The Time-Resolved Fluorescence-Based PCA3 Test on Urinary Sediments after Digital Rectal Examination; a Dutch Multicenter Validation of the Diagnostic Performance

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Abstract

Purpose: To improve the specificity in prostate cancer diagnosis and to prevent unnecessary prostate biopsies, especially in the serum prostate-specific antigen (PSA) “gray zone” between 3 and 15 ng/mL, the implementation of prostate cancer–specific markers is urgently needed. The recently discovered prostate cancer antigen 3 (PCA3) is such a promising prostate cancer marker. In a previous single institution study, the PCA3 urine test clearly proved to be of diagnostic value. Therefore, the diagnostic performance of the PCA3 urine test was validated in a multicenter study.

Experimental Design: The first voided urine after digital rectal examination was collected from a total of 583 men with serum PSA levels between 3 and 15 ng/mL who were to undergo prostate biopsies. We determined the PCA3 score in these samples and correlated the results with the results of the prostate biopsies.

Results: A total of 534 men (92%) had an informative sample. The area under the receiver-operating characteristic curve, a measure of the diagnostic accuracy of a test, was 0.66 for the PCA3 urine test and 0.57 for serum PSA. The sensitivity for the PCA3 urine test was 65%, the specificity was 66% (versus 47% for serum PSA), and the negative predictive value was 80%.

Conclusions: In this multicenter study, we validated the diagnostic performance of the PCA3 urine test in the largest group studied thus far using a PCA3 gene-based test. This study shows that the PCA3 urine test, when used as a reflex test, can improve the specificity in prostate cancer diagnosis and could prevent many unnecessary prostate biopsies.

Nowadays, prostate cancer (PCa) is by far the most common cancer in men in the United States (excluding basal and squamous cell skin cancers and in situ carcinoma except urinary bladder). In 2006, PCa will be diagnosed in ~234,460 American men, and 27,350 American men will die of this disease (1). In Europe, in 2002, an estimated 225,227 men were newly diagnosed with PCa, and about 83,066 died from this disease; for the Netherlands, these numbers were 7,112 and 2,529, respectively (2).

Serum prostate-specific antigen (PSA) is regarded as the standard diagnostic PCa marker. PCa awareness, leading to widespread use of PSA testing, has led to a stage shift, i.e., a lower tumor stage and grade at the time of diagnosis. However, PSA is not cancer specific, resulting in a high negative biopsy rate. Moreover, its use is associated with certain drawbacks, e.g., the diagnosis of clinically irrelevant tumors (i.e., overdiagnosis) and potential overtreatment (3).

As a result, there is an urgent need for PCa-specific markers that can improve the specificity in PCa diagnosis and can prevent unnecessary prostate biopsies, especially in the serum PSA “gray zone” between 3 and 15 ng/mL, in which nowadays many newly diagnosed men are.

PCa antigen 3 (PCA3) is a prostate-specific noncoding mRNA that is highly overexpressed in more than 95% of primary PCa specimens and PCa metastases (4, 5). Hessels et al. found that the median up-regulation of PCA3 in PCa tissue compared with normal prostate tissue was 66-fold (6). Moreover,
a median 11-fold up-regulation was found in prostate tissues containing <10% of PCA cells. As PCA3 is a noncoding mRNA, a dual time-resolved fluorescence (TRF)-based reverse transcription-PCR (RT-PCR) assay was developed to identify it as a diagnostic marker in prostate cells in urine sediments after digital rectal examination (DRE). This second-generation test showed a negative predictive value of 90% in a population of men, admitted for prostate biopsies based on a serum PSA value >3 ng/mL. Therefore, PCA3 clearly showed to have diagnostic value and great potential in reducing the number of unnecessary biopsies (6). Using the first-generation UPtM3 RT-PCR assay, a qualitative nucleic acid sequence–based amplification technology, two independent studies confirmed these results (7, 8). Earlier this year, Groskopf et al. introduced the quantitative automated probe transcription-mediated amplification PCA3 urine test, a third-generation PCA3 assay that uses transcription-mediated amplification, a RNA transcription amplification system using RNA polymerase and reverse transcriptase to drive the isothermal reaction that allows the reaction to be carried out in a single tube format (9).

Because of the promising results of our previous single institution study (6), the aim of this study was to validate the diagnostic performance of the TRF-based PCA3 assay in a multicenter setting. Therefore, we correlated the PCA3 score in urine sediments after DRE with the presence of PCA in subsequent prostate biopsies. Here, the results of the TRF-based PCA3 urine test are reported in a group of 583 men, the largest group studied thus far, using a PCA3 gene-based test.

Materials and Methods

In the urological outpatient clinics of five Dutch hospitals (one university hospital and four community hospitals), the first voided urine after DRE was collected from a total of 583 men with serum PSA levels between 3 and 15 ng/mL (extremes included) who were to undergo ultrasound-guided, transrectal, prostate biopsies as a result of local management. Beforehand, all men had received study information and had signed their informed consent.

As part of standard clinical practice, both serum PSA and the fraction of free serum PSA had already been determined in most men. All other samples and data were collected prospectively. The DRE was done according to a standard protocol: by applying firm pressure to the prostate (enough to depress the surface) from the base to apex and from the lateral to the median line for each lobe. The men were asked to void, and the first voided urine was collected.

Following urine collection, the urologist measured the prostate by transrectal ultrasonography and did the prostate biopsy according to a standard protocol (at least three biopsies from the left peripheral zone, at least three biopsies from the right peripheral zone, one biopsy from the left transition zone, and one biopsy from the right transition zone, plus additional biopsies from other areas suspicious for PCA when present). All biopsy cores were treated lege artis and examined for the presence of PCA.

The first voided urine after DRE was collected in a coded container with 4 mL 0.5 mol/L EDTA. All samples from the four community hospitals were immediately cooled to 4°C and were mailed in batches with cold packs to the laboratory in the university hospital. The samples were processed within 48 h after the sample was acquired to guarantee good sample quality. The samples taken at the university hospital were processed within 1 h. Upon centrifugation at 4°C and 700 ×g for 10 min, urine sediments were obtained. These urinary sediments were washed twice with ice-cold PBS (at 4°C and 700 ×g for 10 min), snap-frozen in liquid nitrogen, and stored at −70°C. The urinary sediments were added to 20 μg of *Escherichia coli* tRNA as a carrier (Roche Diagnostics, Almkerk, the Netherlands). Total RNA was extracted from these urine sediments, using the TRIzol reagent (Invitrogen, Breda, the Netherlands).

To correct for the number of prostate cells present, the PSA transcripts were quantitatively determined in the urinary sediments using the dual TRF-based, quantitative RT-PCR protocol for PSA described by Ylikoski et al. (10, 11) and modified by Hessels et al. (6). Similarly, the PCA3 transcripts were quantitatively determined in the same urinary sediments using a dual TRF-based, quantitative RT-PCR protocol for PCA3. Briefly, *in vitro* transcribed PCA3 RNA and internal standard (IS) PCA3 RNA were used as templates for cDNA synthesis using the first-strand cDNA synthesis Kit (Amersham, Buckinghamshire, United Kingdom). PCA3 and IS-PCA3 RNA were diluted in 0.2 mg/mL *E. coli* tRNA (Roche Diagnostics), which is used as carrier RNA. For the preparation of a calibration curve, 5,000 copies of IS-PCA3 RNA were mixed with a variable amount (50 to 10,000,000 copies) of PCA3 RNA. For the quantification of PCA3 RNA in a sample, 5,000 copies of IS-PCA3 RNA were added to each RNA sample before the reverse transcriptase reaction. The RNA samples were heated for 10 min at 65°C followed by reverse transcription for 1 h at 37°C, using 0.2 mg of universal oligo-d(T)18 primer, 2 mmol/L DTT and 5 mL of a bulk first-strand reaction mixture (Amersham). For PCR amplifications, the following PCA3-specific primers were used: forward, 5′-TGCGAACGACCTGATGATACA-3′ (nucleotides 97-108 of exon 1 of the PCA3 cDNA, GenBank accession number AF103907) and reverse, 5′-CCCCAAGGATCTCGTGGTCTT-3′ (nucleotides 459-477, spanning exons 3 and 4 of the PCA3 cDNA). After 35 cycles of PCR, the amplification products were quantified by the TRF-based hybridization assays on streptavidin-coated microtiter wells. For the target-specific detection, a PCA3 detection probe (30 pg/mL) labeled with Eu³⁺ and an IS-PCA3 detection probe (30 pg/mL) labeled with Tb³⁺ were used. The amount of PCA3 mRNA in the sample was calculated by comparing the PCA3/IS-PCA3 fluorescence ratio in the sample with that of the samples in the calibration curve. The ratio PCA3/PSA mRNA was then calculated by dividing the number of PCA3 mRNA copies by the number of PSA mRNA copies obtained in a given sample. The PCA3 score was defined as the ratio PCA3/PSA mRNA × 1,000.

Data were analyzed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL) version 12.0.1 for Microsoft Windows. To test for differences in PCA3 score between men with a negative biopsy result and men with a positive biopsy result, we used the two-tailed Mann-Whitney U test. We also used the two-tailed Mann-Whitney U test to test for differences in PCA3 score between the biopsy-negative university hospital population and the biopsy-negative community hospital population, as well as between the biopsy-positive university hospital population and the biopsy-positive community hospital population. A P value <0.05 was considered significant.

Results

The total number of urine samples that could be analyzed successfully (i.e., was positive for PSA mRNA expression) was 534 out of the 583 that were collected. Therefore, the analytic performance of the PCA3 urine test was 92% (534/583) overall; 95% (295/312) in the university hospital; and 88% (239/271) in the community hospitals.

The mean age at the time of biopsy of the 534 men in the study population was (mean ± SD) 64.3 ± 7.2 years; the mean serum PSA value was 7.49 ± 2.93 ng/mL; the mean fraction of free serum PSA (determined in 173/534 = 32%) was 0.19 ± 0.11; and the mean total prostate volume (measured in 250/534 = 47%) was 48.2 ± 28.4 mL.
Of the 534 men who yielded informative specimens, 174 (33%) had PCa in their biopsies, and the remaining had negative biopsies. The PCA3 scores obtained for both subject groups were summarized in a box plot (Fig. 1). The median score for men with a negative biopsy result was 24 (range, 0-1,862; the median score for men with a positive biopsy result was 90 (range, 0-4,088). The difference between both groups was highly significant ($P = 1 \times 10^{-9}$).

The diagnostic efficacy of the PCA3 test is visualized by a receiver-operating characteristic curve, in which the test variable was the PCA3 score and the state variable was the biopsy result (Fig. 2). In the absence of an arbitrary cutoff value, we determined a cutoff value of 58 for PCa diagnosis based on this receiver-operating characteristic curve. A lower cutoff value would not have increased the sensitivity of the test, but would have only resulted in a loss of specificity. A similar procedure was described earlier (6). The area under the curve (AUC), a measure of the diagnostic accuracy of a test, was 0.66 [95% confidence interval (95% CI), 0.61-0.71] for the PCA3 test. The diagnostic value of the serum PSA test is also visualized (Fig. 2); for serum PSA, the AUC was 0.57 (95% CI, 0.52-0.63). For the serum free PSA test, the AUC was 0.58 (95% CI, 0.48-0.68; data not shown).

Using this cutoff of 58 for the detection of PCa by the PCA3 urine test, we calculated the sensitivity, specificity, and the negative predictive value. The sensitivity was 65%; the specificity was 66%; and the negative predictive value was 80% (Table 1). For the serum PSA test at the same sensitivity (65%), the specificity was 47% (Fig. 2).

A higher PCA3 score also correlated with a higher probability of a positive biopsy result (Fig. 3).

The diagnostic performance of the TRF-based PCA3 urine test was compared between the university hospital population and the community hospital population.

In the university hospital, the median PCA3 score in men with a negative biopsy was 11 (range, 0-1,779); the median PCA3 score in men with a positive biopsy was 73 (range, 0-1,432). The AUC for the PCA3 test was 0.65 (95% CI, 0.58-0.72). Using the cutoff value of 58 (determined in the total multicenter population), the sensitivity for the detection of PCa...
of the PCA3 urine test in the university hospital population was 59%, the specificity was 74%, and the negative predictive value was 79%. The AUC for the serum PSA test was 0.55 (95% CI, 0.48-0.62).

In the four community hospitals, together, the median PCA3 score in men with a negative biopsy was 39 (range, 0-1,862), the median PCA3 score in men with a positive biopsy was 107 (range, 0-4,088) and the AUC for the PCA3 test was 0.67 (95% CI, 0.60-0.75). Again, a cutoff value of 58 was used, and in the community hospital population, the sensitivity was 72%, the specificity was 56%, and the negative predictive value was 80%. The AUC for the serum PSA test was 0.61 (95% CI, 0.53-0.68).

The differences in PCA3 score between the biopsy-negative university hospital population and the biopsy-negative community hospital population (P < 0.001), as well as between the biopsy-positive university hospital population and the biopsy-positive community hospital population (P < 0.01), were significant. The differences in PCA3 score between the biopsy-negative university hospital population and the biopsy-negative community hospital population (P < 0.001), as well as between the biopsy-positive university hospital population and the biopsy-positive community hospital population (P < 0.01), were significant.

**Discussion**

Because of the promising results of our previous single institution study, the aim of this study was to validate the diagnostic performance of the TRF-based PCA3 urine test in a multicenter setting. Therefore, the PCA3 score in urinary sediments after DRE was correlated with the presence of PCa in subsequent prostate biopsies. In our previous study, the sensitivity for the detection of PCa by the PCA3 test in urine was 67%, the specificity was 83%, and the negative predictive value was 90% (6). In this study, these values were 65%, 66%, and 80%, respectively. The high negative predictive value in particular suggests that the PCA3 test can be used to reduce the number of unnecessary biopsies. We have summarized the results of all reports on PCA3 gene-based urine testing, including our present study (Table 2). Most test performance characteristics in this multicenter study are somewhat lower than in the other studies, in particular when compared with our previous study. A possible explanation for this could be the fact that 55% of our study population consisted of mostly prescreened men who were referred to a university hospital. In this population, the AUC for the PCA3 urine test was lower than in the community hospital population and consequently brought down the overall test performance. Noteworthy is the fact that in the university hospital population, the PCA3 urine test still did much better than the serum PSA test because the latter was no better than the flip of a coin.

Formally, it was statistically incorrect to calculate the sensitivity and specificity for the serum PSA test because a serum PSA value between 3 and 15 ng/mL was a requirement to enter the study. However, for reasons of comparison, we showed that at an equal sensitivity of 65%, the specificity for the serum PSA test was only 47%, compared with 66% for the PCA3 urine test. This suggests that the PCA3 test can be used to improve the specificity in PCa diagnosis.

Overall analytic performance of 92% indicates that the transport and processing of the urine samples in a multicenter setting is feasible. The difference in analytic performance of the PCA3 urine test between the university hospital (95%) and the community hospitals (88%) could not be attributed to RNA degradation that could have occurred during transport because the median copy numbers of PCA3 and PSA mRNA in the samples from the community hospitals were higher when compared with the samples from the university hospital (data not shown).

The median PCA3 scores in both the biopsy-negative and the biopsy-positive groups of the community hospitals were significantly higher than the scores in both groups of the university hospital, respectively. As a result, when using the cutoff value that was determined in the total multicenter study population, the sensitivity of the TRF-based PCA3 urine test was higher (72%) for the study population of the community hospitals when compared with the sensitivity for the study population of the university hospital (59%). Consequently, the specificity was lower (56% versus 74%). The differences observed were not attributable to RNA degradation during transport. A possible explanation is the difference in study population between the university and the community hospitals. A clue in this direction is the fact that the serum PSA test did better in the community hospital study population, with an AUC of 0.61 instead of 0.55 for the university hospital study population. The median serum PSA value was also significantly higher in the community hospital study population (data not shown). Therefore, the university hospital study

<p>| Table 2. The performance in prostate cancer diagnosis of PCA3 gene-based analysis in urine samples following DRE |</p>
<table>
<thead>
<tr>
<th>PCA3 urine test method</th>
<th>Total number of urine samples obtained</th>
<th>Number of informative urine samples</th>
<th>Analytical performance, %</th>
<th>Number of men with prostate cancer (%)</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Negative predictive value, %</th>
<th>Area under the receiver-operating characteristic curve</th>
</tr>
</thead>
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<td>TRF-based</td>
<td>NA</td>
<td>108</td>
<td>NA</td>
<td>24 (22)</td>
<td>67</td>
<td>83</td>
<td>90</td>
<td>0.72</td>
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<tr>
<td>uPM3</td>
<td>517</td>
<td>443</td>
<td>86</td>
<td>152 (34)</td>
<td>66</td>
<td>89</td>
<td>84</td>
<td>0.86</td>
</tr>
<tr>
<td>uPM3</td>
<td>201</td>
<td>158</td>
<td>79</td>
<td>62 (39)</td>
<td>82</td>
<td>76</td>
<td>87</td>
<td>0.87</td>
</tr>
<tr>
<td>Automated probe</td>
<td>70</td>
<td>68</td>
<td>97</td>
<td>16 (24)</td>
<td>69</td>
<td>79</td>
<td>89</td>
<td>0.75</td>
</tr>
<tr>
<td>transcription-mediated amplification</td>
<td>583</td>
<td>534</td>
<td>92</td>
<td>174 (33)</td>
<td>65</td>
<td>66</td>
<td>80</td>
<td>0.66</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not applicable.
population could have been more prescreened. The variance in the median value of the PCA3 score between different study populations and the fact that a higher score correlated with a higher probability of a positive biopsy result also suggest that it would be better to use the result of the PCA3 urine test as a continuous risk variable instead of strictly applying a certain cutoff value, resulting in a test with a dichotomous result.

In the group of men with a negative biopsy outcome, there were several men with a high PCA3 score (the outliers, 25/360 = 7% and extremes, 18/360 = 5% in Fig. 1). We speculate that in this group, a substantial number of men actually have PCa, but in whom it was missed on biopsy. It is well known that some 10% to 20% of men with a previous negative biopsy will be diagnosed with PCa upon repeat biopsy (12). Moreover, several men included in the study published by Hessels et al. (6) who had negative biopsies but a positive PCA3 urine test were shown to have PCa upon repeated biopsies. The follow-up data of our study population will have to show if indeed the PCA3 urine test was able to “predict” the presence of PCa.

In conclusion, this Dutch multicenter study showed that to improve the specificity in PCa diagnosis and reduce the number of unnecessary biopsies, the TRF-based PCA3 urine test is of value as a reflex test in the serum PSA “gray zone” between 3 and 15 ng/mL. At a cutoff value of 58, the sensitivity for the detection of PCa by the PCA3 test in urine was 65%, the specificity was 66% (compared with 47% for the serum PSA test), and the negative predictive value was 80%. A higher PCA3 score correlated with a higher probability of a positive biopsy result.

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