Frequent Methylation of RASSF1 and RARB in Urine Sediments From Patients with Early Stage Prostate Cancer

Kristina Daniūnaitė1, Artūras Berezniakovas1, Feliksas Jankevičius2,3, Aavydas Laurinavičius2,4, Juozas R. Lazutka1, Sonata Jarmalaitė1
1Faculty of Natural Sciences, Vilnius University, 2Faculty of Medicine, Vilnius University, 3Vilnius University Hospital Santariškių Klinikos, 4National Center of Pathology, Vilnius, Lithuania

Key words: prostate cancer; DNA methylation; urine sediments; RASSF1 gene, RARB gene.

Summary. Background. Prostate cancer (PCa) is the second most prevalent malignancy among males, characterized by high mortality rates. Aberrant DNA methylation in promoters of tumor suppressor genes is an early and frequent event during prostate carcinogenesis. Modern techniques allow a sensitive detection of DNA methylation biomarkers in bodily fluids from cancer patients offering a noninvasive tool for PCa monitoring. Our study aimed at the analysis of DNA methylation in urine sediments from PCa patients for the selection of most informative noninvasive biomarkers.

Material and Methods. Real-time methylation-specific polymerase chain reaction was used for the detection of methylated RASSF1, RARB, and GSTP1 genes in catheterized urine specimens from 34 patients with biopsy-proven early or medium stage PCa.

Results. At least one gene was methylated in urine sediments from 28 cases with PCa, with a sensitivity of the test reaching 82%. RASSF1 was methylated in 71% (24 of 34), RARB in 44% (15 of 34), and GSTP1 in 3% (1 of 34) of the specimens. High level of methylation (>50%) in RARB and RASSF1 genes was detected in 40% and 20% of cases, respectively. A significant association was observed between high level of RARB methylation and Gleason score (P=0.01), while methylation of at least one gene occurred more frequently in urine DNA of older patients (P=0.02).

Conclusions. Results of our study show a high sensitivity of DNA methylation biomarkers, especially RASSF1 and RARB, for the early and noninvasive detection of PCa.

Introduction
Prostate cancer (PCa) is the second most common cancer and the fifth leading cause of death from cancer among men worldwide (1). In 2009, more than 3000 new cases of PCa were diagnosed in Lithuania. In our country, it is the most prevalent cancer and the second leading cause of death among men (2). PCa can be effectively treated if it is diagnosed in its early stages, when the tumor is still confined to the prostate. Currently, prostate-specific antigen (PSA) is the only molecular biomarker routinely used for PCa detection and monitoring of disease recurrence (3). When the level of PSA is increased, prostate biopsy is recommended, with 4 ng/mL of PSA being the usual threshold level. However, this protein is specific for prostate tissue, but not for PCa. Such non-cancerous conditions as benign prostatic hyperplasia (BPH), prostatitis, or prostatic ischemia can cause the elevated levels of PSA (1, 4). The low specificity of PSA leads to a high number of unnecessary biopsies, causing avoidable patient discomfort. Therefore, the development of a minimally invasive, yet specific, tool to aid in the early detection of PCa is needed.
tumors have revealed a panel of genes frequently hypermethylated in PCa (3, 5). The most common epigenetic alteration in prostate carcinogenesis is hypermethylation in the promoter region of the glutathione-S-transferase P1 (GSTP1) gene that leads to decreased expression of an intracellular detoxification enzyme. GSTP1 promoter methylation is present in up to 90% of prostate cancer tissues and two-thirds of intraepithelial neoplasia tissues but rarely is present in BPH tissue (7). Methylation of GSTP1 is also detectable, but with the lower frequencies, in plasma, urine, and ejaculates of PCa patients (7–16). Other genes that are commonly found methylated in prostate cancer include RASSF1, RARB, APC, MDR1, PTGS2, TIMP3, and CDH1 (3, 5). Most investigations of epigenetic changes in PCa have mainly focused on the assessment of prostate tissue, while only a limited number of studies (9, 15–18) have analyzed a panel of DNA methylation biomarkers in urine sediments from PCa.

In urine specimens from 34 cases with biopsy-proven prostate adenocarcinoma, a panel of most commonly methylated genes in PCa that are associated with various cellular processes, particularly, GSTP1 (glutathione S-transferase pi 1; detoxification of carcinogens), RARB (retinoic acid receptor beta; transcription regulation), and RASSF1 (Ras association (RalGDS/AF-6) domain family member 1; cell cycle regulation), were analyzed. Real-time (quantitative) methylation-specific polymerase chain reaction (QMSP) was used for the sensitive detection of methylated DNA in catheterized urine specimens. Associations between aberrant promoter methylation of these genes and patients’ demographic as well as clinical characteristics were examined. In addition, for comparison, the data on DNA methylation status of the analyzed genes in tumor tissues was available for the same set of cases from our previous study that showed high methylation frequencies of genes GSTP1, RARB, and RASSF1 by means of methylation-specific polymerase chain reaction (MSP) (Danjuainait et al., unpublished data). The main aim of our study was to determine the sensitivity and clinical utility of this novel noninvasive test based on the detection of methylated DNA in catheterized urine of cancer patients.

Materials and Methods

Patients and Samples. Urine sediments were collected from 34 previously untreated cases who were enrolled into the study of PCa biomarkers (LSSSF project No. C03/2007) under the approved protocol for biopsy-proven prostate adenocarcinoma. Biosamples were collected from January 2008 to August 2009 in the Department Urology, Vilnius University Hospital Santariškių Klinikos, after the approval of study protocol by the local Bioethics Committee, and all patients gave informed consent for participation in the study. Tumor and nonmalignant prostate tissues were also available from most of the patients involved in this study.

The cases were diagnosed with early- (pT2, n=21) or medium-stage (pT3, n=13) prostate carcinoma of score 6 (n=24) or 7 (n=10) according to the Gleason system. No cases had pelvic lymph node involvement or clinical information of distant metastases. The mean age of the patients was 63 years (range, 52–77 years).

Thirty milliliters of urine was collected into urine collection cups by catheterization during radical prostatectomy. Biosamples were centrifuged at 1000 rpm for 15 minutes at 4°C and stored at –70°C before DNA extraction.

DNA Extraction and Quantitative Detection of DNA Methylation. Urine sediments (2 mL) were centrifuged at 1500g for 20 minutes and washed with 500 μL of PBS twice. The supernatant was decanted, and DNA was extracted by digestion with proteinase K, followed by a standard phenol-chloroform purification and ethanol precipitation. Sodium bisulfite conversion of unmethylated (but not methylated) cytosine residues to uracil was performed as described previously (19). Briefly, 720 ng of genomic DNA was denatured with 3 M NaOH for 15 minutes at 37°C and then exposed to bisulfite modification with 2.3 M sodium bisulfite (pH 5.0) and 10 mM hydroquinone at 50°C for 16 h (all reagents from Sigma-Aldrich Inc., Gillingham, Dorsel, UK). Modified DNA was purified with the Wizard DNA Clean-up System (Promega, Madison, WI) and desulphonated with 3 M NaOH, and then precipitated with ethanol.

The bisulfite-modified DNA was then used as a template for QMSP. For the RARB, GSTP1, and RASSF1 genes, primers and hydrolysis probes (biomers.net, Ulm Donau, Germany) were designed according to published sequences (20, 21) to specifically amplify bisulfite-converted fully methylated DNA. A passive reference dye 6-carboxy-X-rhodamine (ROX) (Fermentas, Vilnius, Lithuania) was used for the normalization of fluorescence signal, in order to compensate for non–PCR-related variations in fluorescence. To normalize for DNA input in each sample, a myogenic differentiation gene 1 (MYOD1; primers from [21]) was used as a reference.

Fluorescence-based real-time PCR assays were carried out in a reaction volume of 20 μL consisting of 300 nM of each primer, 50 nM of probe, 1x Maxima Probe qPCR Master Mix (Fermentas, Vilnius, Lithuania), 30 nM of ROX, and 1 μL of bisulfite-converted DNA. PCR was performed in separate wells for each primer/probe set. Each sample was run in duplicate. Leukocyte DNA collected from healthy individuals was methylated in vitro using bacterial SsI methylase (New England BioLabs...
Inc., Beverly, MA) and was included in each assay as a positive control (methylated control, MC). Additionally, multiple nontemplate controls (NTCs) were included in each assay. All amplifications were carried out in 8-well strips (Applied Biosystems, Piscataway, NJ) on a Mx3005P system (Agilent Technologies Inc., Santa Clara, CA) under the following conditions: 95°C for 10 minutes followed by 50 cycles of 95°C for 30 s and 60°C for 1 minute. Fluorescence data were collected at the end of each 60°C step.

A run was considered valid when routinely included MCs gave a positive signal and NTC controls were not amplified. A urine sample was classified as valid if the quantification cycle (Cq) of MYOD1 did not exceed 40, and amplification was detected in all replicates for a particular gene. The results were generated using MxPro v4.0 software (Agilent Technologies Inc., Santa Clara, CA) (Fig. 1).

Amplification-based threshold was used to determine Cq values. The percentage of methylated reference DNA (PMR) for each locus was calculated by dividing the GENE:MYOD1 ratio of a sample by the GENE:MYOD1 ratio of fully methylated control DNA and multiplying by 100. In this study, a sample was considered positive for methylation for a specific gene if PMR was >0 and there was no amplification in NTC controls.

**Statistical Analysis.** The two-sided Fisher exact test was used for comparison of categorical variables. The difference was considered significant if P value was <0.05. Odds ratio (OR) and Mantel-Haenszel 95% confidence intervals (CI) for two binomial samples were calculated using the approximation of Woolf. PMR value of each methylated sample was estimated automatically by the MxPro v4.0 software.

**Results**

Of the 34 samples of urine sediments from PCa patients, at least one gene of the three genes (RARB, GSTP1, and RASSF1) included in the analysis was methylated in 28 cases (sensitivity of detection 82%), and 12 (35%) were positive for methylation of at least two genes. The frequencies of aberrant promoter methylation for RASSF1, RARB, and GSTP1 were 71% (24 of 34), 44% (15 of 34), and 3% (1 of 34), respectively. PMR values ranged from 0.62% to 100% for RASSF1 and from 6.35% to 100% for RARB (Fig. 2). High level of methylation (PMR≥50%) was found in 6 (40%) of the 15 cases for RARB and in 5 (20%) of the 25 cases for RASSF1. The only case with aberrant methylation for GSTP1 was 100% hypermethylated.

In further analysis, gene methylation status in urine sediments was compared to DNA methylation

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**Fig. 1.** Amplification plots of real-time methylation-specific polymerase chain reaction assay, generated with the MxPro v4.0 software

**Fig. 2.** Representative results of PMR values for RASSF1, RARB, and GSTP1 in urine sediments from patients with diagnosed prostate cancer.

MC, in vitro fully methylated leukocyte DNA used as a positive control; S1–S7, samples of urine sediments. DNA was partly methylated in samples S1, S4–S7 for RASSF1 and samples S5 and S7 for RARB. Samples S1 and S2 were fully methylated for GSTP1 and RASSF1, respectively, while no methylation for any of the genes was detected in sample S3.
status in tumor tissue from the same patient (Fig. 3). For such comparison, DNA methylation data collected by means of methylation-specific PCR were available for 32 prostate carcinomas (Daniūnaitė et al., unpublished data). Hypermethylation of RARB and GSTP1 was more common in carcinomas than urine sediments, while RASSF1 was more frequently methylated in urine sediments (Fig. 3). However, all the differences for the individual genes were statistically insignificant.

For 28 (88%) of the 32 patients, at least one gene with an identical methylation status in prostate carcinoma and urine was documented. When a gene was found to be methylated in prostate carcinoma, it was also methylated in urine sediments in 47% of cases for RARB, 100% of cases for RASSF1, and 5% of cases for GSTP1. For RASSF1 and RARB, in some cases (47% and 13%, respectively), a gene was found to be methylated in urine but not in carcinoma tissue. Next, we compared hypermethylation frequencies in urine samples according to demographic and clinical characteristics. Methylated RARB gene was more frequently detected in urine sediments from patients with higher prostate weight and in tumors of greater Gleason score; however, only quantitative measures of methylation showed statistically significant associations. High level of methylation (PMR≥50%) of RARB was significantly more common in patients with score 7 prostate tumors as compared to score 6 (5/6 and 1/9, respectively; P=0.01; OR, 40.0; 95% CI, 2.0–794.9). There were no other significant associations between promoter hypermethylation of a particular gene and patient's age, tumor size or stage, Gleason score, preoperative serum PSA level, and prostate weight (Table). However, hypermethylation of at least one gene was more frequently observed in patients aged >63 years than patients aged ≤63 years (18/18 and 11/16, respectively; P=0.02; OR, 17.7; 95% CI, 0.9–351.2).

**Discussion**

Examination of epigenetic alterations in prostate cancer tissues has previously identified several potential biomarkers; however, for routine clinical application, noninvasive tests are preferable. Our pilot study in a set of 34 cases with early or medium stage prostate adenocarcinoma showed a high sensitivity (82%) of a panel of three DNA methylation markers (RARB, GSTP1, and RASSF1) for noninvasive detection of PCa using DNA from catheterized urine specimens. However, for further validation of diagnostic utility and specificity of these biomarkers, the assessment of DNA methylation changes in age-matched control group is of primarily importance.

Hypermethylation of GSTP1 is an epigenetic biomarker most extensively studied in PCa. This alter-

**Table. Aberrant Promoter Methylation in Urine Sediments From Prostate Cancer Patients According to Clinical and Demographic Characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RASSF1</th>
<th></th>
<th>RARB</th>
<th></th>
<th>GSTP1</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>n/N</td>
<td>%</td>
<td>n/N</td>
<td>%</td>
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<td>Age</td>
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<tr>
<td>≤63 years</td>
<td>63</td>
<td>10/16</td>
<td>38</td>
<td>6/16</td>
<td>0</td>
<td>0/16</td>
</tr>
<tr>
<td>&gt;63 years</td>
<td>83</td>
<td>15/18</td>
<td>50</td>
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<td></td>
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<tr>
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<td>81</td>
<td>17/21</td>
<td>48</td>
<td>10/21</td>
<td>5</td>
<td>1/21</td>
</tr>
<tr>
<td>T3</td>
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<td>38</td>
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<td>Gleason score</td>
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<tr>
<td>6</td>
<td>75</td>
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<tr>
<td>&lt;8 ng/mL</td>
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<td>16/22</td>
<td>41</td>
<td>9/22</td>
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<tr>
<td>≥8 ng/mL</td>
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<td>8/11</td>
<td>31</td>
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<tr>
<td>Tumor size</td>
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<td>&lt;12 cm³</td>
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<td>≥12 cm³</td>
<td>64</td>
<td>9/14</td>
<td>43</td>
<td>6/14</td>
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<td>1/14</td>
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<tr>
<td>Weight of prostate**</td>
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<td></td>
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<td></td>
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<tr>
<td>&lt;62 g</td>
<td>68</td>
<td>15/22</td>
<td>32</td>
<td>7/22</td>
<td>5</td>
<td>1/22</td>
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<tr>
<td>≥62 g</td>
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<td>8/10</td>
<td>60</td>
<td>6/10</td>
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<td>0/10</td>
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</table>

PSA, prostate-specific antigen; N, number of analyzed cases; n, number of cases with methylated gene. *No data for one case; **no data for two cases. The mean value was used as a cut-off to stratify continuous variables into discrete values.
methylation has been detected in around 90% of malignant prostate tissues and, in a lower frequency, in bodily fluids from PCa patients (4, 5). Suh et al. were ones of those who first reported the presence of methylated GSTP1 promoter in ejaculates from 4 of the 9 patients with PCa (11). Later, other studies reported the presence of methylated GSTP1 in urine samples from patients with PCa (7, 12). Including the most recent studies, hypermethylation frequency of GSTP1 in urine sediments ranges from 18% to 83% (8, 10, 12–14, 16, 18). In our study, hypermethylation frequency of GSTP1 was only 3%, which was lower than frequencies indicated in previous studies. The discrepancy could be explained by the different methods used in various studies to obtain urine sediment samples and to detect DNA methylation. It has been noted before that prostate massage could increase the shedding of prostate cells and, thus, the amount of methylated DNA in urine (10, 16), while in our study, the samples were collected by catheterization during prostatectomy aiming to avoid additional procedures for the patients. Moreover, Jeronimo et al. (10) reported that conventional MSP was more sensitive than real-time PCR in the detection of GSTP1 hypermethylation in bodily fluids. In agreement with this notion, our assessment of DNA methylation by means of MSP (Fig. 3) rendered quite high GSTP1 methylation frequencies in prostate adenocarcinomas. In addition, rare detection of methylated GSTP1 in urine sediments can be impacted by predominance (69%) of the cases with early stage PCa in our study. The increased detection of GSTP1 methylation in bodily fluids from patients with PCa of pronounced aggressiveness was shown in several studies (14, 22).

Although the reported frequencies of GSTP1 methylation in urine sediments from PCa patients are comparably high, the combined analysis of several genes has a clear potential to increase the sensitivity of the test and minimize the false-positive rate. In urine sediments from PCa, the sensitivity of four-gene panel was 86%–87% in two studies (9, 16) that used real-time PCR for DNA methylation detection, while the large panel of 9 or 10 genes (9, 16) had a sensitivity of almost 100%. In our cohort of PCa cases, the combined sensitivity of 3 genes was 82%, and the most informative biomarkers of PCa were hypermethylated promoters of RASSF1 and RARB. Up to date, only three previous studies have included the RASSF1 and RARB genes in a panel of biomarkers analyzed in urine from PCa patients (9, 16, 17). The hypermethylation frequencies reported in these studies were 73%–78% and 35%–73% for RASSF1 and RARB, respectively (9, 16, 17). In present study, RASSF1 was determined to be most commonly methylated in our gene panel, with a frequency of 71%. The hypermethylation frequency of RARB (44%) also fits into the reported range. In agreement with other studies (7, 9, 12), our study demonstrated an association between DNA methylation status in urine sediments and tumor. In 88% of the patients, at least one gene with an identical methylation status in prostate carcinoma and urine was observed, confirming the origin of methylated DNA in urine from exfoliated tumor cells. However, in some cases, a gene was methylated in urine but not in carcinoma. It could be explained by technical differences in DNA methylation detection by MSP and QMSP assays, but mainly is attributable to multifocality of prostate cancer.

Several studies have shown not only a diagnostic but also prognostic potential of noninvasive DNA methylation tests. Associations have been detected between hypermethylation of several gene promoters in noninvasive specimens (serum, urine) from PCa patients and Gleason score, stage, or PSA value (14, 15, 23). Moreover, the presence of GSTP1 hypermethylation in serum DNA from PCa patients was shown to be a significant predictor of PSA recurrence (22). In our study, high level (≥50%) of RARB hypermethylation was significantly more common in urine from patients with PCa of greater Gleason score. Similarly, the quantitative measurement of DNA methylation in cancerous tissue revealed significant associations between increasing levels of methylation and advanced stage or greater score of PCa (24). In concern with previous data (17), our study detected more frequent DNA methylation in urine from older patients in support of the notion that aberrant methylation may accumulate during aging. Indeed, the age-related increase of DNA methylation levels in promoter region of the GSTP1, RASSF1, and RARB genes was shown in normal prostate tissues (25); however, age-matched prostate tumors contained significantly higher levels of methylation. Further analysis in urine samples from PCa patients and age-matched controls are of high importance to determine the aging-related and cancer-related levels of DNA methylation.

Conclusions

Results of our pilot study suggest that aberrant promoter methylation can be detected with high sensitivity in catheterized urine specimens from patients at early PCa stages. In addition, it shows that the panel of most informative genes may differ in various populations. In our cohort of patients with localized prostate cancer, RASSF1 and RARB, but not GSTP1, genes are the most sensitive biomarkers of PCa. We continue our analysis in a larger set of cases and controls to evaluate the specificity of DNA methylation biomarkers for PCa. After further validation of these biomarkers in larger-scale studies involving the patients from different study centers, the detection of aberrant methylation in urine DNA may become a promising tool for the noninvasive...
Dažnai metilinimo intensyvumas (≥50 proc.) nustatytas atitinkamai 1 proc. ir 20 proc. atvejų. Nustatytas statistiškai patikimas (p=0,01) ryšys tarp geno metilinimo ir ankstyvąjai neinvazinės priešinės liaukos vėžio diagnostikai.

**Raktažodžiai:** priešinės liaukos vėžys, DNR metilinimas, šlapimo nuosėdos, RASSF1, RARB.

**Santrauka:** Priešinės liaukos vėžys yra antra pagal dažnumą vyrų onkologinė liga, kuriai būdingos didelis mirtingumas. DNR metilinimo pokyčiai nustatytas 28 liaukos vėžių šlapimo nuosėdose (tyrimo jautrumas – 82 proc.). Genas RASSF1 buvo metilintas 71 proc. (24 iš 34), RARB – 44 proc. (15 iš 34), GSTP1 – 3 proc. (1 iš 34) atvejų. Didelis genų RARB ir RASSF1 metilinimo intensyvumas (>50 proc.) nustatytas atitinkamai 40 proc. ir 20 proc. atvejų. Nustatytas statistiškai patikimas (p=0,01) tarp geno RARB metilinimo ir naviko diferenciacijos lygio pagal Gleason skalę. Bent vieno geno metilinimas nustatytas tarp geno RARB metilinimo ir naviko diferenciacijos lygio pagal Gleason skalę. Bent vieno geno metilinimas nauzinius ligos biožymenį.

**Rezultatai:** Bent vieno geno metilinimas nustatytas 28 liaukos vėžių šlapimo nuosėdose (tyrimo jautrumas – 82 proc.). Genas RASSF1 buvo metilintas 71 proc. (24 iš 34), RARB – 44 proc. (15 iš 34), GSTP1 – 3 proc. (1 iš 34) atvejų. Didelis genų RARB ir RASSF1 metilinimo intensyvumas (>50 proc.) nustatytas atitinkamai 40 proc. ir 20 proc. atvejų. Nustatytas statistiškai patikimas (p=0,01) tarp geno RARB metilinimo ir naviko diferenciacijos lygio pagal Gleason skalę. Bent vieno geno metilinimas nauzinius ligos biožymenį.

**Išėdos:** Tyrimas rodo didelį DNR metilinimo biožymenų, ypač RASSF1 ir RARB genų, metilinimo jautrumą ir tinkamumą ankstyvajai neinvazinės priešinės liaukos vėžio diagnostikai.

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**Statement of Conflict of Interest**

The authors state no conflict of interest.

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