine after the prostate massage was detected only in a minor subset of cases, particularly exclusively in the group of patients with PCa (16%) of all ISUP grades. Likewise in PCA3, there was a significant difference in mean TMPRSS2:ERG expression in urine between subgroups of csPCa and ciPCa. The differentiation between clinically significant and clinically insignificant PCa using the threshold of TMPRSS2:ERG expression in the urine of 29 RU was possible with a sensitivity of 94% and specificity of 100 %, while the accuracy of the assay was the highest among all markers investigated in this study (AUC = 0.980, 95% CI = 0.941–0.100, $p < 0.001$). Such data to some extent correlates with previous studies and reflects the potentially important role of TMPRSS2:ERG in the detection of high-grade PCa, especially in cases when PSA or PCA3 tests are doubtful.

The main limitations of our study were: confirmation of PCa diagnosis based only on TRUS-Bx results and verification of CP diagnosis based only on clinical and laboratory data.

5. Conclusions

In our study PCA3 score detected in urine demonstrated moderate sensitivity and good specificity in differentiation between PCa and non-PCa and high sensitivity and specificity in differentiation between clinically significant PCa and non-PCa. Both HOXC6 and TMPRSS2:ERG markers could be used only in combination with PSA or PCA3 tests when their results are doubtful, with TMPRSS2:ERG preferred as it has shown the best indicators of sensitivity and specificity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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