Prostate cancer antigen 3 (PCA3) RNA detection in blood and tissue samples for prostate cancer diagnosis

Abstract

Background: The non-coding prostate cancer antigen 3 (PCA3) RNA is currently the most specific biomarker for prostate cancer (PCa) diagnosis. Although its clinical value has been validated in a urine assay after intensive prostatic massage, few studies have been conducted to establish its diagnostic value in the peripheral blood (PBL). The aim of the present study was to examine the PCA3 expression in blood as a diagnostic tool, and to provide an additional strategy to improve PCa diagnosis.

Methods: PCA3 transcripts were detected by RT-PCR in PBL and prostatic tissues from patients. PBL sampling also included a group of young healthy volunteers. The relationship between the PCA3 RNA detection and clinical characteristics was analyzed.

Results: PCA3 detection in blood presented 94% specificity and 32% sensitivity, and its combined detection in tissues significantly improved diagnostic parameters. However, PCA3 RNA detection in blood was also associated with PSA levels $\geq 10$ ng/mL, and their combination provided a sensitivity of 60% and specificity of 93%.

Conclusions: Detection of the PCA3 RNA in patients’ blood is an efficient tool for PCa diagnosis because it allows a routine collection procedure, which is also supported by the ongoing screening marker, prostate-specific antigen (PSA). We propose its combined use with PSA levels $\geq 10$ ng/mL, which improves accuracy, and prevents overdiagnosis and overtreatment.

Keywords: benign hyperplasia; gene expression; peripheral blood; prostate cancer; prostate cancer antigen 3; prostatic tissue; RT-PCR assay.
in vitro transcription and translation has not provided evidence that a small protein is produced from this gene yet [7]. Considering tumor biomarker specificity, PCA3 gene presents high expression in PCa and is not found in other types of cells or tissues [7, 8]. Significantly increased RNA levels in prostatic cancer cells can be detected in important targets for the clinical analyses as prostatic biopsies [7–9], urine after prostate massage [10–12] and PBL [13–15]. Although the PBL is routinely used in laboratory tests on a large scale, there is a lack of studies involving PCA3 detection in this biological sample.

Molecular technologies, such as the Polymerase Chain Reaction (PCR), have significantly facilitated the detection of blood markers in several types of cancer, which includes the detection of circulating tumor cells. Gene expression analyses for PCa have also been performed by both qualitative and quantitative Reverse Transcription PCR (RT-PCR) assays in PBL to establish associations with the presence of mRNA and the cancer occurrence, or disease recurrence and metastasis potential [14–19].

In our study, we aimed to evaluate PCA3 RNA expression, using RT-PCR technique, in prostatic tissues (PT) and PBL of patients, and associate them with disease clinical characteristics. Our work indicates that molecular monitoring of PCA3 RNA in PBL of patients can be used as an auxiliary test to correctly discriminate BPH from PCa, and may consequently reduce overtreatment due to the low specificity of the PSA-based screening assays.

Materials and methods

Materials

Ethidium bromide, agarose, Marine Moloney Leukemia Virus Reverse Transcriptase (MMLV-RT), RNase inhibitor, desoxribonuclease (dNTP), and DYEnamic ET Dye Terminator Cycle Sequencing kit were purchased from GE Healthcare Life Sciences (Sao Paulo, Brazil). Taq Platinum DNA Polymerase, TRIZol® Reagent, set of primers synthesized, hexamer random primers, PCR Enhancer System, MgCl₂, Topo TA Cloning™ kit, 50 and 100-bp ladder, and Top 10 bacteria were obtained from Invitrogen-Gibco (Carlsbad, CA, USA). Ammonium chloride, ammonium bicarbonate, Diethyl pyrocarbonate (DEPC), guanidine thiocyanate, and phenol were purchased from Sigma-Aldrich (Sao Paulo, Brazil) and chloroform was from Vetec (Rio de Janeiro, Brazil). All solutions were prepared using DEPC water.

Clinical samples

The biopsies were carried out by transrectal-guided ultrasonography (TRUS) in the Urology Service of the Clinics Hospital University of Uberlandia. Six fragments of each prostate lobe were collected for pathological analysis. All specimens were surgically resected between 2004 and 2006, and the final diagnosis was pathologically confirmed. The Institutional Research Ethics Board has approved this investigation under the number 005/2001, and informed consent was obtained from all patients and controls.

Tissue fragments were obtained from 57 PCa patients submitted to radical prostatectomy and 12 BPH patients submitted to transurethral resection (TURP) and were frozen in –80°C before RNA extraction. For PCa patients, the tumor stage was classified according to Gleason score and TNM (tumor-node metastasis) staging.

PBL samples from 179 individuals (69 patients and 110 healthy volunteers) were collected in two 5 mL Vacutainer™ tubes containing K₂EDTA 7.2 mg and maintained at 4°C. PSA levels were obtained through the IMMULITE 1000 System for quantitative detection (Siemens Healthcare Diagnostics Inc.), considering normal values between 0 and 4.0 ng/mL. All patients were free from preoperative chemotherapy, radiation, or hormonal therapy. Recruited healthy individuals (blood donors with no disease symptoms) presented median age of 23 years (ranging from 18 to 30), and PSA levels were not evaluated. BPH cases presented mean serum PSA levels of 5.6 ng/mL (ranging from 1.1 to 10.1) and mean age of 71 years (ranging from 47 to 82). The PCa cases were stratified according to their TNM, Gleason score and PSA levels. TNM T2 stage and Gleason score <7 represented the majority of cases (Table 1). The PCa cases presented general PSA mean of 16.1 ng/mL (ranging from 3.2 to 92.9) and mean age of 67 years (ranging from 50 to 87 years).

RNA isolation

Five milliliters of PBL samples were obtained from each patient before surgery at primary diagnosis. The total RNA procedure was performed using refrigerated centrifugation at 4°C. Serum was separated and discarded after separation at 2500 rpm (approximately 1000g) for 10 min and cells were precipitated using a salt solution containing nine parts of 0.144 M ammonium chloride to one part of 0.01 M ammonium bicarbonate. After homogenization and incubation at 70°C, the mixture was centrifuged at 3000 rpm for 15 min. The next steps were performed according to the instructions of TRIzol® reagent.

Table 1 Clinical pathological feature of patients.

<table>
<thead>
<tr>
<th>Individuals</th>
<th>TNM staging, n, %</th>
<th>Gleason score, n, %</th>
<th>Serum PSA levels, ng/mL Average±SD</th>
<th>Age, years Average±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteers</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>23.1±4.4</td>
</tr>
<tr>
<td>BPH</td>
<td>–</td>
<td>–</td>
<td>5.6±3.0</td>
<td>70.5±9.4</td>
</tr>
<tr>
<td>PCa</td>
<td>4/57 (7)</td>
<td>–</td>
<td>7.5±1.5</td>
<td>68.0±4.2</td>
</tr>
<tr>
<td></td>
<td>32/57 (56)</td>
<td>–</td>
<td>9.9±5.1</td>
<td>63.4±8.1</td>
</tr>
<tr>
<td></td>
<td>21/57 (37)</td>
<td>–</td>
<td>11.6±5.0</td>
<td>66.4±7.9</td>
</tr>
<tr>
<td>Gleason &lt;7</td>
<td>–</td>
<td>30/57 (53)</td>
<td>13.4±11.9</td>
<td>66.4±7.4</td>
</tr>
<tr>
<td>≥7</td>
<td>–</td>
<td>27/57 (47)</td>
<td>20.9±25.9</td>
<td>67.6±8.3</td>
</tr>
</tbody>
</table>

SD, standard deviation.
step for total RNA isolation was carried out using guanidine thiocyanate and phenol chloroform according to procedures described elsewhere [20]. The RNA was precipitated for 16 h with isopropanol at -20°C, washed with ethanol 75% and resuspended in 25 μL of DEPC water. One microgram of PT was homogenized using Trizol™ reagent according to manufacturer’s procedures for total RNA extraction. The RNA concentration and quality were analyzed in 1.5% agarose gel stained with ethidium bromide and its concentration was obtained spectrophotometrically by absorbance readings at 260 and 280 nm.

**Semiquantitative RT-PCR**

The high levels of PCA3 RNA in tissue have been reported by Real-time PCR [9, 21] and semiquantitative methods [22]. In this work, we have chosen the semiquantitative analysis for PCA3 expression in tissues because it is an efficient method for screening small samples sizes, simple, fast, and providing similar results to those provided by quantitative real-time PCR as observed elsewhere [23].

One microgram of total RNA from PT was reverse transcribed in a 20 μL final volume containing 40 U of MMLV-RT, 10 U of RNase inhibitor, 250 μM of each dNTP and 126 μM of hexamer random primers. The reactions were incubated at 37°C for 1 h and heated at 95°C for 5 min. The PCR reactions were performed with 2 μL of each RT product (cDNA) in 20 μL final volume containing 1.0 U of Taq Platinum DNA polymerase, 1X polymerase buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3), 1.5 mM MgCl₂, 200 μM of each dNTP and 5.0 pmoles of each primers. The primers sequences used for PCA3 were described elsewhere [22]. β-Microglobulin (B2M) transcripts were used for normalization of amplification reactions to validate and to further characterize RNA quality. The size of PCR amplicons for PCA3 and B2M genes were 277-bp (base pairs) and 534-bp, respectively. A set of primers for B2M was: forward 5′- AGCAGAGAATGGAAAGTCAAA-3′, and reverse 5′- ATCGATGACCCAAGATGGCG − 3′ (Genbank access No NM000484.2) [7]. The reaction was incubated for 29 cycles at 95°C/50 s, 94°C/40 s, 59°C/40 s, 72°C/50 s and final extension at 72°C/5 min. The number of PCR cycles was chosen to fall into the exponential phase of the amplification reaction, thus enabling a relative quantification.

**Nested RT-PCR**

Two micrograms of total RNA from PBL was reverse transcribed in a 20 μL final volume containing 40 U of MMLV-RT, 10 U of RNase inhibitor, 250 μmol of each dNTP and 126 μM of hexamer random primers. The reactions were incubated at 37°C for 60 min and heated at 95°C for 5 min. The first reaction were performed with 3 μL of cDNA in 20 μL final volume containing 1.0 U of Taq Platinum DNA polymerase, 1X Polymerase buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3), 1.5 mM MgCl₂, 200 μM of each dNTP, 10.0 pmoles of each primers.

The primers sequences for PCA3 were: forward 5′- AGATTTGTTGAGTGGACCACGAG-3′ (located at exon 1) and reverse 5′- TCCCTCGGACTCTTAAAGG − 3′ (located at exon 4) [7]. The reaction was incubated for 30 cycles at 95°C/40 s, 94°C/40 s, 59°C/40 s, 72°C/40 s and final extension at 72°C/5 min.

The nested-PCR reactions were performed with 5 μL of amplions from the first reaction in 20 μL final volume containing 1.0 U of Taq Platinum DNA polymerase, 1X PCR Enhancer System, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 200 μM of each dNTP, 10.0 pmoles of each primer. The primers sequences for PCA3 were: forward 5′- CCGAGGGAGACCCAGAAGAT − 3′ (located at exon 1) and reverse 5′- ATCGATGACCCAAATGTCGCG − 3′ (located at exon 4) [22]. The reaction was incubated for 35 cycles at 95°C/5 min, 94°C/40 s, 59°C/40 s, 72°C/50 s and final extension at 72°C/5 min.

The PCR reaction for B2M transcripts were performed separately, with 2 μL of cDNA in 20 μL final volume containing 1.0 U of Taq Platinum DNA polymerase, 1X polymerase buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3), 1.5 mM MgCl₂, 200 μM of each dNTP, 5.0 pmoles of B2M primers. The reaction was incubated for 35 cycles at 95°C for 5 min, at 94°C for 40 s, 59°C for 40 s, 72°C for 50 s and final extension at 72°C for 10 min.

**RT-PCR analyses**

The amplicons obtained for PCA3 and B2M transcripts in PT were analyzed and quantified according to their densitometric readings and were normalized as described previously [22]. Densitometric readings of signal intensity were performed using the ImageMaster VDS Software, version 2.0 (Amersham Biosciences). Signal intensities of amplicons were measured and the PCA3/B2M ratio was calculated. The cut-off ratio was established according to sensitivity and specificity diagnostic parameters, and values equal or higher than 0.2 were considered positive for PCA3 expression in PT. In the PBL, the expression was represented by 277-bp fragment detection in agarose gel.

**Cloning and sequencing**

To confirm that amplified fragments represent the PCA3 RNA sequence, two random bands with expected size of 277-bp were purified from agarose electrophoresis gel using 7.5 M ammonium acetate. The purified product was cloned using Topo TA Cloning™ kit and transformed into competent Top 10 bacteria following the manufacturer’s instructions. After plasmid extraction, sequencing was performed in an automatic capillary sequencer (MegaBace 1000) using the DY-Enamic ET Dye Terminator Cycle Sequencing kit. Three sequencing reactions were carried out for each fragment, using the universal M13 bacteriophage primer (5′- GGTACGTTACGACGTTGTA-3′). These reactions were injected twice to minimize sequencing artifacts. The sequencing results were aligned using Blast from National Center for Biotechnology Information (NCBI).

**Statistical analysis**

The frequencies of PCA3 expression between two groups were compared using Mann-Whitney’s U-test or the Fisher’s exact test when appropriate. Spearman’s correlation analyses were performed according to clinical features and PCA3 expression. Stratification of Gleason scores (<7 and ≥7) and TNM staging (T1-T2 and T3-T4) were performed for statistical analyses. Serum PSA levels were also dichotomized, with a cut-off value of 10 ng/mL. Odds ratio (OR) and ROC curves were calculated considering the cut-off values. Probability values below 0.05 were considered statistically significant. All data were analyzed using GraphPad Prism software (San Diego, CA, USA).
Results

The agarose gel electrophoresis for PCA3 RT-PCR reactions is represented in Figure 1, for which sequencing reactions have confirmed the transcript sequence specificity. The observed frequencies for PCA3 positivity detection in a PBL + PT combined data from PCa group were 44 out of 57 (77.2%) and four out of 57 (7.0%) of the patients presented detection in PBL only. No simultaneous PCA3 detection in PBL + PT was observed for BPH group and its positivity frequencies were represented for three out of 12 (25%) patients (PBL + PT- plus PBL - PT +) (Figure 2). The PCA3 was detected in six out of 110 (5.4%) healthy individuals in the PBL.

The results obtained for analysis in PBL and PT samples showed that PCA3 gene expression is significantly higher in PCa (p<0.0001). No difference was found in PCA3 nested RT-PCR between BPH and healthy volunteers groups. These samples were analyzed in the blood as PCa negative controls.

The PCA3 combined expression in PBL and PT for PCa cases was stratified into: PBL-PT-, PBL-PT+, PBL-PT- and PBL-PT+, and their clinical pathological features were considered (Table 2). No significant difference was observed among subgroups and Gleason score < or ≥ 7 and TNM classification system. However, a positive association was observed between frequencies of patients classified as PBL + PT+ and serum PSA levels ≥ 10 ng/mL (p=0.015). Other correlations were not found in this study.

The PCA3 expression presented a significant area under the curve (AUC), sensitivity and specificity for PBL or PT, or for their combination (p<0.006) (Table 3). In the PBL, the PCA3 detection showed sensitivity of 32% and specificity of 94%. The sensitivity was further improved by combining the PCA3 expression in both PBL and PT, with an accuracy of 82.1%. The PCA3 positivity in PBL associated with PSA levels ≥10 ng/mL also improved

### Table 2

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>PCA3 RNA expression</th>
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<tbody>
<tr>
<td></td>
<td>PBL-PT-</td>
</tr>
<tr>
<td>PSA &lt;10 ng/mL (n=29)</td>
<td>8 (27.6%)</td>
</tr>
<tr>
<td>≥10 ng/mL (n=28)</td>
<td>5 (17.9%)</td>
</tr>
<tr>
<td>p=0.015</td>
<td></td>
</tr>
<tr>
<td>Gleason &lt;7 (n=30)</td>
<td>7 (23.3%)</td>
</tr>
<tr>
<td>≥7 (n=27)</td>
<td>6 (22.2%)</td>
</tr>
<tr>
<td>p=0.369</td>
<td></td>
</tr>
<tr>
<td>TNM T1–T2 (n=36)</td>
<td>8 (22.2%)</td>
</tr>
<tr>
<td>T3 (n=21)</td>
<td>5 (23.8%)</td>
</tr>
<tr>
<td>p=0.751</td>
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</table>

Figure 1 Quantitative and nested RT-PCR for PCA3 expression analysis.

Duplicated assays for seven PCa and six BPH tissue samples are represented in (A) and (B), respectively. RT-PCR for B2M endogenous control (C) and Nested RT-PCR for PCA3 detection in peripheral blood (D). Amplified fragments for B2M and PCA3 presenting 534-bp and 277-bp, respectively. Black arrows indicate bands with 300-bp (A, B and D: 100-bp ladder, C: 50-bp ladder).
sensitivity to 60%, with a slight decrease in the specificity (93%) because one BPH patient presented PSA higher than 10 ng/mL. Considering that the young healthy controls present low levels of PSA, they were also included into the estimate of specificity for PBL + PSA ≥10 ng/mL (Table 3).

The estimated OR for cancer occurrence with positive PCA3 detection in PT was 11.76 (95% CI 2.3–59.5, p=0.002), and for PBL was 7.6 (95% CI 2.9–19.5, p=0.000). The OR for the combination of PCA3 expression in both PBL and PT was 44.8 (95% CI 18.3–114.8, p<0.001), and for PBL + PSA ≥10 ng/mL was 21.07 (95% CI 8.6–51.3, p<0.001).

Discussion

This study shows a simple strategy to molecularly detect the non-coding PCA3 RNA in the PBL of patients as an auxiliary tool for PCa diagnosis. Currently, diagnosis is based on a previous triage using a PSA-based screening; however, the PSA test has been severely criticized for the excess false-positives, due to its inability to discriminate benign diseases, inflammation and infection from cancer, which has led to overdiagnosis and overtreatment [5]. Furthermore, PSA is not even an ideal biomarker for organ-confined or advanced tumor stages, since it is not correlated with morphological changes or molecular alterations in tumor cells [3]. Therefore, other strategies for diagnosis are urgently needed.

Molecular analyses with many biomarkers, mainly using RT-PCR, have been performed in the PBL of PCa patients, and also in prostatic benign inflammatory diseases; however, controversial results and interpretations have been obtained, which prevented their use. Among several factors that might explain these divergent results, we can include: use of different primers sets; variation in RT-PCR protocols; improper patients’ stratification and disease staging, which are based only on morphological alterations without considering the molecular status; molecular detection performed shortly after TRUS biopsy that may detect transient or circulating prostate cells in the PBL; high levels of false-positive results in the blood based on PSA levels, illegitimate transcripts of non-prostate tissues; evaluation of only one prostate-related transcript or a unique tissue (PBL, lymph node, prostatic biopsies, or bone marrow) [16–19]. However, the non-coding RNA of the PCA3 gene has been recently approved as a biomarker for molecular diagnosis, which has been demonstrated to be prostate and tumor-specific [7], and it is basically a urine-based test that previously required an intense prostatic massage [10].

Differently from the previous assay, our investigation demonstrates the usefulness of the PCA3 detection in the PBL assay developed by our group [13], which minimizes the invasiveness and improves specificity, reaching similar accuracy levels as the previous urine assay [8–11, 14, 15]. It is important to note that controversies in PCa diagnosis using PCA3 urine assay and PSA levels still exist [12].

The PCA3 RNA is associated with PCa and is specific to this gland with no expression in lung, esophagus, ileum, colon, pancreas, testis, breast and bladder cancers [9]. The PCA3 detection in PBL for PCa diagnosis was first demonstrated by our research group using a nested RT-PCR assay [13], and in the present work we have further corroborated its usefulness by introducing the diagnostic parameters for both PBL and biopsies. In these samples, PCA3 transcript levels were significantly higher in PCa in comparisons to BPH. By combining PBL and PT results from the same patients the diagnostic parameters were enhanced, but not significantly different from the PBL alone. This could be explained by the heterogeneous and multifocal nature of the tumor, further complicated by sampling problems in biopsies, which are divided for pathological and molecular analyses. Besides, morphological evaluations do not correlate with molecular data because molecular changes precede morphological alterations. Our data indicate

<table>
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<tr>
<th>Clinical parameters</th>
<th>PCA3 RT-PCR</th>
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<tbody>
<tr>
<td></td>
<td>PBL</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>31.6% (18/57)</td>
</tr>
<tr>
<td>Specificity</td>
<td>94.3% (115/122)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>74.3% (133/179)</td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>0.63 (0.54–0.72)</td>
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</tbody>
</table>

Table 3 Diagnostic parameters for PCA3 detection by RT-PCR assay in peripheral blood.

CI, confidence interval.
that the PCA3 detection in the blood can be employed in routine diagnosis of PCa, and could be a useful auxiliary tool for clinical decisions to carry out further analysis with biopsies. A negative result does not imply an absence of cancer, but prevents overdiagnosis with a specificity of 94%. The lack of sensitivity of the PCA3 assay in PBL can be improved by monitoring patients that have reached PSA levels over 10 ng/mL.

Regarding the PCA3 positivity in PBL of BPH patients, an occult focal cancer cannot be excluded, which may have been misdiagnosed during the biopsy analysis, and may further corroborate that molecular alterations are not accompanied by morphological changes. Moreover, the positivity observed in healthy young individuals may possibly indicate that these may be future PCa patients, but a clinical follow-up should be carried out to verify this possibility, although it may cause significant psychological stress for these specific individuals. Interesting to note that more than 90% of PCa are sporadic and are associated with environmental factors, and <10% are conferred by hereditary factors [24]. Therefore, the 5% PCA3 positivity observed in those healthy individuals could be originated from familial PCa, but this should be also an investigation that must be carried on.

The diagnostic parameters obtained in our study are similar to those validated for the urine assay, but it is superior if considered that it is less invasive, and could be readily used in the routine blood collection without the overview of specialized clinicians; while the urine collection requires a prior prostatic massage, which has led to a great discomfort to the patient [12].

It is worth mentioning that other strategies have been used with PCA3 in PBL, which include the capture of circulating tumor cells with anti-EpCAM coupled to magnetic particles, or in combination with anti-PSMA, but it was not able to detect the PCA3 expression in hormone-independent cancer patients [15]. Another study that attempted PCA3 detection in PBL using real-time qPCR was not effective, and showed a very low sensitivity, with expression observed only in metastatic patients [14]. This could be due to the specific target site, primers’ set, or even the strategy of using specific buffer conditions, such as the enhancer system used in this study. The small number of patients and controls used in this investigation preconizes additional studies to further validate our findings in the PBL, but our results present a significant robustness when considering the careful clinical analysis and stratification of patients.

Stratifying the sensitivity parameter within the combined assay (PSA/PCA3), we can conclude that PSA levels >10 ng/mL contribute to PCa detection with 16 additional cases (28%). The combined results led us to propose their concomitant use for PCa diagnosis, without losing specificity.

Therefore, our results also suggest combined analyses of PCA3 expression and PSA levels in the blood, which may be further explored not only for diagnostics, but also for future prognostic studies. This is supported in our study by the positive association between higher preoperative serum PSA levels and the positivity of PCA3 in both PBL and PT. This result may be linked to a possible PCa recurrence after treatment or surgery, suggested by the presence of higher number of circulating tumor cells in PBL that further increases PSA levels. Patients with PSA over than 10 ng/mL present higher chances for biochemical failure in postoperative analysis, and must be treated with adjuvant or neo-adjuvant approaches and/or prostatectomy for prevention of disease recurrence [19]. So, it is possible that triple detection of high PSA and PCA3 positivity in PBL and PT may be a good prognostic strategy that should be investigated.

In summary, the PCA3 qualitative detection in PBL can be efficiently used as an auxiliary tool in PCa diagnosis with excellent specificity, and its combination with PSA levels ≥10 ng/mL has further improved sensitivity and accuracy parameters. The PBL analysis does not modify the current procedures for sample collection, and may prevent overdiagnosis, reducing the unnecessary procedures related to decisions based on the PSA-based screening assay.

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Conflict of interest statement

Authors’ conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Financial support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication. Research funding: None declared. Employment or leadership: None declared. Honorarium: None declared.

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