Received: 16 April 2023 | Accepted: 12 August 2023

DOI: 10.1002/pros.24615

ORIGINAL ARTICLE

The Prostate WILEY

Combination of DNA methylation biomarkers with multiparametric magnetic resonance and ultrasound imaging fusion biopsy to detect the local spread of prostate cancer

Augustinas Matulevičius MD[1,2,3] | Kristina Žukauskaitė MSc[1,2] | Rugilė Gineikaitė BSc[2] | Darius Dasevičius MD[4] | Mantas Trakymas MD, PhD[2] | Ieva Naruševičiūtė BSc[2] | Jurgita Ušinskienė MD, PhD[2] | Albertas Ulys MD, PhD[2] | Feliksas Jankevičius MD, PhD[3,5] | Sonata Jarmalaitė PhD[1,2]

[1]Institute of Biosciences, Life Sciences Center, Vilnius University, Vilnius, Lithuania

[2]National Cancer Institute, Vilnius, Lithuania

[3]Vilnius University Hospital Santaros Klinikos, Vilnius, Lithuania

[4]National Centre of Pathology, Affiliate of Vilnius University Hospital Santaros Klinikos, Vilnius, Lithuania

[5]Faculty of Medicine, Vilnius University, Vilnius, Lithuania

Correspondence

Sonata Jarmalaitė, PhD, Institute of Biosciences, Life Sciences Center, Vilnius University, 10257 Vilnius, Lithuania.
Email: sonata.jarmalaite@gf.vu.lt

Funding information

2014–2020 European Union Structural Funds, Grant/Award Number: J05-LVPA-K-04–0029

Abstract

Background: This study aimed to investigate the extent of field cancerization adjacent to index lesions in prostate cancer (PCa) by measuring DNA methylation of selected tumor suppressor genes in the perifocal tissue of PCa not visible on multiparametric magnetic resonanse imaging (mpMRI) for the safe zone of focal therapy identification.

Methods: A total of 272 patients were enrolled in this study, 44 patients' tissue biosamples were included in the field cancerization research, and 272 urine samples were included in the urine-based test development. Targeted biopsies were performed using the mpMRI/ultrasoundimage fusion system.

Results: Quantitative analysis revealed significantly higher DNA methylation levels of *RARB*, *RASSF1*, *GSTP1* & *APC* genes in the index lesion compared with perifocal tissue samples 10 mm away from it ($p < 0.0001$). Notably, the *RARB*, *GSTP1* & *APC* and *RARB*, *RASSF1*, *GSTP1* & *APC* biomarker combinations exhibited the highest sensitivity and specificity comparing the extent of DNA methylation in index lesions and noncancerous prostate tissues 20 mm away (both area under the curve [AUC] = 0.98; $p < 0.0001$). The analysis of the potential urinary biomarkers showed that the combination of all four DNA methylation biomarkers with prostate-specific antigen (PSA) or PSA density (PSAD) in the blood significantly improves the detection of clinically significant PCa (csPCa). The combination of the four-biomarker test with PSAD allowed the identification of csPCa with ≥90% sensitivity and specificity.

Conclusion: Thus, this study suggests that for focal therapy by region target hemi-ablation, the safe distance from the index lesion is no less than 10 mm. Noninvasive urine DNA methylation tests in combination with PSAD could be used for further follow-up of the patients, but larger prospective studies with external validation are needed.

KEYWORDS

epigenetic biomarkers, mpMRI/US, PCa focal therapy, prostate cancer

1 | INTRODUCTION

Prostate cancer (PCa) is a dominant oncological disease of males in Western Europe, including Lithuania.[1] It is known that PCa is characterized by wide clinical, histomorphological, and genetic heterogeneity.[2] Since prostate multiparametric magnetic resonance imaging (mpMRI) was started evaluating according to the PIRADS 2.1 score system the detection rate of clinically significant PCa (csPCa) foci was improved.[3] The most aggressive lesions detected by mpMRI can be reflected in index lesions on the final histopathology specimen and could be treated by focal therapy. On the other hand, mpMRI can only show the most aggressive PCa and clinically insignificant PCa can be missed. Based on the literature, magnetic resonance, and ultrasound imaging (MRI/US) fusion biopsy diagnosed up to 80% of index lesions and 20% mpMRI missed foci with potentially lethal cancer could come up to cancer progression in the near future.[4]

Even in the most contemporary nerve-sparing robotic prostatectomy series, severe incontinence and erectile dysfunction are reported at detectable rates that are not seen in any of the focal therapy cohorts. For many intermediate-risk patients for whom the risk of progression with active surveillance (AS) is unacceptably high, but for whom quality of life with whole-gland treatment would be too low, focal therapy offers a possible solution. However, many questions remain unanswered for every mode of focal therapy for PCa. High radical treatment-free survival is seen with focal cryotherapy over a medium-term period, but it has been reported as low as 70% at 5 years. Many patients receiving both cryotherapy and high-intensity focused ultrasound will need repeat focal treatment for definitive cancer control. The major argument against focal therapy lies in the undertreatment of patients with clinically significant disease. The consequences of improperly designating a patient for focal therapy may be profound. Patient selection criteria for focal therapy continue to evolve—mpMRI guidance, targeted fusion biopsy.[5] Due to diagnostic inconsistency, additional evidence-based parameters are needed for patient selection.

Nowadays evidence is increasing that molecular biomarkers can complement existing standard clinic diagnostic and prognostic tools, especially when a liquid biopsy is exploited. DNA methylation is the earliest, most stable, and most frequent alteration in the PCa genome and has been investigated in detail as a source of molecular biomarkers.[6–8] Frequent DNA hypermethylation of promoter region of PCa-associated tumor suppressor genes is a prominent feature observed during early prostate tumorigenesis, as well as during its local progression and potential metastatic development, and perhaps occurs much more frequently and consistently than genetic mutations.[9–11] This study was initiated to better understand the field cancerization effect next to PCa index lesions and to evaluate the DNA methylation extent of target tumor suppressor genes in perifocal tissue on mpMRI-nonvisible PCa and compare it to the normal prostate tissue and mpMRI visible PCa.

Current strategies for organ-preserving PCa ablative therapy have varied in their eligibility criteria but also in the amount of tissue targeted for destruction and/or preservation: nerve-sparing prostate ablation, hemi-ablation, hockey stick, and target focal therapy. Diseases (ICUD)/*Societ Internationale de Urologie* (SIU) defined image-guided focal therapy success as the eradication of the tumor focus on the short term, also the absence of clinically significant cancer in the intermediate to long-term. Within the untreated or out-field area for the development of clinically significant cancer. In the short term, this out-field cancer focus likely represents selection failure but in the intermediate-to-long-term, this may mean de novo cancer.[12]

Gene selection was based on the genetic pathway significance in prostate carcinogenesis and our previous experience on PCa biomarker research.[13,14] DNA methylation changes of selected genes were analyzed by quantitative means in tumor foci and perifocal prostate tissue in comparison to normal prostate tissue in PCa cases with mpMRI-diagnosed PCa. Therefore, we undertake to determine the safe area of focal therapy (10–20 mm), to evaluate which area needs to be affected to obtain long-term good results, believing that hypermethylation in the cancerization field is important in the progression of PCa.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

In total, 272 patients have been included in the study, 10 cases were excluded due to clinical and anatomical reasons (data of these cases are not shown). Forty-four patients with csPCa were included in the field cancerization research, and these patients were under radical prostatectomy (RP), all the rest were involved in the urine-based test validation. Clinical and radiological characteristics of all study cohort were presented in.[15] Clinical and radiological characteristics of the radical prostatectomy cohort are presented in Table 1.

2.2 | A multiparametric magnetic resonance imaging (mpMRI)

3 T mpMRI scans have been performed in the same institution and the PI-RADSv2.1 scoring system was used to report mpMRI results.[3] Contouring of the prostate margins and target lesions was performed by two experienced radiologists using the transverse T2 TSE images. The targeted prostate biopsies have been performed with the "BioJet" fusion system (D&K Technologies) using the transperineal approach by a single interventional radiologist. For all patients, lesion-targeted and systematic biopsies have been performed and every core taken was documented in three dimensions and registered to the mpMRI protocol (Figure 1).

Lesion-targeted biopsy of two to four cores have been taken from each PI-RADS three to five lesion, followed by a systemic 12-core biopsy ignoring index lesions, and additional four biopsy cores were taken from distinct range 10 and 20 mm from the index lesion for DNA methylation analyses. Four tissue specimens were collected

TABLE 1 Clinical and radiological characteristics of the radical prostatectomy cohort.

Variable		Patients (n = 44)
Age, years	Mean SD	62.92 7.29
PSA, ng/mL	Mean SD	8.24 6.68
PSA density, ng/mL/cc	Mean SD	0.19 0.18
Prostate volume, mL	Mean SD	46.64 20.71
mpMRI targets quantity, *n*	Mean SD	1.54 0.66
The number of index lesions after RP in the pathohistological protocol, *n*	Mean SD	2.72 1.82
mpMRI target volume, mL	Mean SD	1.37 1.69
mpMRI ADC value $\mu m^2/s$	Mean SD	543.70 250.64
PI-RADS V2.1 score, *n* (%)	4 5	25 (56.82%) 19 (43.18%)
ISUP grade group, *n* (%)	GG1 GG2 GG3 GG4	15 (34.1%) 19 (43.2%) 9 (20.5%) 1 (2.3%)

Note: CSPCa was defined as ISUP grade group ≥ 2.[16] The Vilnius Regional Biomedical Research Ethics Committee approved the study (No. 2019/11-166-654 and 2020-LP-68), and informed consent was obtained from all participants.

Abbreviations: ADC, apparent diffusion coefficient; GG, prostate cancer grade group; ISUP, The International Society of Urological Pathology; mpMRI, multiparametric magnetic resonance imaging; PSA, prostate-specific antigen; RP, radical prostatectomy.

for each patient using a 22 mm automatic punching device: two samples from index lesions, one from perifocal 10 mm away tissue, and another one (control sample)—20 mm away from index lesion; 20 mm away from the focus were obtained from another lobe of the prostate (Figure 2).

All biopsy samples and RP specimens have been evaluated by dedicated uropathologists (D. D.) from the National Center of Pathology (Vilnius, Lithuania) and reported according to the The European Association of Urology recommendations.[17] Formalin-fixed paraffin-embedded (FFPE) prostate tissues, after accurate histological evaluation, were sent to the National Cancer Institute, Laboratory of Genetic Diagnostics in Vilnius, Lithuania for DNA purification procedure and further genetic analysis. The RP specimen was fixed in 10% neutral buffered formalin for 24 h. After fixation tissue was serially sectioned at 3–5 mm intervals into perpendicular sections (about 4 mm thick). Sections were dehydrated and embedded in paraffin wax. Hematoxylin and eosin (H&E)-stained sections (5 µm thick) were produced for microscopic evaluation using whole slide images. Whole-mount histopathology (Figure 3) of RP specimens was evaluated and compared with mpMRI images in the same slices.[18] This technique was used to specify the location of prostate biopsies and to precisely evaluate the distances of the perifocal field biopsies from the index lesion to perform a qualitative analysis of hypermethylated genes.

2.3 | DNA extraction and bisulfite conversion

FFPE prostate tissues were pretreated with 1 mL of xylene (Carl Roth) for 10 min at 55°C twice. Xylene was removed from the samples by incubating them with 1 mL of ≥96% ethanol at 55°C for 10 min. Samples were further treated with 25 µL of proteinase K (Thermo Scientifc™, Thermo Fisher Scientific) for 18 h at 55°C and

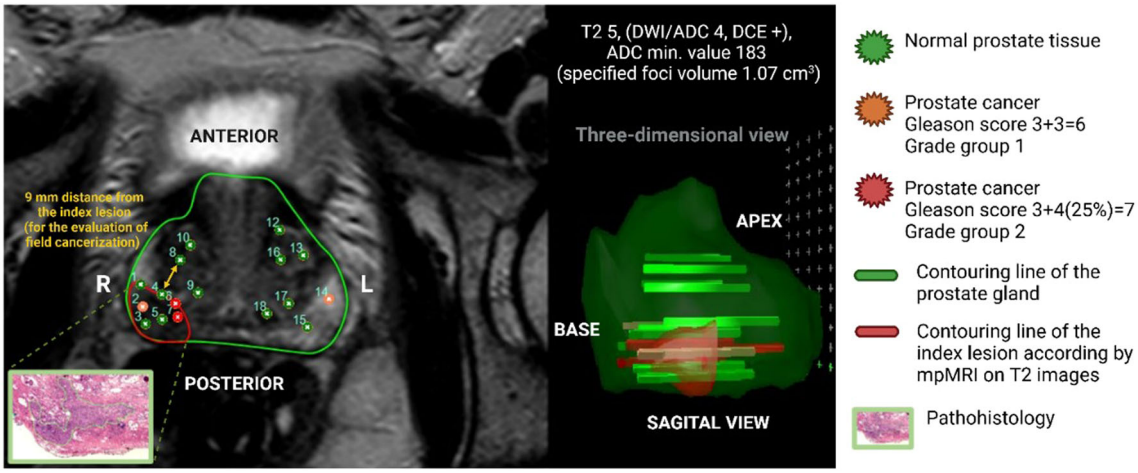


FIGURE 1 A three-dimensional picture of prostate gland multiparametric magnetic resonance imaging (mpMRI) after prostate biopsy with mapping of histopathology. Original image modified by BioRender.com. [Color figure can be viewed at wileyonlinelibrary.com]

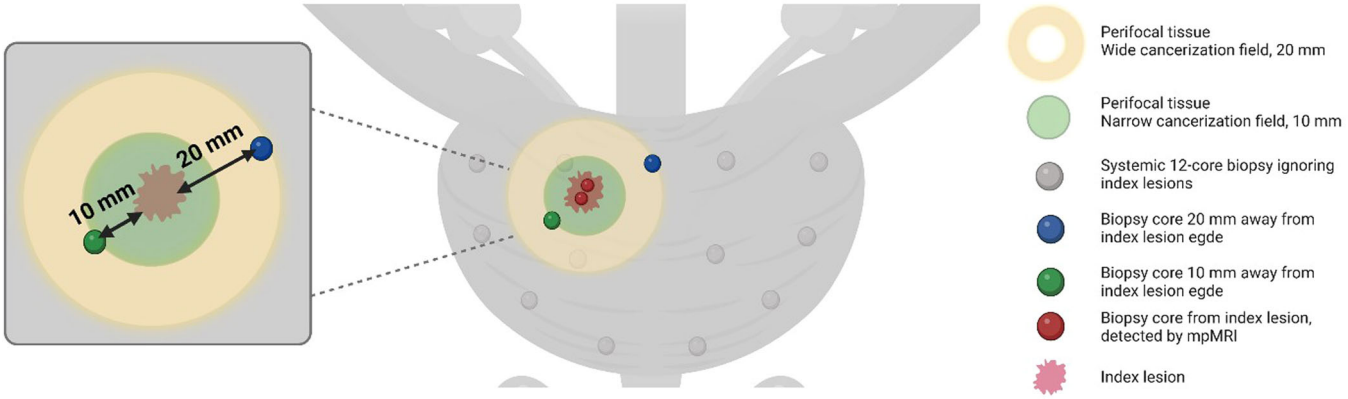


FIGURE 2 Prostate biopsy technique and field cancerization scheme. Created with BioRender.com. [Color figure can be viewed at wileyonlinelibrary.com]

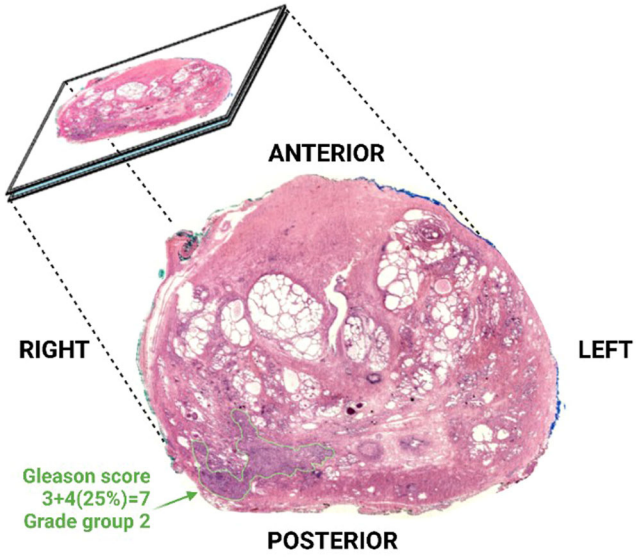


FIGURE 3 Whole-mount prostate histopathology image. Prostate moderately differentiated (Gleason 3 + 4(25%) = 7, Grade Group 2) acinic adenocarcinoma (with intraductal spread). Original image modified by BioRender.com. [Color figure can be viewed at wileyonlinelibrary.com]

500 µL of lysis buffer, consisting of 50 mM Tris–HCl pH 8.5, 1 mM EDTA, 0.5% Tween-20 (all from Carl Roth).

All voided urine samples ($n = 272$) were prepared and further processed according to the standard protocol.[13] For DNA extraction from urine sediment samples lysis buffer consisting of 10 mM Tris–HCl pH 8.0, 1% Sodium dodecyl sulfate, and 75 mM NaCl (all from Carl Roth) was used. DNA was extracted using standard phenol-chloroform purification and ethanol precipitation method.

For qualitative DNA methylation analysis, up to 400 ng of purified DNA were modified with bisulfite, using EZ DNA Methylation™ Kit (Zymo Research) according to the manufacturer's protocol, except that the initial incubation of samples was performed at 42°C for 15 min, instead of 37°C.

2.4 | Quantitative DNA methylation-specific PCR (qMSP)

The qMSP primers and probes specific for methylated DNA for genes *RARB*, and *GSTP1* were designed using Methyl Primer Express® Software v1.0 (Applied Biosystems™, Thermo Fisher Scientific) and ordered from Metabion. Primer sequences for *APC*, *RASSF1*, and *ACTB* were obtained from previous publications[19,20] (Supporting Information: 1). *ACTB* was used in each run for the normalization of the DNA input. The qMSP was performed in triplicates for each set of primers in separate wells. The reaction mix (20 µL in total) consisted of 1× TaqMan® Universal Master Mix II, no UNG (Applied Biosystems™), 300 nM of each primer, 50 nM of the probe, and ~ 10 ng of bisulfite-converted DNA. All assays were carried out under the following conditions: 95°C for 10 min followed by 45–50 cycles of 95°C for 15 s and 60°C for 1 min, using QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™). Only those runs where no-template control (NTC) gave no amplification products, and artificially methylated control (MC) gave a positive signal were considered valid. The background-based threshold algorithm was applied for the estimation of the cycle of quantification (C_q) value. The methylation level of a particular gene was estimated based on the $_{\Delta\Delta}C_q$ algorithm and expressed as a percentage of the MC.[14]

2.5 | Statistical analysis

Statistical analysis and data visualization were performed with MedCalc® v12.7 software (MedCalc Software) and GraphPad Prism v8.0.1 software (GraphPad Software, Inc.). The Mann–Whitney *U* test was used for two-group comparisons of continuous data. The ability of the biomarkers to distinguish groups was evaluated by performing receiver operating characteristic (ROC) curve analysis and estimating the area under the curve (AUC) values. A *p*-value of <0.050 was considered significant.

3 | RESULTS

3.1 | Epigenetic biomarkers in FFPE tissue samples

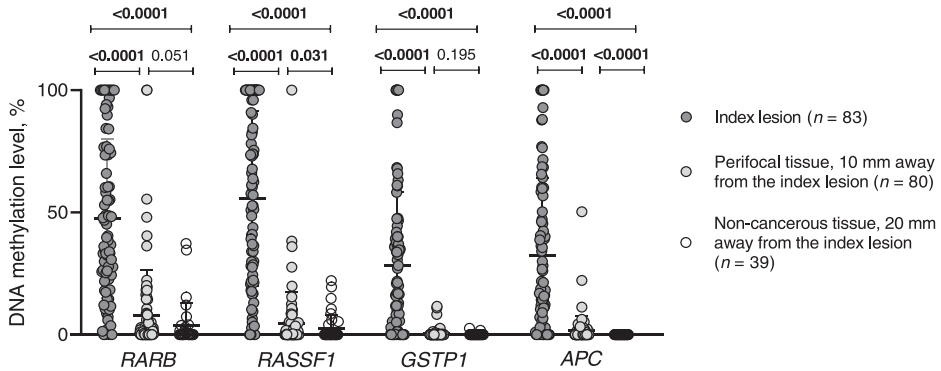
To evaluate the field cancerization extent, DNA methylation level in the *RARB*, *RASSF1*, *GSTP1*, and *APC* genes was analyzed in biopsy sample from PCa index lesion ($n = 83$), perifocal tissue ($n = 80$), and control noncancerous tissue ($n = 39$) from 44 patients. All patients included in this study underwent MRI/US image-guided prostate biopsy.

Quantitative analysis revealed that the DNA methylation level of all studied genes was statistically significantly higher in the index lesion compared with the perifocal samples 10 mm distant from the index lesion (all $p < 0.0001$; Figure 4). Moreover, noncancerous tissues taken 20 mm away from the index lesion showed even lower levels of DNA methylation as compared with perifocal samples or the samples from the index lesion.

On average, the level of *RARB* methylation in index lesions was six times higher compared with perifocal tissue and 13 times higher than in noncancerous tissue. The average level of *RASSF1* methylation in index lesions was 13 times higher compared with perifocal tissue and 23 times higher than in noncancerous tissue. The average methylation level of *GSTP1* and *APC* in the index lesion compared with perifocal tissue was 60 and 21 times higher, respectively. The average methylation level of *GSTP1* was 234 times higher than in noncancerous tissue, while *APC* methylation in noncancerous tissue was not detected at all. This shows the accuracy of MRI/US image-guided prostate biopsy in identifying index lesions and the safety of focal PCa therapy considering the spread of PCa cells beyond the PCa foci.

To gather further insights about the spread of epigenetic changes in prostate tissue, heatmap analysis was performed which allowed us to map DNA methylation marks of each sample (Figure 5). In some of the cases (B20-19937, B20-26292, B20-33989, B20-5479, B20-36740, B20-33707, B20-20740) quite extensive DNA methylation was observed in perifocal samples, and even in noncancerous samples (B20-33707, B21-3263, B20-20740). In most of the cases with two samples from index lesion involved in analysis ($n = 34$) the extent of epigenetic alterations was rather uniform, but in a few cases (B20-20740; B20-33707; B20-36740; B20-2088; B20-33989, B20-7272) some disagreement was noted.

Discrepant cases were additionally checked by the pathologist and radiologist. B20-20740 and B20-2088 cases showed high *RARB*, *GSTP1*, *APC*, and *RASSF1* DNA methylation levels in the PeriF samples (Supporting Information: 2). The distance from index lesion was corrected and the actual distance was 6 and 5 mm accordingly, instead of 10 mm. B20-33707 case also showed high *RARB*, *GSTP1*, *APC*, and *RASSF1* DNA methylation levels in PeriF samples and according to pathologist opinion this sample contained some grade group 1 (GG1) prostate adenocarcinoma cells, that possibly impacted epigenetic findings. This field cancerization is invisible on mpMRI images but detectable by genetic screening. According to the final pathology protocol (Gleason 3 + 4(5%) = 7, GG2) acinic adenocarcinoma of the prostate, pT3a (extra prostatic spread 6 mm long on the right), high methylation levels could be related to the large volumes of locally advanced disease. Similar situation was with the B20-33989 case. While the B20-36740 case also showed high *RARB*, *GSTP1*, *APC*, and *RASSF1* DNA methylation levels in PeriF samples, and this sample has high-grade prostatic intraepithelial neoplasia (PIN) diagnosis by histology protocol and the distance from the index lesion was 6 mm. Of interest, the B20-7272 case had the low methylation level in all studied genes, instead of *GSTP1*, at the sample from index lesion, even though it was the (Gleason 3 + 3 = 6, GG1) acinic adenocarcinoma of the prostate, 30% (4.0 mm).



Average DNA methylation level, %	*RARB*			*RASSF1*			*GSTP1*			*APC*		
	Index lesion	Perifocal tissue	Non-cancerous	Index lesion	Perifocal tissue	Non-cancerous	Index lesion	Perifocal tissue	Non-cancerous	Index lesion	Perifocal tissue	Non-cancerous
	47.65	7.98	3.75	55.78	4.45	2.48	28.43	0.48	0.12	32.48	1.57	0.00

FIGURE 4 DNA methylation levels of the regulatory regions of *RARB*, *RASSF1*, *GSTP1*, and *APC* genes in formalin-fixed paraffin-embedded (FFPE) prostate tissue samples according to their histology. Lines indicate a median with 95% confidence intervals. The table shows the average level of DNA methylation in index lesions, 10 mm away from them in perifocal tissue samples, and in noncancerous tissue samples, 20 mm away from the index lesion.

FIGURE 5 Heatmap analysis of biomarkers, distinguishing between prostate cancer index lesions (IL), perifocal tissue samples (PeriF), and noncancerous prostate tissue (No PCa). It represents the average DNA methylation levels of each gene in the sample. [Color figure can be viewed at wileyonlinelibrary.com]

After this additional assessment, it is apparent that the shorter distance from the index lesion, the greater hypermethylation intensity in histologically normal prostate tissue. It means that hypermethylation is proportional to the distance from the index lesion, which is also observed in control samples. Also, high methylation levels could be related to larger volumes of locally advanced disease or the presence of GG1 PCa, which is invisible on mpMRI images.

3.2 | Characteristics of diagnostic tool

For the selection of the most appropriate biomarkers for molecular diagnostics of PCa and evaluation of field cancerization extent, the ROC curve analysis was performed. When the index lesion was compared with noncancerous tissues, the highest sensitivity and specificity were reached by analyzing *RARB*, *GSTP1* & *APC* (AUC = 0.98; $p < 0.0001$), and *RARB*, *RASSF1*, *GSTP1* & *APC* (AUC = 0.98; $p < 0.0001$) biomarker combinations (Figure 6, Table 2). When assessing the diagnostic potential of each of the studied genes, the gene *RASSF1* had the highest sensitivity and specificity (AUC = 0.94; $p < 0.0001$).

Similarly, when PCa index lesions were compared with perifocal tissues, *RARB* & *GSTP1* and *RASSF1* & *GSTP1* (both AUC = 0.94; $p < 0.0001$) combinations had the largest area under the curve (Figure 7, Table 3). The same AUC (AUC = 0.94; $p < 0.0001$) was reached with three (*RARB*, *RASSF1* & *GSTP1*, and *RARB*, *GSTP1* & *APC*, and *RASSF1*, *GSTP1* & *APC*) or all four-biomarker panel. As previously,

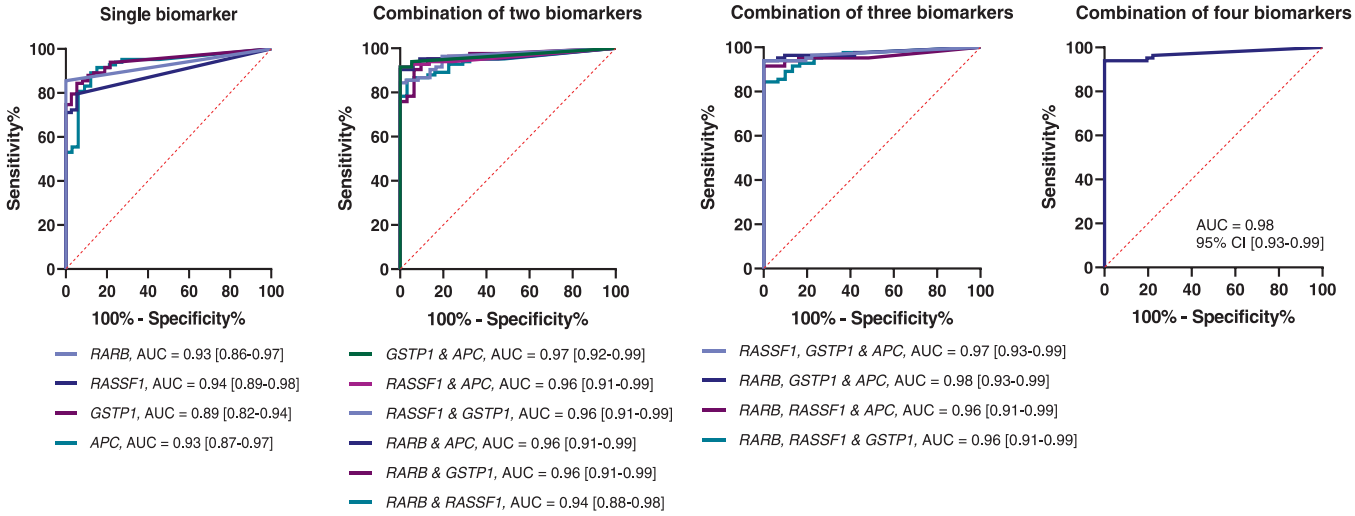


FIGURE 6 Analysis of receiver operating characteristic (ROC) curves for single biomarkers and combinations of two to four biomarkers, distinguishing between prostate cancer index lesions and noncancerous tissue samples. The 95% confidence interval is indicated in the brackets. AUC, area under the curve. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Analysis of ROC curves for single biomarkers and combinations of two to four biomarkers in distinguishing between prostate cancer index lesions and noncancerous tissue samples.

Biomarker	AUC	95% CI	*p* value	Specificity, %	Sensitivity, %	Youden index *J*	ACV	PPV, %	NPV, %
Single biomarker									
RARB	0.93	0.86–0.97	<0.0001	87.88	89.16	0.77	>7.40	93.99	79.23
RASSF1	0.94	0.89–0.98	<0.0001	94.59	84.34	0.79	>14.90	97.07	73.98
GSTP1	0.89	0.82–0.94	<0.0001	94.59	79.52	0.74	>0	96.90	68.49
APC	0.93	0.87–0.97	<0.0001	100	85.54	0.86	>0	100	76.50
The panel of two biomarkers									
RARB & RASSF1	0.94	0.88–0.98	<0.0001	96.77	85.54	0.82	>0.76	98.25	75.90
RARB & GSTP1	0.96	0.91–0.99	<0.0001	93.55	90.36	0.84	>0.48	96.75	82.04
RARB & APC	0.96	0.91–0.99	<0.0001	100	90.36	0.90	>0.75	100	83.00
RASSF1 & GSTP1	0.96	0.91–0.99	<0.0001	100	84.34	0.84	>0.72	100	75.03
RASSF1 & APC	0.96	0.91–0.99	<0.0001	100	91.57	0.92	>0.53	91.57	84.81
GSTP1 & APC	0.97	0.92–0.99	<0.0001	100	91.57	0.92	>0.23	91.57	84.81
The panel of three biomarkers									
RARB, RASSF1 & GSTP1	0.96	0.91–0.99	<0.0001	100	84.34	0.84	>0.83	100	75.03
RARB, RASSF1 & APC	0.96	0.91–0.99	<0.0001	100	91.57	0.92	>0.55	100	84.81
RARB, GSTP1 & APC	0.98	0.93–0.99	<0.0001	100	93.98	0.94	>0.60	100	88.66
RASSF1, GSTP1 & APC	0.97	0.93–0.99	<0.0001	100	93.98	0.94	>0.31	100	88.66
The panel of four biomarkers									
RARB, RASSF1, GSTP1 & APC	0.98	0.93–0.99	<0.0001	100	93.98	0.94	>0.46	100	88.66

Abbreviations: ACV, associated criterion value; AUC, area under the curve; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic.

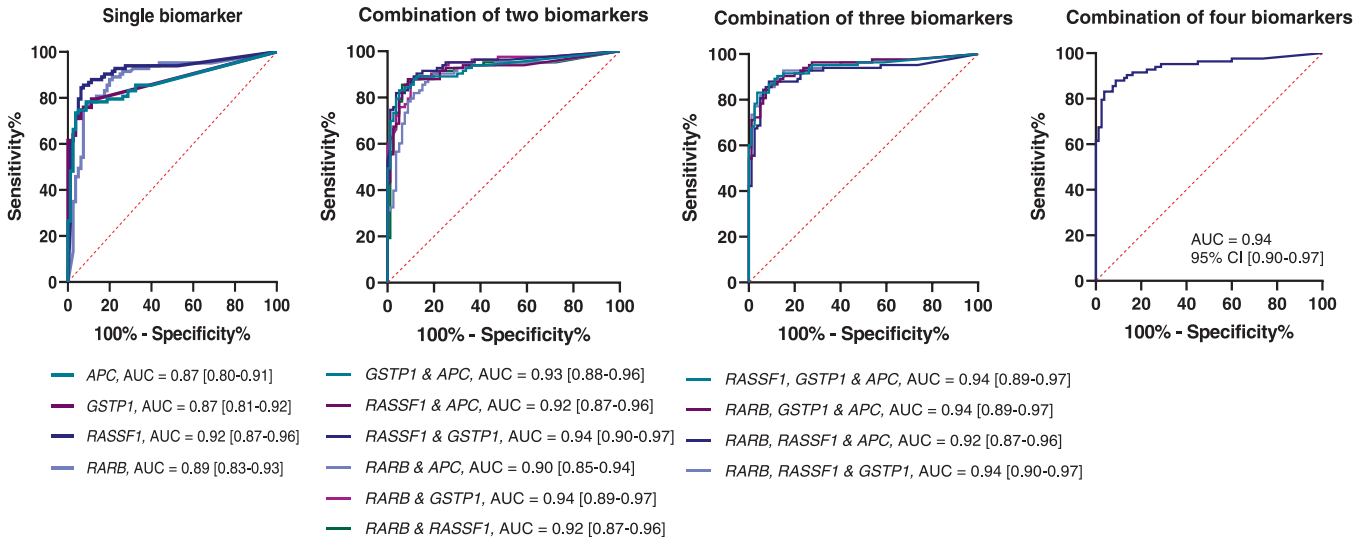


FIGURE 7 Analysis of receiver operating characteristic (ROC) curves for single biomarkers and combinations of two to four biomarkers, distinguishing between prostate cancer index lesions and perifocal tissue samples. The 95% confidence interval is indicated in the brackets. AUC, area under the curve. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Analysis of ROC curves for single biomarkers and combinations of two to four biomarkers in distinguishing between prostate cancer index lesions and perifocal tissue samples.

Biomarker	AUC	95% CI	*p* value	Specificity, %	Sensitivity, %	Youden index *J*	ACV	PPV, %	NPV, %
Single biomarker									
RARB	0.89	0.83–0.93	<0.0001	92.50	75.90	0.68	>22.26	91.33	78.67
RASSF1	0.92	0.87–0.96	<0.0001	93.75	84.34	0.78	>15.44	93.35	85.19
GSTP1	0.87	0.81–0.92	<0.0001	93.75	74.70	0.68	>1.32	92.56	78.07
APC	0.87	0.80–0.91	<0.0001	96.25	73.49	0.70	>6.28	95.33	77.72
The panel of two biomarkers									
RARB & RASSF1	0.92	0.87–0.96	<0.0001	93.75	85.54	0.79	>0.41	93.44	86.17
RARB & GSTP1	0.94	0.89–0.97	<0.0001	90.00	87.95	0.78	>0.27	90.16	87.77
RARB & APC	0.90	0.85–0.94	<0.0001	88.75	81.93	0.71	>0.37	88.34	82.51
RASSF1 & GSTP1	0.94	0.90–0.97	<0.0001	96.25	81.93	0.78	>0.56	95.79	83.65
RASSF1 & APC	0.92	0.87–0.96	<0.0001	91.25	87.95	0.79	>0.31	91.28	87.92
GSTP1 & APC	0.93	0.88–0.96	<0.0001	95.00	83.13	0.78	>0.40	94.54	84.40
The panel of three biomarkers									
RARB, RASSF1 & GSTP1	0.94	0.90–0.97	<0.0001	92.50	85.54	0.78	>0.35	92.23	86.00
RARB, RASSF1 & APC	0.92	0.87–0.96	<0.0001	91.25	87.95	0.79	>0.31	91.28	87.92
RARB, GSTP1 & APC	0.94	0.89–0.97	<0.0001	92.50	84.34	0.77	>0.41	92.13	85.02
RASSF1, GSTP1 & APC	0.94	0.89–0.97	<0.0001	96.25	83.13	0.79	>0.47	95.85	84.57
The panel of four biomarkers									
RARB, RASSF1, GSTP1 & APC	0.94	0.90–0.97	<0.0001	96.25	83.13	0.79	>0.56	95.85	84.57

Abbreviations: ACV, associated criterion value; AUC, area under the curve; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

the gene *RASSF1* had the highest sensitivity and specificity (AUC = 0.92; $p < 0.0001$) as a single biomarker of PCa.

3.3 | Epigenetic biomarkers in urine sediments

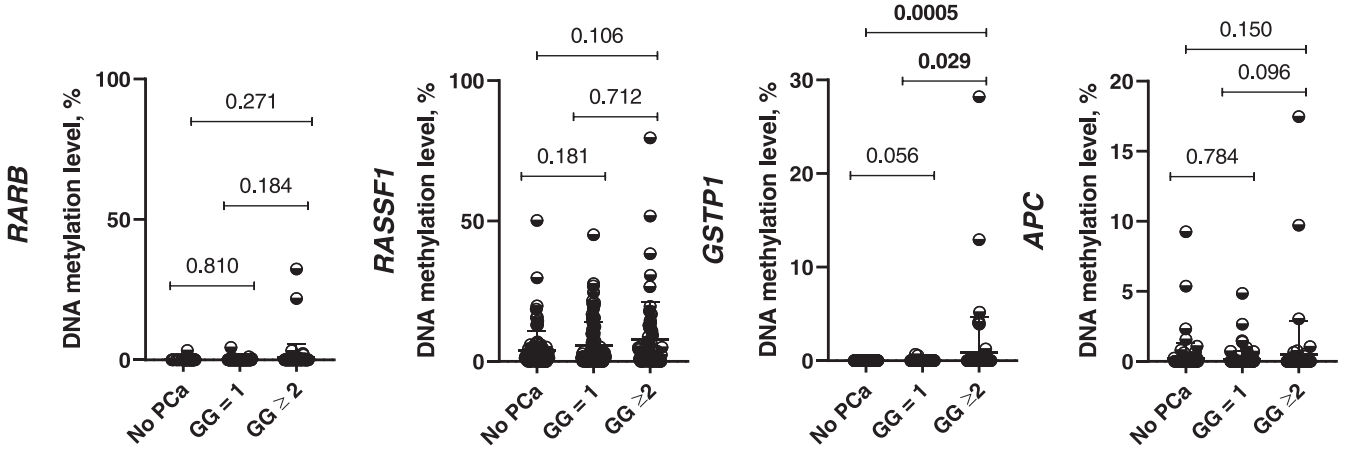
Aiming at the assessment of suitability of selected biomarkers for noninvasive detection of csPCa quantitative methylation analysis of genes *RARB*, *RASSF1*, *GSTP1*, and *APC* was performed by the qMSP method in urine samples of PCa patients with clinically significant ($n = 71$), clinically insignificant ($n = 97$) PCa, and patients without PCa ($n = 104$). using urine samples. In this comparison, all cases from the MRI/US-guided biopsy cohort were included and compared with the control group without PCa. Out of four genes involved in the analysis, only *GSTP1* promoter methylation level was able to discriminate PCa cases from controls ($p = 0.0005$) and even the csPCa cases from the clinically insignificant cases ($p = 0.029$, Figure 8).

Cell-free DNA methylation levels in urine were apparently lower than in tissue samples. However, despite the low DNA methylation levels in urine, all tested biomarkers showed the same tendency—the lowest methylation levels were detected in the control group, and it was the highest in the csPCa group (GG ≥ 2). The analysis of the characteristics of the potential urinary biomarkers showed that the combination of all four biomarkers with prostate-specific antigen (PSA) or PSA density (PSAD) in blood, significantly increases sensitivity and specificity in distinguishing csPCa from the control (noncancerous) group (Figure 9, Supporting Information: 3). The combination of four-biomarker test with PSAD allowed the identification of csPCa with ≥90% sensitivity and specificity, showing a strong potential to use these noninvasive measures during follow-up the cases after focal therapy.

4 | DISCUSSION

PCa is characterized by a multifocal phenomenon and 67%–96% of prostatectomy specimens display more than one tumor focus. This suggests the presence of a field cancerization, whereby factors underlying carcinogenesis result in molecular changes in large areas of the prostate grand. Such changes are detectable in histologically normal or inflammatory cells and in PIN tissue which generally precedes carcinogenesis. Studies of tumor-adjacent benign tissue have found alterations in gene expression, telomere DNA content, and gene copy number that mimics a malignant phenotype. Additionally, there is an increased prevalence of methylation in genes such as *APC*, *GSTP1*, *RASSF1*, and *RARB* known to be methylated in PCa. DNA methylation biomarkers in combination with PSA level or PSAD could be used to stratify risk for latent cancer, monitoring disease progression, and in combination with radiological prostate imaging can assist in proper and safe treatment selection.[21]

Prostate mpMRI is a proven imaging modality that can detect clinically significant foci of PCa with a high degree of accuracy. However, it lacks the precision to separate csPCa from clinically insignificant disease. This is due to the pitfalls in the interpretation and technical evaluation of MRI images. A failure to recognize these issues can result in suboptimal patient treatment in low-grade PCa.[22] The main routine clinical and pathological variables (PSA levels, Grade group/International Society of Urological Pathology, tumor clinical, and radiological staging)[23,24] are useful in the diagnostic assessment of the tumor, but they lack sensitivity and specificity in classifying the risk of the disease.[25,26] Thus, mpMRI alone may not be enough to choose an individualized treatment for PCa patients.



Average DNA methylation level, %	*RARB*			*RASSF1*			*GSTP1*			*APC*		
	No PCa	GG = 1	GG ≥ 2	No PCa	GG = 1	GG ≥ 2	No PCa	GG = 1	GG ≥ 2	No PCa	GG = 1	GG ≥ 2
	0.04	0.08	0.98	3.52	4.36	7.69	0.00	0.01	0.88	0.23	0.15	0.50

FIGURE 8 DNA methylation level of regulatory regions of genes *RARB*, *RASSF1*, *GSTP1*, and *APC*, in DNA samples extracted from urine sediments. GG, grade group; PCa, prostate cancer. Lines indicate median with 95% confidence intervals.

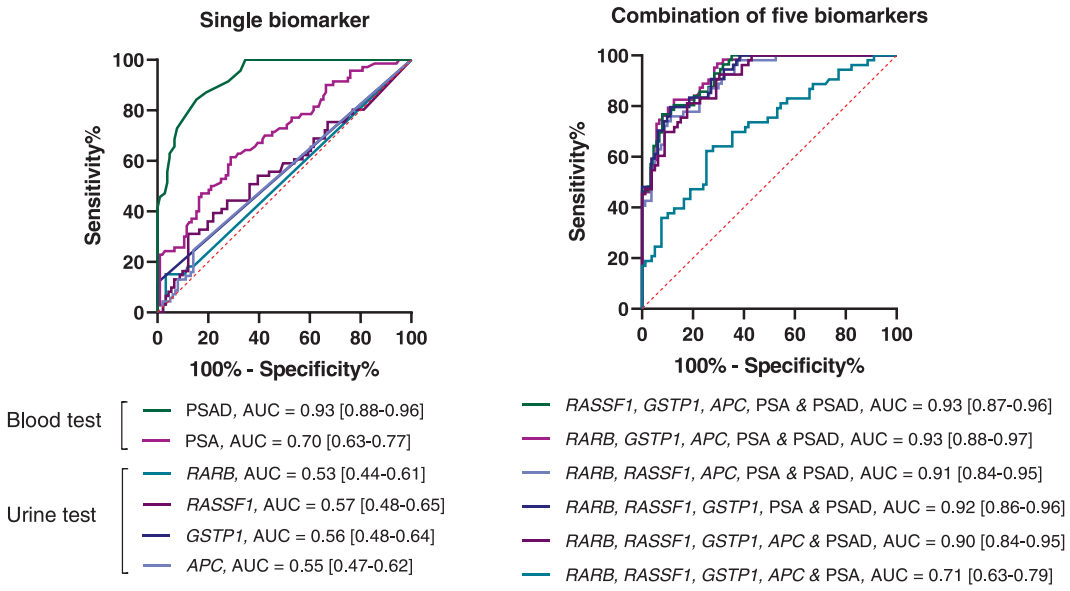


FIGURE 9 Analysis of receiver operating characteristic (ROC) curves for single biomarkers and combinations of five biomarkers, distinguishing between control group and clinically significant prostate cancer. The 95% confidence interval is indicated in the brackets. AUC, area under the curve. [Color figure can be viewed at wileyonlinelibrary.com]

DNA methylation biomarker discovery has accelerated rapidly with the development of whole-genome profiling techniques.[27] The clinically added value of biomarkers in the era of upfront MRI and targeted biopsies remains unclear.[28]

In this study, we tried to evaluate the DNA methylation extent of target tumor suppressor genes in perifocal tissue on mpMRI-nonvisible PCa and compare it to the normal prostate tissue and mpMRI visible PCa. It was hypothesized that DNA methylation biomarkers can subsequently be used as a sensitive measure to detect the local spread of PCa. Based on previous studies, we chose to study these genes that best reflect the PCa-specific changes and local spread of PCa in prostate tissue.[13,29,30] *GSTP1*, *RASSF1*, *APC*, and *RARB* genes are the most frequently methylated genes in PCa, with a frequency range from 70% to almost 100% in different studies.[31–33] Our results revealed that the DNA methylation level of all studied genes was statistically significantly higher in the index lesion, compared with the perifocal samples and histopathologically normal tissues. Also, the four-gene biomarker combination showed the highest sensitivity and specificity to PCa with 94% sensitivity and 100% specificity. The main finding of this study is that although from a radiological point of view by mpMRI, the perifocal prostate tissue and the area 20 mm away from the index lesion did not differ, at the genetic level significant differences were determined. This leads to the assumption that ≥10 mm area seems safe enough for focal PCa therapy based on molecular mapping of extent of field cancerization.

In the study conducted by Hanson et al.,[34] *GSTP1* and *RARB* methylation were detected in some of the histologically normal-appearing epithelial samples from two and four patients, respectively. Additionally, normal stromal samples from one and two patients, respectively, also exhibited methylation. Most of these samples displayed partial methylation, although two samples showed methylation levels exceeding 51%. These findings indicated the potential presence of a field effect, where gene methylation extends beyond the tumor region.[34] In study conducted by Mehrotra et al.,[35] the sensitivity and specificity of methylation markers were evaluated by analyzing 51 pairs of histologically malignant and nonmalignant tissues from prostatectomies. Additionally, one prostatectomy sample without matching normal tissue was included in the analysis. The study observed the presence of *GSTP1* methylation in 62% of cancer tissues compared with 2% of nonmalignant tissues. *APC* methylation was found in 69% of cancer tissues and none of the nonmalignant tissues. *RARB* methylation was detected in 58% of cancer tissues but was absent in nonmalignant tissues. Similarly, *RASSF1A* methylation was identified in 58% of cancer tissues and 11% of nonmalignant tissues.[35] Similar DNA methylation levels in PCa foci were found in the study by Serenaite et al.,[30] it was shown that *GSTP1*, *RASSF1*, and *RARB* were also frequently (>75%) methylated and they also showed a high level of methylation (21%–36%). Thus, DNA methylation levels and sensitivity of tested biomarkers found in this study are similar to the findings of other researchers. However, it is necessary to consider that various methods and experimental conditions in different studies can influence the results.

To date, focal prostate therapy is based only on the results of histological examination, and we do not have a comparative molecular analysis technique. Our study showed that epigenetic tests have the potential to be combined with radiomics, to increase the precision of focal prostate therapy to avoid local progression of cancer in the future. Moreover, mpMRI misses up to 20% of foci with potentially csPCa. In such case, the liquid biopsy-based tests can be used to improve the diagnostic value of MRI. The epigenetic test used in field cancerization analysis was also informative on cell-free

DNA extracted from urine of the patients. Despite the low DNA methylation levels, all tested biomarkers showed the same tendency as in tissues, and the highest DNA methylation levels were detected in the csPCa group (GG ≥ 2). These values were significantly improved by combining urinary biomarkers with blood test parameters such as PSA and PSAD. Noninvasive urine test Select MDx (MDxHealth) is recommended for preselection of aggressive PCa cases before the mpMRI of the prostate,[36] while our study suggest possible benefit of such noninvasive testing for follow-up of the patients after focal therapy. However, to prove the concept additional prospective study is needed in the group of cases treated by focal PCa therapy.

5 | CONCLUSIONS

Our study shows, that the DNA methylation level of all studied genes was statistically significantly higher in the index lesion compared with the perifocal samples, and hypermethylation significantly decreases after reaching a distance of >10 mm. Based on this, we can conclude that the safe distance from the index lesion is no less than 10 mm and possible focal therapy for intermediate-risk PCa could be performed by region target hemi-ablation. Noninvasive urine tests are a highly valuable for further follow-up of patients after PCa treatment, however, larger prospective studies with external validation are needed.

ACKNOWLEDGMENTS

The authors thank Aistė Gerulaitytė (National Cancer Institute, Vilnius, Lithuania) for the technical assistance with DNA extraction and qMSP analysis. Her work greatly contributed to the results presented in this publication. This work was funded by the 2014–2020 European Union Structural Funds according to the activity "Intelligence. Joint science-business projects" grant No. J05-LVPA-K-04–0029.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Augustinas Matulevičius http://orcid.org/0009-0006-6109-6597

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Matulevičius A, Žukauskaitė K, Gineikaitė R, et al. Combination of DNA methylation biomarkers with multiparametric magnetic resonance and ultrasound imaging fusion biopsy to detect the local spread of prostate cancer. *The Prostate*. 2023;83:1572-1583. doi:10.1002/pros.24615