VILNIUS UNIVERSITY

Arnas
BAKAVIČIUS

The Value of Clinical and Molecular Markers in Preoperative Diagnosis of Prostate Cancer

SUMMARY OF DOCTORAL DISSERTATION

Medicine and Health Sciences,
Medicine (M 001)

VILNIUS 2020

This dissertation was written between 2015 and 2019 at Vilnius University. The research was supported by Research Council of Lithuania, Lithuanian State Science and Studies Foundation and Vilnius University Faculty of Medicine.

Academic supervisor:
Prof. Feliksas Jankevičius, PhD (Vilnius University, Medicine and Health Sciences, Medicine – M 001).

Academic consultant:
Prof. Sonata Jarmalaitė, PhD (Vilnius University, Natural Sciences, Biology – N 010).

Dissertation Defence Panel:
Chairman – **Prof. Kęstutis Strupas, PhD** (Vilnius University, Medicine and Health Sciences, Medicine – M 001);

Members:
Prof. Mindaugas Jievaltas, PhD (Lithuanian University of Health Sciences, Medicine and Health Sciences, Medicine – M 001);

Marius Kinčius, PhD (National Cancer Institute, Medicine and Health Sciences, Medicine – M 001);

Ass. Prof. Vilnis Lietuvietis, PhD (Riga Stradiņš University, Medicine and Health Sciences, Medicine – M 001);

Prof. Gintaras Valinčius, PhD (Vilnius University, Natural Sciences, Biochemistry – N 004).

The dissertation will be defended at a public meeting of the Dissertation Defence Panel at 10 a.m. on 22 June 2020 in the Red Auditorium (E122) at the Vilnius University Hospital Santaros Klinikos. Address: Santariškių Str. 2, 08661 Vilnius, Lithuania.

The text of this dissertation can be accessed through the Library of Vilnius University as well as on the website of Vilnius University at *www.vu.lt/lt/naujienos/ivykiu-kalendorius*

VILNIAUS UNIVERSITETAS

Arnas
BAKAVIČIUS

Klinikinių ir molekulinių žymenų reikšmė priešoperacinėje prostatos vėžio diagnostikoje

DAKTARO DISERTACIJOS SANTRAUKA

Medicininos ir sveikatos mokslai,
Medicina (M 001)

VILNIUS 2020

Disertacija rengta 2015– 2019 metais Vilniaus universitete.
Mokslinius tyrimus rėmė Lietuvos mokslo taryba, Lietuvos valstybinis mokslo ir studijų fondas ir Vilniaus universiteto Medicinos fakultetas.

Mokslinis vadovas:
prof. dr. Feliksas Jankevičius (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

Mokslinė konsultantė:
prof. dr. Sonata Jarmalaitė (Vilniaus universitetas, gamtos mokslai, biologija – N 010).

Gynimo taryba:
Pirmininkas – **prof. habil. dr. Kęstutis Strupas** (Vilniaus universitetas, Medicinos ir sveikatos mokslai, Medicina – M 001);

Nariai:
Prof. dr. Mindaugas Jievaltas (Lietuvos sveikatos mokslų universitetas, Medicinos ir sveikatos mokslai, Medicina – M 001);

Dr. Marius Kinčius (Nacionalinis vėžio institutas, Medicinos ir sveikatos mokslai, Medicina – M 001);

Doc. dr. Vilnis Lietuvietis (Rygos Stradinio universitetas, Medicinos ir sveikatos mokslai, Medicina – M 001);

Prof. dr. Gintaras Valinčius (Vilniaus universitetas, Gamtos mokslai, Biochemija – N 004).

Disertacija ginama viešame disertacijos Gynimo tarybos posėdyje 2020 m. birželio mėn. 22 d. 10 val. Vilniaus universiteto ligoninės Santaros klinikų Raudonojoje auditorijoje (E122). Adresas: Santariškių g. 2, 08661 Vilnius, Lietuva.

Disertaciją galima peržiūrėti Vilniaus universiteto bibliotekoje ir VU interneto svetainėje adresu: *https://www.vu.lt/lt/naujienos/ivykiu-kalendorius*

CONTENTS

1. ABBREVIATIONS

BCR – biochemical recurrence
CI – confidence interval
cISUP – clinical ISUP grade group
CSS – cancer-specific survival
cTNM – clinical TNM stage
DNA – deoxyribonucleic acid
EAU – European Association of Urology
FS – Fisher Scientific
GSTP1 – *glutathione S-transferase pi 1* gene
ISUP – International Society of Urological Pathology
MFS – metastases-free survival
MSP – methylation-specific polymerase chain reaction
NPV – negative predictive value
OR – odds ratio
OS – overall survival
PB – prostate biopsy
PCR – polymerase chain reaction
PIN – prostate intraepithelial neaoplasia
pISUP – pathological ISUP grade group
PPV – positive predictive value
PSA – prostate specific antigen
PSM – positive surgical margin
PV – prostate volume
QMSP – quantitative methylation-specific polymerase chain reaction
RARβ – *retinoic acid receptor β* gene
RASSF1 – *RAS association domain family member 1* gene
ROC – receiver operating characteristic
RP – radical prostatectomy
SEM – standard error of mean
TFS – Thermo Fisher Scientific
TNM – Tumour, Node, Metastasis classification
TURP – transurethral resection of the prostate

2. INTRODUCTION

2.1 Relevance of the thesis

Prostate cancer (PCa) is the second most common oncological disease among men all over the world and the first in North America, Europe and Lithuania. It is a particularly heterogeneous disease ranging from indolent localised tumour to aggressive and metastatic cancer causing patients' lethal outcomes. Thus, accurate assessment of disease's aggressiveness at diagnosis is one of the main goals for optimal disease management. It is significantly relevant now, in the era of modern medicine, when most newly diagnosed PCa cases are characterised as low and intermediate-risk, and individualized organ-sparing therapy is becoming a standard treatment option in everyday clinical practice.

Clinical ISUP grade group (cISUP) determined during prostate biopsy (PB), clinical TNM stage (cTNM) and prostate-specific antigen (PSA) are the main clinico-pathological characteristics determining the choice of PCa treatment tactics. According to literature, upgrading or upstaging to advanced disease after pathological examination of radical prostatectomy (RP) material has emerged as a serious issue and is reported in 40-60% of PCa patients. Upgrading and upstaging have been applied to indicate that the method of treatment chosen while planning the course of treatment was not effective enough, thus could negatively influence PCa oncological outcomes. Scientific literature provides evidence of association between upgrading or upstaging and increased risk of biochemical recurrence (BCR) as well as inferior cancer-specific survival results. On the other hand, downgrading and downstaging might be associated with overtreatment, which is related to higher risks of complications and worse functional outcomes.

Over the last years, rapid development of genomic technologies and their application for deciphering cancer genome have offered new diagnostic possibilities for PCa patients. During PCa cancerogenesis, changes in deoxyribonucleic acid (DNA) chains as well as epigenetic alterations are detected, during which the sequence of DNA does not

change, while the normal gene expression is affected. Epigenetic changes occur early in prostate cancerogenesis and are characterised by a greater stability; therefore, they have become a popular object of scientific research. Majority of PCa epigenetic alterations are associated with methylation changes in gene regulatory areas, for example, promoters' regions, which conditions the loss of messenger ribonucleic acids (mRNA) and suppression of protein synthesis. Due to the fact that PCa-specific methylated DNA is easily detectable in body fluids (blood, urine, etc.), contains molecular information from all tumour foci and reflects PCa heterogeneity, analysis of the latter may provide valuable supplementary data for improved diagnosis and timely prediction of PCa aggressiveness.

2.2 Aim of the thesis

The dissertation aims at evaluating suitability of clinico-pathological characteristics presently used in everyday clinical practice for PCa diagnostics, exploring PCa epigenetic profile and evaluating potential molecular markers (*RARβ*, *RASSF1* and *GSTP1*) for timely prediction of accurate PCa diagnosis.

2.3 Objectives of the thesis

1. Based on histopathological examination of primary PB and RP material, to evaluate suitability of clinico-pathological characteristics (cISUP, cTNM and preoperative PSA) to predict PCa aggressiveness, local stage and risk group.
2. Based on results of histopathological examination of RP material, postoperative PSA, postoperative radiological investigations (bone scintigraphy, computed tomography, magnetic resonance tomography) and data from State Register of Death Cases and Their Causes by the Institute of Hygiene, to evaluate frequency of PSM and BCR, MFS, overall (OS) and cancer-specific-survival (CSS), as well as their relationship with upgrading and upstaging.

3. To set frequencies of promoter methylation of potential PCa diagnostic biomarkers (*RARβ*, *RASSF1* and *GSTP1*) in cancerous and noncancerous prostate tissues.
4. To evaluate promoter methylation of *RARβ*, *RASSF1* and *GSTP1* genes in two independent cohorts of PCa patients with urine specimens collected by two different techniques, i.e. voided and catheterized.
5. To evaluate the potential of urine biomarkers (*RARβ*, *RASSF1* and *GSTP1*) for characterization of PCa aggressiveness, local stage and risk group, as well as select an optimal combination of molecular markers with clinico-pathological characteristics for timely and precise prediction of PCa aggressiveness, local stage and risk group.

2.4 Novelty and practical significance of the thesis

A large and well-characterized cohort consisting of more than 1000 subjects with a long postoperative follow-up allowed precise assessment of PCa diagnostic problems associated with clinico-pathological characteristics (cISUP, cTNM and preoperative PSA) presently used in everyday clinical practice and their relationship with PSM, BCR, MFS as well as with OS and CSS.

In scientific literature there are published more than 100 genetic alterations associated with PCa, however the majority of them were investigated in cancerous and noncancerous prostate tissues and were poorly associated with aggressiveness of the disease, which particularly restricts their implementation into clinical practice.

In the process of our research, promoter methylation of *RARβ*, *RASSF1* and *GSTP1* was investigated in urine, an easily accessible biological material. Collection of urine does not require any intervention, thus not causing additional risk for the patient and making it easier to apply the molecular test into clinical practice. During the research, genetic markers were investigated in two independent cohorts with voided and catheterized urine, which allowed us to compare different techniques for substance collection. During the research, the

combination of the molecular biomarkers with PSA was identified, which allows a more accurate prediction of PCa aggressiveness, local stage and risk group.

2.5 Statements to be defended

1. Clinico-pathological characteristics (cISUP, cTNM and preoperative PSA) presently used in everyday clinical practice are not sufficiently reliable diagnostic tools to characterize PCa aggressiveness, local stage and risk group.
2. PCa upgrading and upstaging after RP is associated with PSM, BCR, MFS and inferior CSS.
3. *RARβ*, *RASSF1* and *GSTP1* gene promoter methylation frequencies are significantly different in cancerous and noncancerous prostate tissues, consequently, they are suitable for PCa diagnostics.
4. *RARβ*, *RASSF1* and *GSTP1* gene promoter methylation in urine combined with PSA could be used to predict PCa aggressiveness, local stage and risk group.

3. MATERIAL AND METHODS

The study was approved by the Lithuanian Bioethics Committee (2007-11-23 No. 50 and 2011-09-07 No. 6B-11-275). Patients who were treated at the Centre of Urology of Vilnius University Hospital Santaros Klinikos, met inclusion criteria and did not have any exclusion criteria were included into the study. Molecular analysis was performed at the Institute of Biosciences of Life Sciences Centre.

3.1 Inclusion criteria

- Histologically confirmed PCa by systematic transrectal PB.
- Radical prostatectomy (RP) was performed at Vilnius University Hospital Santaros Klinikos because of PCa.

3.2 Exclusion criteria

- < 10-core systematic PB was performed.
- Diagnosis of neuroendocrine, small cell, sarcomatoid, squamous cell or signet ring cell carcinoma.
- Previously diagnosed PCa with any type of active treatment, including active surveillance and androgen deprivation therapy.
- History of urothelial carcinoma.

3.3 Clinico-pathological characteristics

3.3.1 Data collection

Information about age at diagnosis, preoperative PSA (ng/mL), date of PB, Gleason score and cTNM was collected from prostate biopsy reports. Some PBs were performed at outside institutions and no pathological re-evaluation of these specimens were performed.

Information about date of surgery, Gleason score, pathological TNM stage, surgical margin status, postoperative PSA (ng/mL) and metastases was collected from RP pathology reports and postoperative

follow-up documentation. Gleason score was evaluated according to the 2005 Guidelines of International Society of Urological Pathology (ISUP) and ISUP grade groups were assigned according to ISUP 2014 recommendations.

The data regarding survival were obtained from the State Register of Death Cases and Their Causes, by the Institute of Hygiene under the Ministry of Health of the Republic of Lithuania (2019-10-21 No. (9.20) 01-517).

3.3.2 Upgrading, upstaging and risk change

Upgrading was defined as any increase of ISUP grade group between PB (cISUP) and RP pathology (pISUP), i.e. pISUP > cISUP. Upstaging was confirmed if a patient was pathologically diagnosed with locally advanced PCa (≥ pT3) when clinically unsuspected. *Partin* nomograms were calculated to evaluate a chance of locally advanced disease at RP pathology (≥ pT3).

According to European Association of Urology (EAU) 2019 and National Comprehensive Cancer Network 2019 risk assessment models and clinico-pathological characteristics (ISUP grade group and T stage), all patients were stratified into preoperative and postoperative PCa risk groups: preoperatively – low (cISUP 1 and ≤ cT2a), intermediate (cISUP 2-3 and/or cT2b), high (cISUP 4-5 and/or ≥ cT2c), and postoperatively – low (pathologic ISUP grade group (pISUP) 1 and ≤ pT2c), intermediate (pISUP 2-3 and ≤ pT2c), high (pISUP 4-5 and/or ≥ pT3a). Risk increase was confirmed when a patient postoperatively was diagnosed with higher PCa risk group then clinically suspected, i.e. postoperative risk group > preoperative risk group.

3.3.3 Positive surgical margin and biochemical recurrence

Positive surgical margin (PSM) was defined as the presence of tumour cells at the inked margin of RP material on the inspection under microscopy.

BCR following RP was defined as a postoperative PSA > 0.2 ng/mL with a subsequent confirmatory value.

3.3.4 Metastases-free, overall and cancer-specific survival

Data about metastatic disease were obtained from follow-up documentation, including bone scintigraphy, computed tomography and/or magnetic resonance tomography. Metastases-free survival (MFS) was defined as the time from RP to confirmed evidence of distant metastases on imaging. Metastatic lymph nodes in the pelvis were considered as locally advanced disease.

OS was defined as a time from RP to death from any cause. CSS was defined as a time from RP to death at the time of progressive metastatic PCa. Patients who had died without BCR or with BCR and PSA < 1.0 ng/mL with metastatic-free disease were classified as dying from other causes.

3.4 Biological samples and molecular analysis

3.4.1 Collection and preparation of biological samples

Prostate tissue specimens and urine samples were collected for molecular analysis. Urine samples (30-50 mL) were collected preoperatively by two different techniques: voided and catheterized. Voided urine samples were collected after the prostate massage in the morning before the surgery, while catheterized urine was obtained under general anaesthesia immediately before the surgery. All urine samples were delivered to the laboratory within 30 mins.

Non-fixed prostate gland was delivered to the National Center of Pathology within 30 mins after radical prostatectomy. 8 mm of cancerous (≥ 70% of cancer cells) and noncancerous (0% of cancer cells) prostate tissue samples were immediately dissected and snap frozen for molecular analysis.

Tissue samples were mechanically homogenized into powder. Urine samples were centrifuged for 15 mins at 1000 rpm at 4 °C temperature and collected sediments were washed twice with 1× PBS buffer (Thermo Fisher Scientific (TFS), Waltham, Massachusetts, USA). Prepared samples were stored at -80 °C temperature.

3.4.2 Molecular analysis

3.4.2.1 DNA extraction and bisulfite conversion

DNA was extracted from 10-30 mg of snap frozen and homogenized tissue specimens and 1-2 mL of frozen urine sediments by using standard phenol-chloroform protocol for DNA purification. Tissue samples and urine sediments were treated with proteinase K (TFS) and lysis buffer (for tissue samples: 50 mM of Tris-HCl (Carl Roth, Karlsruhe, Germany), 1 mM of EDTA (Carl Roth), 0.5% Tween-20 (Carl Roth); for urine sediments: 10 mM of Tris-HCl pH 8.0 (Carl Roth), 1% SDS (Carl Roth), 75 mM of NaCl (Sigma-Aldrich, St. Louis, USA)) for up to 18 hours at 55 °C temperature on thermoshaker. The precipitation was performed with ≥ 96% ethanol solution, while elution was done with 40 µL of nuclease-free water. The extracted DNA was evaluated by "NanoDrop 2000" spectrophotometer (Fisher Scientific, FS). Bisulfite modification of 400 ng of DNA was done with "EZ DNA Methylation™ Kit" (Zymo Research, Irvine, California, USA) according to manufacturer's recommendations, except initial incubation (42 °C temperature, 15 mins). The modified DNA was immediately used or stored at -80 °C temperature.

3.4.2.2 DNA methylation analysis in tissue

Methylation analysis of *RARβ*, *RASSF1* and *GSTP1* in prostate tissue samples was performed by using methylation-specific polymerase chain reaction (MSP). Primers were selected from scientific literature. 25 µL of reaction mixture consisted of: 1× "Maxima Hot Start Taq MSP buffer" (TFS), 2.5 mM of $MgCl_2$ (TFS), 1.6 mM of deoxyribonucleoside triphosphate mix (TFS), 1.25 U of polymerase "Maxima® Hot Start Taq DNA Polymerase" (TFS), 1 mM of each primer (Metabion, Munich, Germany) and 1 µL of bisulfite-modified DNA. DNA was amplified for 37-39 cycles of MSP, while the primers were annealed for 45 seconds at 60-62 °C temperature. At each run three controls were used: positive methylation control (*in vitro* methylated DNA of leukocytes), negative methylation control

(DNA of leukocytes from healthy men) and no-template control (reaction mixture without DNA).

3.4.2.3 DNA methylation analysis in urine

Methylation analysis of *RARβ*, *RASSF1* and *GSTP1* in urine samples was performed using quantitative methylation-specific polymerase chain reaction (QMSP). Primers and hydrolysis probes were selected from scientific literature. 20 μL of final reaction mixtures consisted of 1× "TaqMan® Universal Master II no UNG" mix (TFS), 50 nM of hydrolysis probe, 300 nM of each primer (Metabion) and 1 μL of modified DNA. All assays were carried out under the following conditions: 10 mins of incubation at 95 °C, followed by 50 amplification cycles for 15 seconds at 95 °C and 1 min at 60 °C. Positive methylation and no-template controls were included in each assay run. QMSP was considered as valid if amplification of positive methylation control was detected and there was no amplification in no-template control. A sample was classified as valid if amplification of *ACTB* gene was < 40 cycles in all 3 replicas. Methylation level of a particular gene was calculated by using quantification cycle (ΔΔCq) algorithm according to endogenous and positive methylation control and presented as percentages. For the qualitative analysis, samples were dichotomized into methylated and unmethylated considering the 0.1% methylation level as the threshold.

3.5 Statistical analysis

All statistical tests were performed using STATISTICA™ 8.0 (StatSoft, Tulsa, USA), SPSS 23.0 (IBM Corp., Armonk, NY, USA) and MedCalc® 12.7 (MedCalc Software, Ostend, Belgium) software.

Continuous variables are expressed as means with standard error of mean (SEM). Data for categorical variables are presented as frequencies and percentages. Continuous variables were checked for normal distribution by *Shapiro–Wilk* statistics and compared them by the 2-sided *t test* when normally distributed or the *Mann–Whitney*

U test for non-normally distributed variables. *Pearson's χ2* and *Fisher* exact tests were used for comparison of categorical variables, as appropriate.

Kaplan–Meier curves were used and *Log Rank* (*Mantel–Cox*) test was applied for BCR, metastases-free, OS and CSS analyses. To identify predictors for BCR and metastatic disease, univariate and multivariate logistic regression analysis was performed, where odds ratios (OR) and 95% confidence intervals (CI) were calculated. The ability of the biomarkers to distinguish groups was evaluated by receiver operating characteristic (ROC) curve analysis and estimating the area under the curve (AUC) values. The regression analysis was used to calculate the test's performance parameters – sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). P-value of < 0.050 was considered as statistically significant.

3.6. Author's contributions

Arnas Bakavičius (doctoral student) enrolled the patients into the study, collected all the clinical data, collected biological samples and was responsible for postoperative follow-up. The doctoral student performed molecular analysis of 30% of biological samples and analysed the results. Arnas Bakavičius was the main author of the first publication, prepared the second publication and presented the results of the study at scientific congresses.

Molecular analysis of other biological samples was performed by Kristina Daniūnaitė and Kristina Žukauskaitė. Kristina Daniūnaitė also analysed the results and participated in the preparation of the first manuscript. Kristina Žukauskaitė trained the doctoral student to perform the molecular analysis and was the main supervisor in the laboratory.

Prof. Sonata Jarmalaitė was the academic consultant who supervised the analysis of biological samples as well as revised the manuscripts critically for important intellectual content.

Prof. Feliksas Jankevičius was the academic supervisor who designed the research, supervised clinical part of the study and revised the manuscripts critically for important intellectual content.

4. RESULTS

4.1 General characteristics of study participants

Overall, 1056 patients were included into the study. Mean time from PB to RP was 3.8 months (SEM: ± 0.2). In total, 111 fresh-frozen tissue samples of prostate tumours, 16 noncancerous prostate tissues from RP material and 514 urine samples (188 voided and 326 catheterized) were available for molecular analysis. Clinico-pathological characteristics of the study cohort are summarized in Table 1.

Table 1. Clinico-pathological characteristics of the study cohort.

Parameter	All cases (N = 1056)	Tumour tissues (N = 111)	Catheterized urine (N = 326)	Voided urine (N = 188)	P-value*
Age, years[a]					
Mean (± SEM)	62.2 (0.2)	60.6 (0.8)	61.7 (0.4)	62.2 (0.6)	0.382
PSA, ng/mL[b]					
Mean (± SEM)	8.4 (0.3)	10.3 (1.0)	8.6 (0.5)	8.4 (0.7)	0.613
cISUP, N (%)[c]					
1	723 (70.5)	69 (64.5)	226 (81.0)	93 (51.4)	
2	203 (19.8)	21 (19.6)	42 (15.0)	55 (30.4)	
3	58 (5.7)	9 (8.4)	7 (2.5)	24 (13.2)	**<0.001**
4	34 (3.3)	6 (5.6)	3 (1.1)	7 (3.9)	
5	7 (0.7)	2 (1.9)	1 (0.4)	2 (1.1)	
pISUP, N (%)[a]					
1	539 (51.0)	38 (34.2)	207 (67.0)	39 (21.4)	
2	405 (38.4)	50 (45.1)	86 (27.8)	108 (59.3)	
3	76 (7.2)	16 (14.4)	13 (4.2)	28 (15.4)	**<0.001**
4	14 (1.3)	2 (1.8)	1 (0.3)	1 (0.6)	
5	22 (2.1)	5 (4.5)	2 (0.7)	6 (3.3)	
cT stage, N (%)[a]					
≤ cT1c	637 (60.3)	59 (53.2)	189 (61.2)	112 (61.5)	
cT2a	11 (1.1)	2 (1.8)	4 (1.3)	3 (1.7)	
cT2b	128 (12.1)	17 (15.3)	28 (9.0)	30 (16.5)	
cT2c	188 (17.8)	20 (18.0)	73 (23.6)	25 (13.7)	0.597
cT3a	78 (7.4)	13 (11.7)	12 (3.9)	10 (5.5)	
cT3b	13 (1.2)	-	3 (1.0)	2 (1.1)	
cT4	1 (0.1)	-	-	-	

Parameter	All cases (N = 1056)	Tumour tissues (N = 111)	Catheterized urine (N = 326)	Voided urine (N = 188)	P-value*
pT stage, N (%)[a]					
pT2a	80 (7.6)	4 (3.6)	36 (11.7)	3 (1.7)	**0.006**
pT2b	12 (1.1)	-	1 (0.3)	3 (1.7)	
pT2c	698 (66.1)	63 (56.8)	199 (64.4)	122 (67.0)	
pT3a	169 (16.0)	30 (27.0)	48 (15.5)	37 (20.3)	
pT3b	95 (9.0)	13 (11.7)	24 (7.8)	17 (9.3)	
pT4	2 (0.2)	1 (0.9)	1 (0.3)	-	
Preoperative PCa risk, N (%)[c]					
Low	501 (48.9)	44 (41.1)	152 (54.5)	70 (38.7)	0.104
Intermediate	243 (23.7)	29 (27.1)	47 (16.8)	71 (39.2)	
High	281 (27.4)	34 (31.8)	80 (28.7)	40 (22.1)	
Postoperative PCa risk, N (%)[a]					
Low	489 (46.3)	35 (31.5)	183 (59.2)	38 (20.9)	**<0.001**
Intermediate	290 (27.5)	30 (27.0)	52 (16.8)	88 (48.3)	
High	277 (26.2)	46 (41.5)	74 (24.0)	56 (30.8)	
Partin value, %[d]					
Mean (±SEM)	27.2 (0.4)	29.3 (1.3)	26.3 (0.8)	29.1 (1.0)	**0.024**

Abbreviations: cISUP = clinical ISUP grading; cT = clinical T-staging; ISUP = International Society for Urological Pathology; N = number of patients; pISUP = pathological ISUP grading; PSA = prostate-specific antigen; pT = pathological T-staging; PCa = prostate cancer; SEM = standard error of mean; T = local tumour staging according to TNM classification.

[a] Age, pISUP, cT-stage, pT-stage, stage change and postoperative PCa risk missing in 17 patients of catheterized urine cohort, and in 6 patients of voided urine cohort.

[b] PSA missing in 10 patients of all cohort, in 25 patients of catheterized urine cohort, and in 6 patients of voided urine cohort.

[c] cISUP, preoperative PCa risk, grade change and risk change missing in 31 patients of all cohort, in 4 patients of tumour tissue cohort, in 47 patients of catheterized urine cohort, and in 7 patients of voided urine cohort.

[d] *Partin* value missing in 145 patients of all cohort, in 15 patients of tumour tissue cohort, in 63 patients of catheterized urine cohort, and in 17 patients of voided urine cohort.

[*] P-values calculated for comparison of catheterized and voided urine cohorts only. Significant P values are in bold.

To estimate differences between cohorts with voided and catheterized urine, clinico-pathological characteristics of both cohorts were compared with each other. Mean age and preoperative PSA did not

differ between both groups, while higher cISUP ($p < 0.001$), higher pISUP ($p < 0.001$), higher pT ($p = 0.006$) and higher *Partin* value ($p = 0.024$) was detected in patients with voided urine samples (Table 1).

4.2 Upgrading, upstaging and risk change

Upgrading was observed in 27.2% (279/1025) of the patients, while it was not possible to evaluate grade change in 31/1056 patients because of missing data. 20.3% (214/1056) of the patients upstaged post-RP. The total misclassification rate, defined as the change of at least one of the two parameters, i.e. upgrading and/or upstaging, was 39.0% (400/1025).

Among the upgraded cases, 86.4% (241/279) of the patients were initially diagnosed with cISUP 1 disease, the majority of whom (86.3%, 208/241) were upgraded to pISUP 2 disease. ISUP grade change rates are summarized in Figure 1.

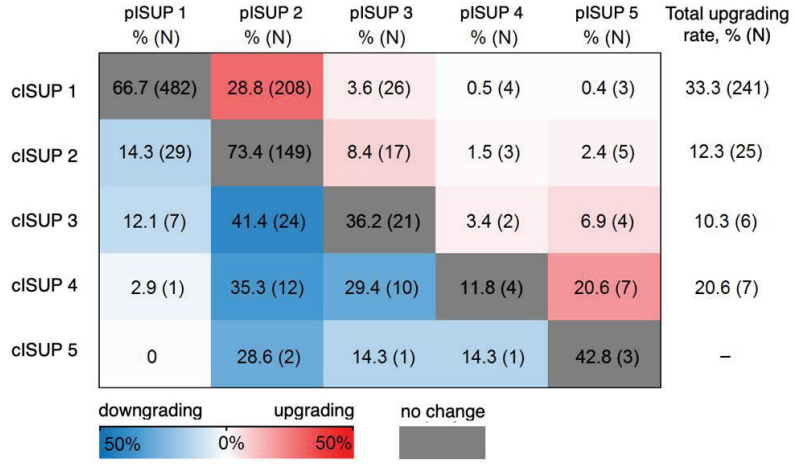

Fig 1. ISUP grade change rates after pathological examination of surgical material. Rates of ISUP change are accompanied with colours, where more intense blue depicts higher rate of downgrading, while more intense red depicts higher rate of upgrading. For visual purposes, the colour scale is capped at 50%.

Abbreviations: cISUP = clinical ISUP grading; ISUP = International Society for Urological Pathology; N = number of patients; pISUP = pathological ISUP grading.

Patients initially diagnosed with the cT1c cancer dominated among the upstaged cases (49.1%; 105/214; Fig. 2).

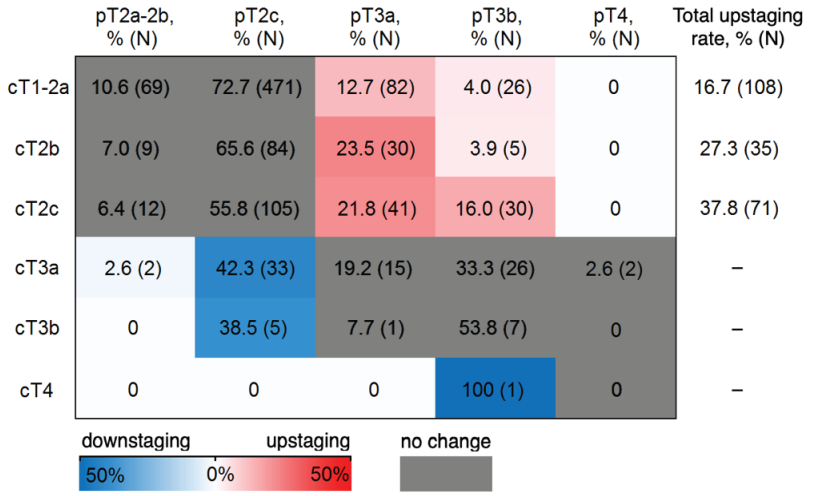

Fig 2. Stage (T) change rates after pathological examination of surgical material. Rates of stage (T) change are accompanied with colours, where more intense blue depicts higher rate of downstaging, while more intense red depicts higher rate of upstaging. For visual purposes, the colour scale is capped at 50%.

Abbreviations: cT = clinical T-staging; N = number of patients; pT = pathological T-staging; T = local tumour staging according to TNM classification.

Histopathological examination of the whole prostate gland after RP revealed that 23.9% (245/1025) of the patients were assigned to a higher postoperative PCa risk group than clinically suspected, while it was not possible to evaluate risk change in 31/1056 patients because of missing data. 69.8% (N = 171) of patients with risk increase had been preoperatively diagnosed with low-risk PCa. Upgrading alone was the major cause of the risk increase (45.3%, N = 111), whereas both grade and tumour stage increase were identified in 22.5% (N = 55) and only stage increase – in 32.2% (N = 79) of the cases.

4.3 Positive surgical margin

In the analysis of PSM, BCR, metastases-free survival (MFS), OS and CSS 676/1056 patients were included with available postoperative follow-up data.

PSM was detected in 32.1% (217/676) of PCa patients undergoing RP. According to prostate anatomy, apex was the most common site for PSM – 56.0% (108/193), followed by postero-lateral position – 48.2% (93/193), base – 15.0% (29/193) and seminal vesicles – 4.7% (9/193). It was not possible to identify the location of PSM in 24/217 patients because of missing information in histopathological reports.

The patients whose cancer was upgraded post RP more commonly had PSM (41.6%, 82/197) as compared to patients with no upgrading (28.2%, 135/479; $p = 0.001$). Upstaging after RP was also associated with PSM, where 44.3% (66/149) of PCa patients with upstaging and 28.7% (151/527) with no upstaging had been reported with PSM ($p < 0.001$).

4.4 Biochemical recurrence

BCR-only was diagnosed to 25.7% (174/676) of PCa patients after RP. At the time of BCR detection 77.3% (126/163) of the patients presented with PSA value < 0.5 ng/mL, 12.3% (20/163) – with PSA 0.5-2.0 ng/mL and 10.4% (17/163) – with PSA > 2.0 ng/mL, while exact PSA value at the time of BCR diagnosis was missing to 11/174 patients. The mean follow-up time of patients without BCR was 46.8 months (SEM: ± 1.63).

BCR was diagnosed to 37.6% (74/197) of PCa patients whose cancer upgraded post RP, while only to 20.9% (100/479) of patients with no upgrading ($p < 0.001$). Mean time to BCR after RP was 2.1 years (SEM: ± 0,2) in upgraded cases and 2.7 years (SEM: ± 0,3) in patients with no upgrading ($p < 0.001$; Figure 3).

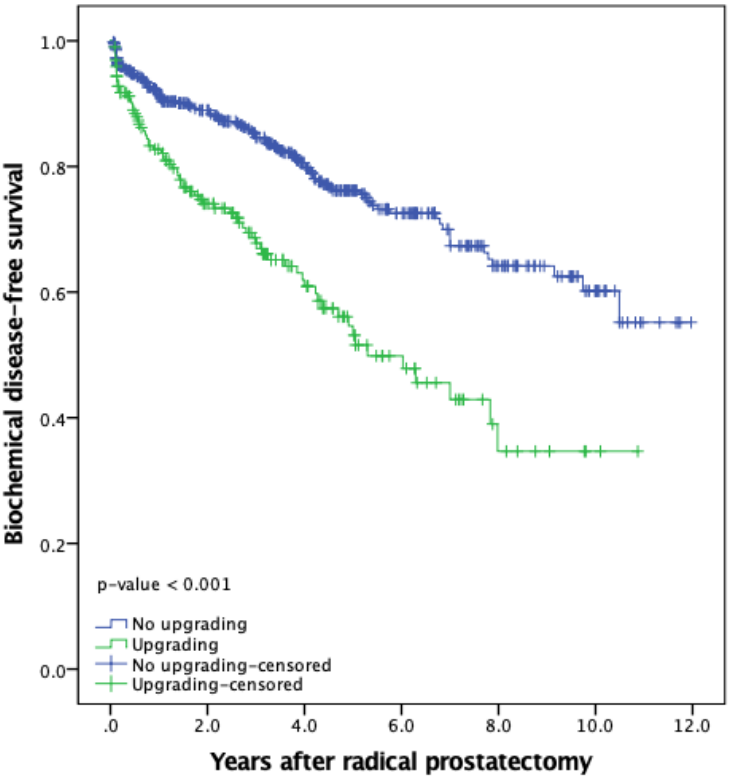

Fig 3. Prostate cancer biochemical disease-free survival rates after radical prostatectomy according to upgrading (all cISUP grade groups).
Abbreviations: cISUP = clinical ISUP grading; ISUP = International Society for Urological Pathology.

Patients who were upgraded from clinically low-risk (cISUP 1) disease showed more favourable BCR rates as compared to patients with clinically diagnosed intermediate or high-risk (cISUP 2-4) PCa ($p < 0.001$; Figure 4).

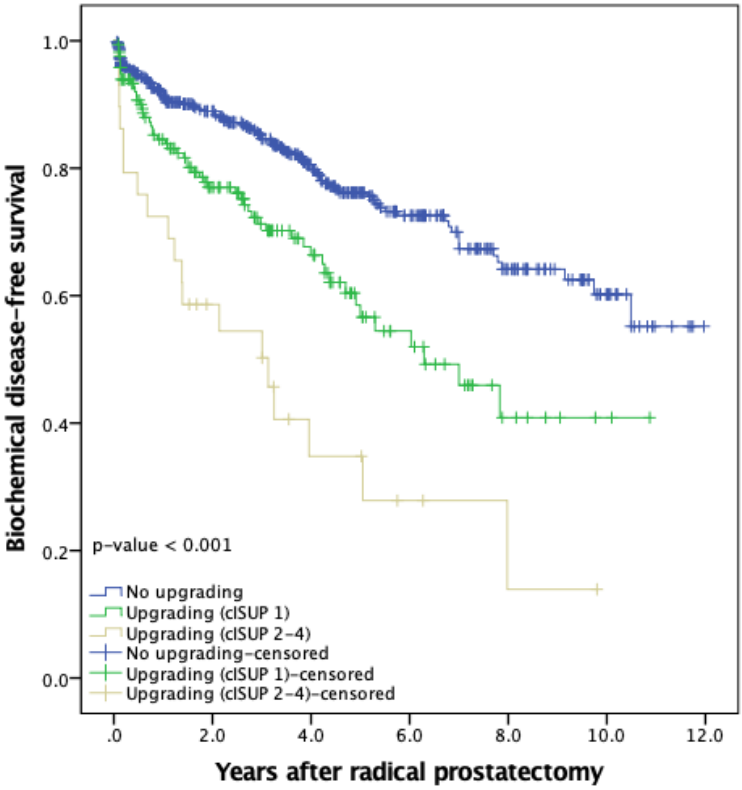

Fig. 4. Prostate cancer biochemical disease-free survival rates after radical prostatectomy according to upgrading, where blue line depicts no upgrading, green line – upgrading from cISUP grade group 1 and yellow line – upgrading from cISUP grade group 2-4.

Abbreviations: cISUP = clinical ISUP grading; ISUP = International Society for Urological Pathology.

Upstaging after RP was also associated with BCR, where 43.6% (65/149) of PCa patients undergoing upstaging in contrast to 20.7% (109/527) of patients without upstaging were diagnosed with BCR ($p < 0.001$). Mean time to BCR after RP was 1.9 years (SEM: ± 0,3) in upstaged and 2.8 years (SEM ± 0,2) in non-upstaged cases ($p < 0.001$; Figure 5).

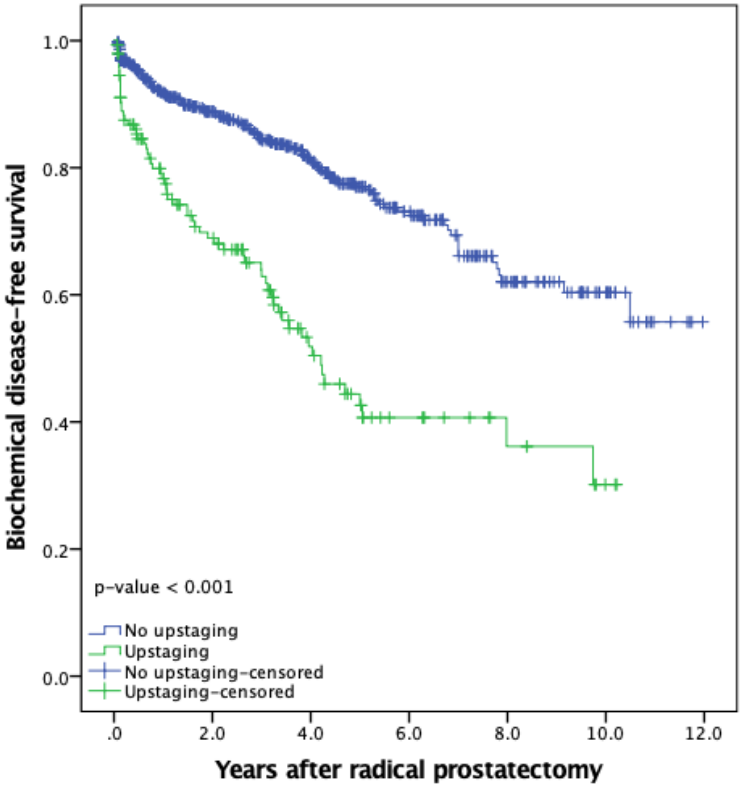

Figure 5. Prostate cancer biochemical disease-free survival rates after radical prostatectomy according to upstaging.

In logistic regression analysis PSM showed the highest OR for BCR (2.29 [1.55-3.40], $p < 0.001$). According to this model, the ORs for upgrading and upstaging were 1.92 [1.29-2.86] and 2.14 [1.39-3.27], respectively (all $p < 0.001$; Table 2).

Table 2. Univariate and multivariate logistic regression analysis of the associations between clinico-pathological characteristics and biochemical recurrence.

Variable	Univariate			Multivariate		
	OR	**95 % CI**	**P-value**	**OR**	**95 % CI**	**P-value**
PSA, ng/mL	1.10	[1.07-1.13]	**< 0.001**	1.09	[1.05-1.13]	**< 0.001**
PSM	3.27	[2.28-4.69]	**< 0.001**	2.29	[1.55-3.40]	**< 0.001**
Upgrading*	2.28	[1.59-3.28]	**< 0.001**	1.92	[1.29-2.86]	**0.001**
Upstaging	2.97	[2.02-4.37]	**< 0.001**	2.14	[1.39-3.27]	**< 0.001**

* All cISUP grade groups were included. Statistically significant p-values ($p < 0.050$) are marked in bold.

Abbreviations: CI = confidence interval; cISUP = clinical ISUP grade group; ISUP = International Society of Urological Pathology; PSA = prostate-specific antigen; PSM = positive surgical margin.

4.5 Metastases-free survival

Metastatic disease was diagnosed to 4.0% (27/676) of PCa patients. According to upgrading and upstaging, metastases were diagnosed to 6.1% (12/197) of PCa patients whose cancer upgraded post RP, while to 3.1% (15/479) of patients with no upgrading ($p = 0.074$). Mean metastasis-free survival was 11.5 (95% CI: 10.9-12.1) and 11.4 (95% CI: 11.1-11.7) years for patients with and without upgrading, respectively ($p = 0.048$; Figure 6).

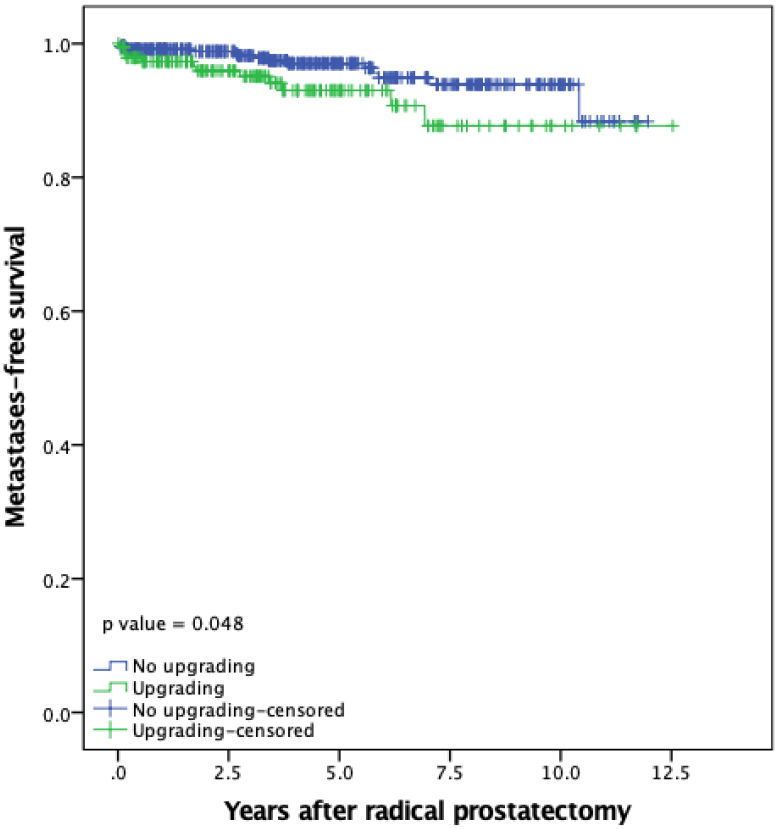

Fig 6. Metastases-free survival after radical prostatectomy according to upgrading.

Upstaging after RP was also associated with metastatic disease, where 8.7% (13/149) of PCa patients undergoing upstaging and 2.7% (14/527) of patients without upstaging developed metastases ($p = 0.001$). Mean metastasis-free survival was 10.3 (95% CI: 9.6-11.1) years for patients with upstaging, as compared with 12.1 (95% CI: 11.8-12.3) years for patients with no upstaging ($p < 0.001$; Figure 7).

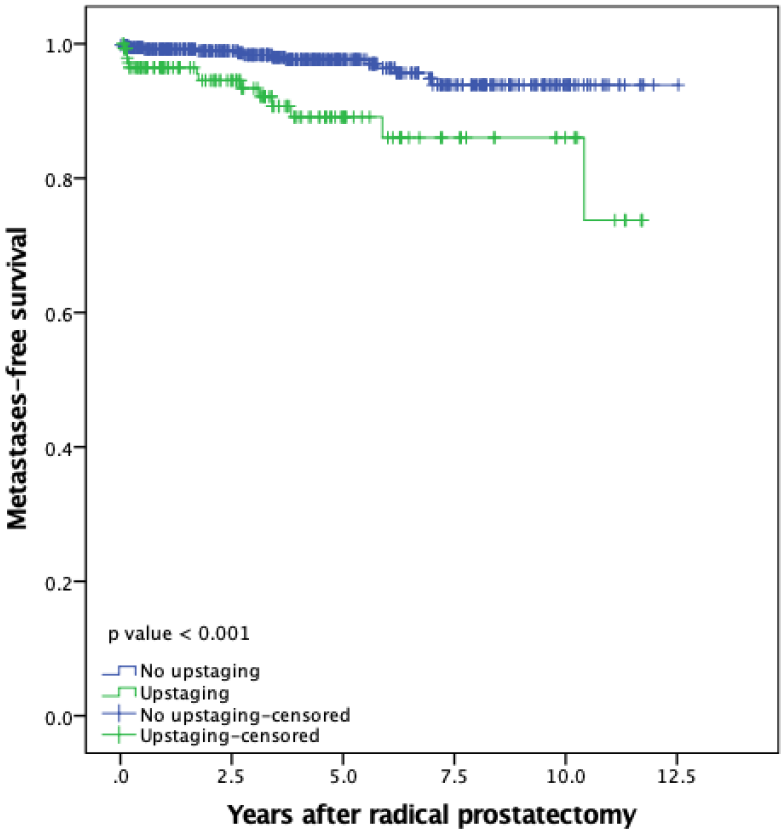

Fig. 7. Metastases-free survival after radical prostatectomy according to upstaging.

In multivariate logistic regression analysis upstaging showed the highest OR for metastatic disease (3.40 [1.52-7.61], p = 0.003), followed by PSA (1.05 [1.01-1.08], p = 0.004), while upgrading was removed from the model.

4.6 Overall and cancer-specific survival

Mean postoperative follow-up – 53.4 months (SEM: ± 1.5). Mean OS for patients with and without upgrading was 10.2 (95% CI: 9.3-11.0) and 9.7 (95% CI: 9.3-10.2) years, while five and ten-year OS rates were comparable in both groups: 88.6%, 66.7% and 90.1%, 67.7%, respectively (p = 0.746; Figure 8).

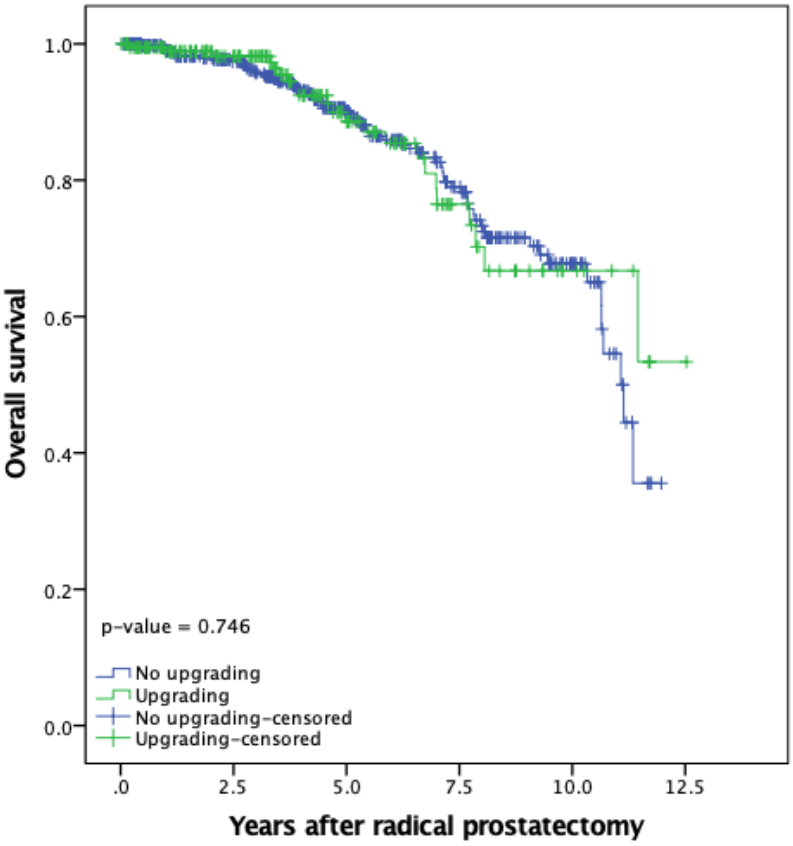

Fig 8. Prostate cancer overall survival rates according to upgrading.

Similar OS results were observed in upstaged and non-upstaged PCa cases, where mean OS was 9.3 (95% CI: 8.5-10.2) and 10.0 (95% CI: 9.5-10.5) years, while five and ten-year OS did not differ significantly: 91.2%, 56.4% and 89.5%, 69.9%, respectively (p = 0.567; Figure 9).

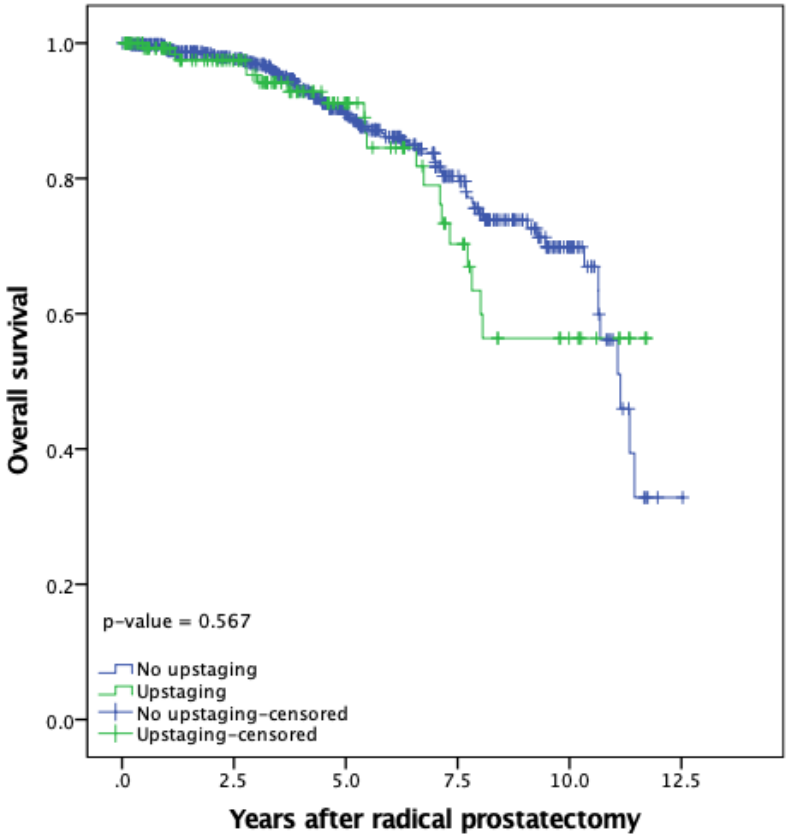

Fig 9. Prostate cancer overall survival rates according to upstaging.

For patients with and without PCa upgrading mean CSS was 11.9 (95% CI: 11.3-12.5) and 11.9 (95% CI: 11.7-12.0) years. Five-year CSS did not differ between both cohorts (99.1% vs. 99.1%), while ten-year CSS rate was significantly lower (88.7% vs. 98.3%) in patients who underwent pathological upgrading after RP (p = 0.039; Figure 10).

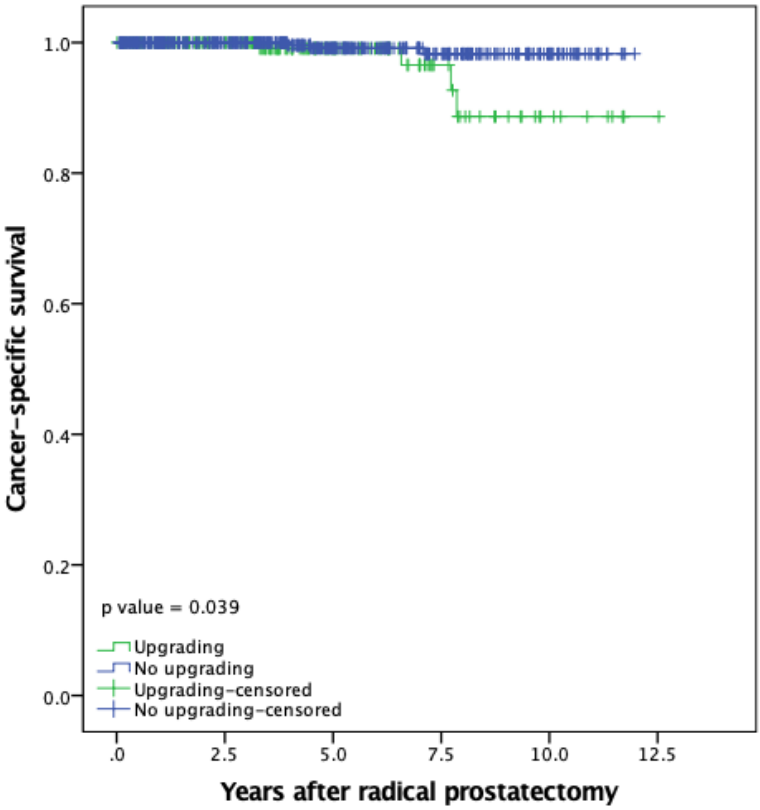

Fig 10. Prostate cancer-specific survival rates according to upgrading.

Mean CSS for upstaged and non-upstaged PCa was 11.1 (95% CI: 10.5-11.7) and 12.4 (95% CI: 12.3-12.5) years. No differences were also observed at five-year mark (98.2% vs. 99.3%), while upstaging was associated with inferior ten-year CSS rates after RP (87.3% vs. 98.3%; p = 0.008; Figure 11).

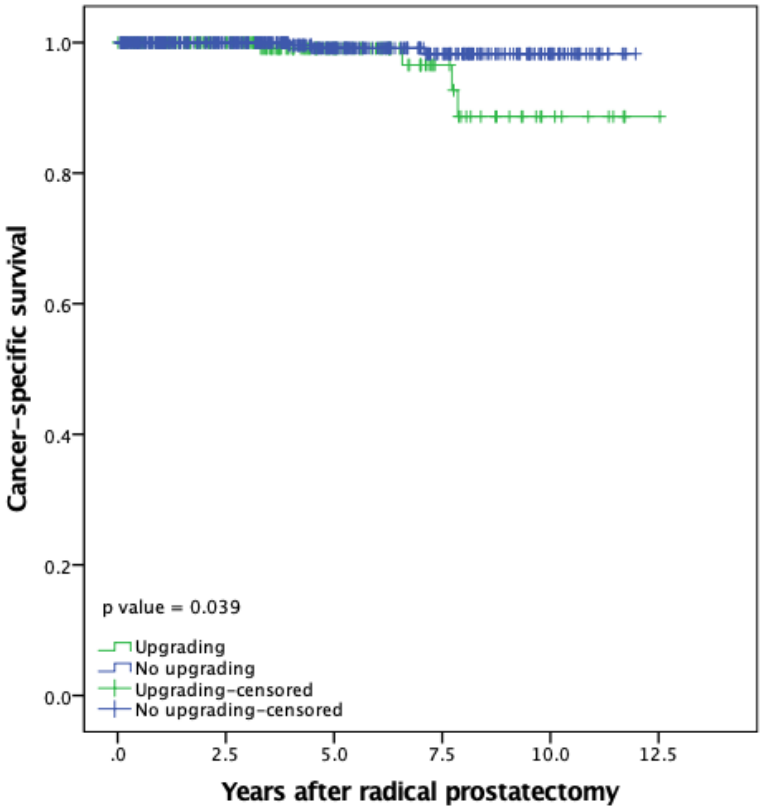

Fig 11. Prostate cancer-specific survival rates according to upstaging.

4.7 DNA methylation analysis in tissue

Methylation status of three tumour suppressor genes (*RARβ*, *RASSF1* and *GSTP1*) were first validated in prostatic tissues to evaluate their suitability for PCa diagnostics. Methylation frequencies of all three genes were significantly higher in PCa as compared to noncancerous prostate tissues (all $p < 0.001$; Figure 12), what encouraged their further analysis in urine.

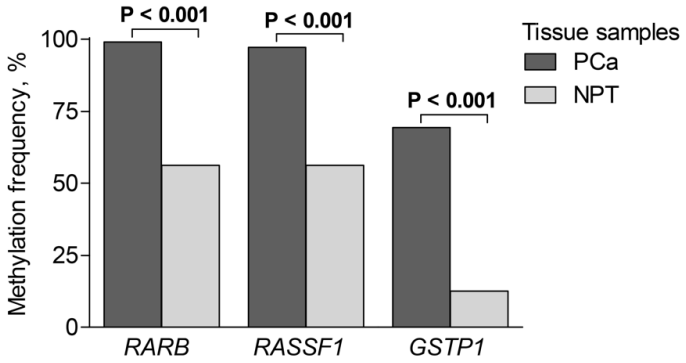

Fig. 12. DNA methylation frequencies of genes *RARβ*, *RASSF1*, and *GSTP1* in cancerous and noncancerous prostate tissue. Statistically significant p-values ($p < 0.050$) are marked in bold.
Abbreviations: NPT = noncancerous prostate tissue; PCa = prostate cancer.

4.8 DNA methylation analysis in urine

In urine samples, methylation frequencies were similar between the voided and catheterized urine cohorts, except for *GSTP1* ($p = 0.016$; Figure 13).

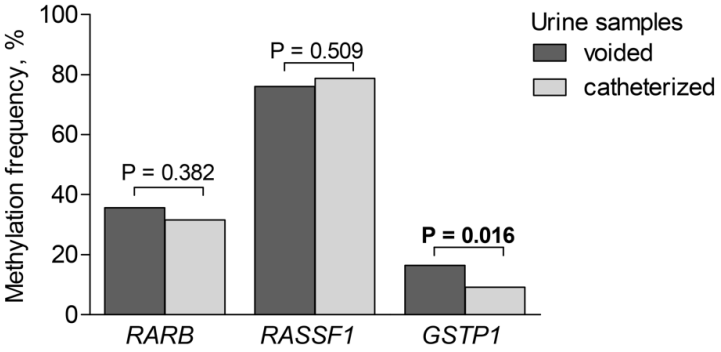

Fig. 13. DNA methylation frequencies of genes *RARβ*, *RASSF1*, and *GSTP1* in urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

Methylation of at least one gene was detected in 80.3% (151/188) of voided and 83.7% (273/326) of catheterized urine. The average methylation levels ranged from 0.6 to 15.1% and were significantly different for *RASSF1* and *GSTP1* between the voided and catheterized urine samples (both p = 0.001; Figure 14).

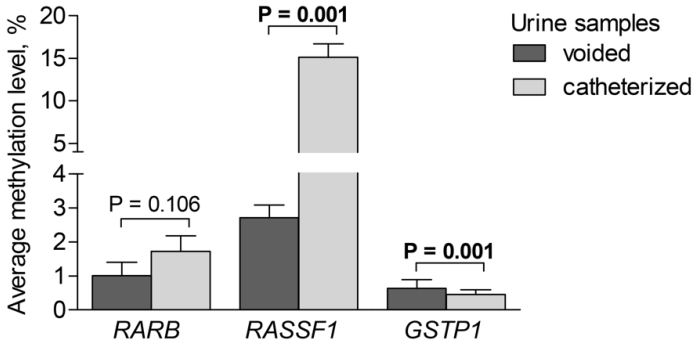

Fig. 14. Average methylation levels of genes *RARβ*, *RASSF1*, and *GSTP1* in urine samples. Statistically significant p-values (p < 0.050) are marked in bold.

4.9 DNA methylation analysis in urine and upgrading

Single genes, two-gene (*RASSF1* and *GSTP1*) and three-gene (*RARβ*, *RASSF1*, and *GSTP1*) panels were further evaluated as potential noninvasive biomarkers for PCa upgrading, upstaging and risk increase.

In the catheterized urine cohort, higher average methylation level of *GSTP1* was detected in the cases with postoperative upgrading (p = 0.022; Figure 15), while no associations were detected in the voided samples (Figure 16).

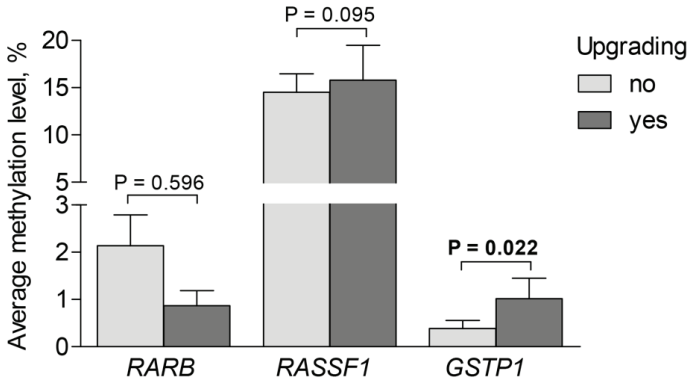

Fig 15. Average methylation levels of *RARβ*, *RASSF1*, and *GSTP1* according to ISUP change in catheterized urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

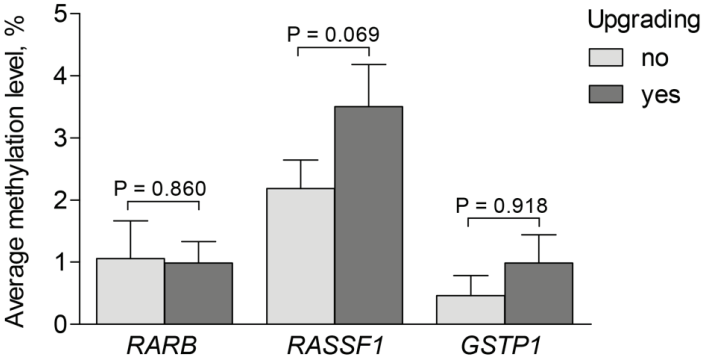

Fig. 16. Average methylation levels of *RARβ*, *RASSF1*, and *GSTP1* according to ISUP change in voided urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

During ROC curve analysis none of the genes separately (Table 3) revealed significant results in predicting PCa upgrading. Meanwhile, three-gene (*RARβ*, *RASSF1* and *GSTP1*) panel in both cohorts (Figure 17 and 18) and PSA in catheterized urine (Figure 18) as well as its combinations with single genes (Table 3) or two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1*, *GSTP1*) panels (Figure 17 and 18) were predictive for PCa upgrading (all $p < 0.050$).

Table 3. ROC curve values of particular gene methylation alone and in combination with PSA as biomarkers of upstaging, upgrading, and risk increase.

Gene	Voided urine (N = 188)				Catheterized urine (N = 326)			
	Gene methylation only		Gene methylation + PSA		Gene methylation only		Gene methylation + PSA	
	AUC	P value	AUC	P value	AUC	P value	AUC	P value
Upgrading								
RARβ	0.508	0.862	0.532	0.470	0.521	0.600	0.682	**<0.001**
RASSF1	0.581	0.074	0.600	**0.025**	0.571	0.082	0.692	**<0.001**
GSTP1	0.503	0.919	0.517	0.704	0.550	0.065	0.715	**<0.001**
Upstaging								
RARβ	0.551	0.330	0.596	0.053	0.554	0.154	0.626	**0.002**
RASSF1	0.586	0.106	0.608	**0.029**	0.540	0.313	0.632	**0.001**
GSTP1	0.575	0.062	0.608	**0.033**	0.533	0.174	0.627	**0.018**
Risk increase								
RARβ	0.558	0.194	0.518	0.692	0.510	0.816	0.589	**0.045**
RASSF1	0.538	0.434	0.537	0.426	0.523	0.599	0.597	**0.025**
GSTP1	0.539	0.189	0.521	0.634	0.557	**0.047**	0.623	**0.004**

Statistically significant p-values ($p < 0.050$) are marked in bold.
Abbreviations: N = number of patients; PSA = prostate-specific antigen.

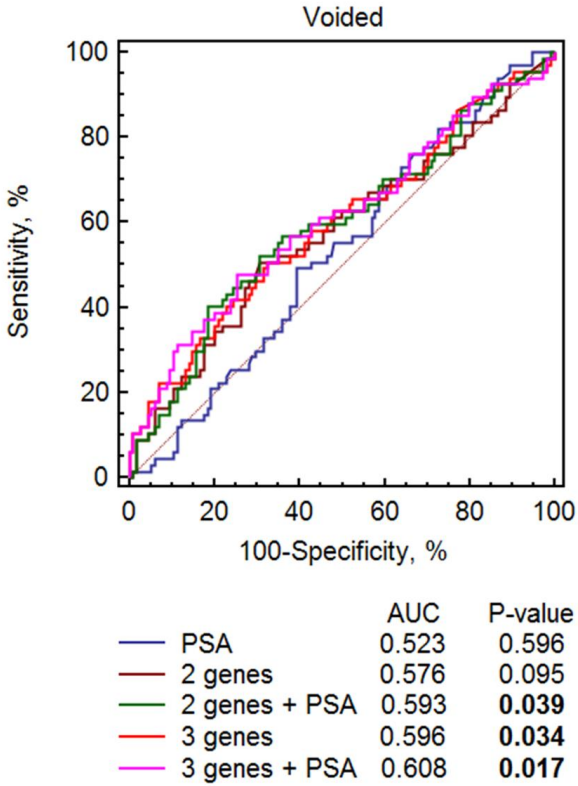

Fig. 17. ROC curve analysis of two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1* and *GSTP1*) panels as well as their combinations with PSA for prediction of PCa upgrading in voided urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

Abbreviations: AUC = area under the curve; ROC = receiver operating characteristic; PSA = prostate-specific antigen.

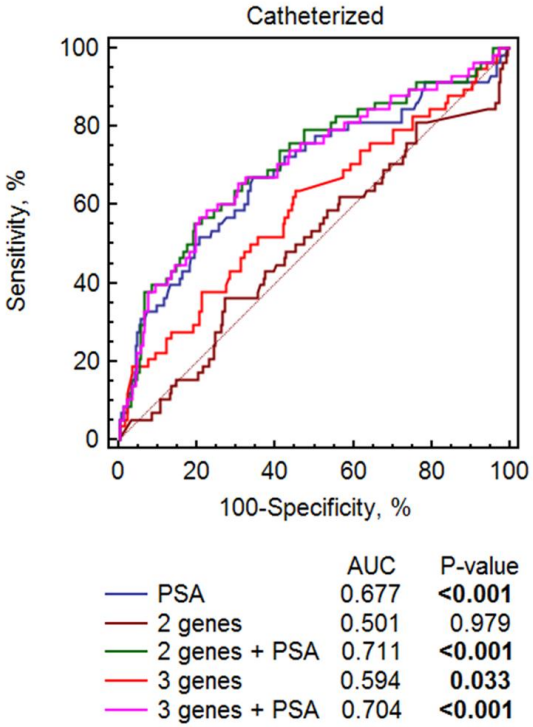

Fig 18. ROC curve analysis of two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1* and *GSTP1*) panels as well as their combinations with PSA for prediction of PCa upgrading in catheterized urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

Abbreviations: AUC = area under the curve; ROC = receiver operating characteristic; PSA = prostate-specific antigen.

NPV of two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) panels as well as their combinations with PSA was 38-83%, while PPV – 25-51%, sensitivity – 40-85% and specificity – 55-82% (Table 4).

During multivariate logistic regression analysis (Table 5) three-gene (*RARβ, RASSF1* and *GSTP1*) panel in combination with PSA showed the highest OR for upgrading in voided (OR = 111.5; 95% CI: 3.0-4077.9; $p = 0.004$) and catheterized (OR = 213.0; 95% CI: 13.6-3339.4; $p < 0.001$) urine.

Table 4. Sensitivity, specificity, PPV and NPV of the DNA methylation biomarkers (*RARβ*, *RASSF1*, and *GSTP1*) and PSA for predicting PCa upgrading, upstaging and risk group increase.

Parameter	Upgrading				Upstaging				Risk increase			
	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Sensitivity, %	Specificity, %	PPV, %	NPV, %	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Voided urine (N = 188)												
PSA	82,6	27,0	29,7	80,6	86,7	27,9	23,4	89,1	85,0	27,4	26,9	85,3
RARβ, RASSF1, GSTP1	50,7	67,5	36,9	78,6	52,3	72,5	32,6	85,6	45,8	71,3	33,4	80,7
RARβ, RASSF1, GSTP1 + PSA	47,8	74,6	41,2	79,3	56,8	65,2	29,4	85,6	89,8	27,0	27,9	89,4
RASSF1, GSTP1	50,7	69,3	35,7	78,1	50,0	73,9	32,8	85,3	45,8	71,3	33,4	80,7
RASSF1, GSTP1 + PSA	40,3	81,6	45,0	78,5	54,5	66,7	27,7	84,6	89,8	27,0	27,9	89,4
Catheterized urine (N = 326)												
PSA	66,1	64,8	41,2	87,3	73,1	51,4	27,7	88,2	58,2	62,6	32,8	82,7
RARβ, RASSF1, GSTP1	63,8	54,8	34,5	80,2	68,7	43,7	23,7	84,6	30,2	82,4	35,0	79,0
RARβ, RASSF1, GSTP1 + PSA	56,9	79,2	50,5	83,1	60,9	63,3	29,7	86,4	56,6	62,1	31,2	81,4
RASSF1, GSTP1	84,5	73,6	24,7	38,4	67,2	53,9	27,1	86,6	35,8	75,8	31,7	79,0
RASSF1, GSTP1 + PSA	55,2	80,5	51,4	82,8	65,6	64,9	27,5	86,5	58,5	61,2	32,2	82,4

Abbreviations: N = number of patients; NPV = negative predictive value; PPV = positive predictive value; PCa = prostate cancer; PSA = prostate-specific antigen.

Table 5. Multivariate logistic regression analysis for predicting upgrading, upstaging and risk increase

Gene	Voided urine (N = 188)			Catheterized urine (N = 326)		
	OR	95% CI	P value	OR	95% CI	P value
Upgrading						
PSA	1.0	[0.9; 1.0]	0.385	1.1	[1.0; 1.1]	**< 0.001**
RARβ, *RASSF1*, *GSTP1*	110.6	[2.8; 4422.2]	**0.005**	199.7	[1.2; > 10000.0]	**0.040**
RARβ, *RASSF1*, *GSTP1* + PSA	111.5	[3.0; 4077.9]	**0.004**	213.0	[13.6; 3339.4]	**< 0.001**
RASSF1, *GSTP1*	69.9	[0.4; > 10000,0]	0.092	68.7	[0.1; > 10000.0]	0.192
RASSF1, *GSTP1* + PSA	69.7	[0.6; 8442.0]	0.075	171.0	[10.4; 2801.1]	**< 0.001**
Upstaging						
PSA	1.0	[1.0; 1.0]	0.857	1.1	[1.0; 1.1]	**< 0.001**
RARβ, *RASSF1*, *GSTP1*	120.1	[2.2; 6545.0]	**0.009**	1742.6	[0.1; > 10000.0]	0.078
RARβ, *RASSF1*, *GSTP1* + PSA	122.2	[2.3; 6556.6]	**0.008**	169.1	[8.6; 3335.0]	**< 0.001**
RASSF1, *GSTP1*	113.1	[2.2; 5717.0]	**0.009**	585.6	[<0.1; > 10000.0]	0.252
RASSF1, *GSTP1* + PSA	115.2	[2.3; 5742.5]	**0.008**	143.0	[7.4; 2773.5]	**< 0.001**
Risk increase						
PSA	1.0	[0.9; 1.0]	0.401	1.0	[1.0; 1.1]	0.130
RARβ, *RASSF1*, *GSTP1*	84.5	[0.8; 9115.3]	0.059	416.0	[1.4; > 10000.0]	**0.037**
RARβ, *RASSF1*, *GSTP1* + PSA	83.5	[1.0; 7048.2]	**0.046**	280.4	[2.7; > 10000.0]	**0.018**
RASSF1, *GSTP1*	85.2	[0.8; 9215.9]	**0.045**	118.5	[0.1; > 10000.0]	0.171
RASSF1, *GSTP1* + PSA	84.5	[1.0; 7164.5]	**0.045**	103.5	[0.7; > 10000.0]	0.074

Statistically significant p-values ($p < 0.050$) are marked in bold.

Abbreviations: CI = confidence interval; N = number of patients; OR = odds ratio; PSA = prostate-specific antigen.

4.10 DNA methylation analysis in urine and upstaging

In the voided urine cohort, higher average methylation level of *GSTP1* was detected in the cases with postoperative upstaging (p = 0.033; Figure 19), while no associations were detected in the catheterized samples (Figure 20).

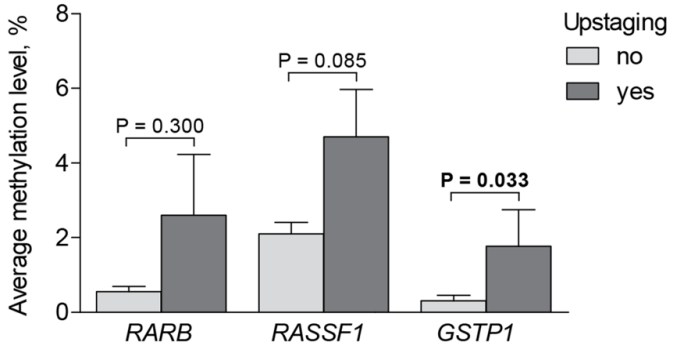

Fig 19. Average methylation levels of *RARβ*, *RASSF1*, and *GSTP1* according to upstaging in voided urine samples. Statistically significant p-values (p < 0.050) are marked in bold.

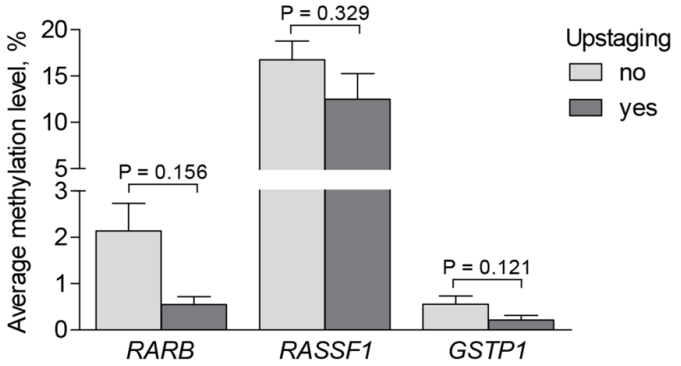

Fig 20. Average methylation levels of *RARβ*, *RASSF1*, and *GSTP1* according to upstaging in catheterized urine samples.

During ROC curve analysis none of the genes separately (Table 3) or any of their combinations with each other (Figure 21 and 22) did not reveal any statistically significant result in predicting PCa upstaging. Meanwhile, PSA alone in catheterized urine (Figure 22) and its combinations with single genes (Table 3) as well as with two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) panels (Figure 21 and 22) were predictive for PCa upstaging in both cohorts (all $p < 0.050$).

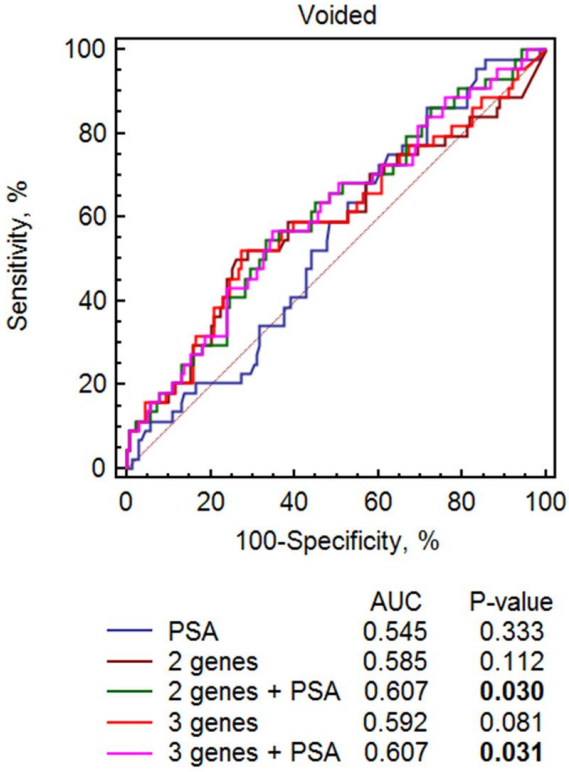

Fig 21. ROC curve analysis of two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1* and *GSTP1*) panels as well as their combinations with PSA for prediction of PCa upstaging in voided urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

Abbreviations: AUC = area under the curve; ROC = receiver operating characteristic; PSA = prostate-specific antigen.

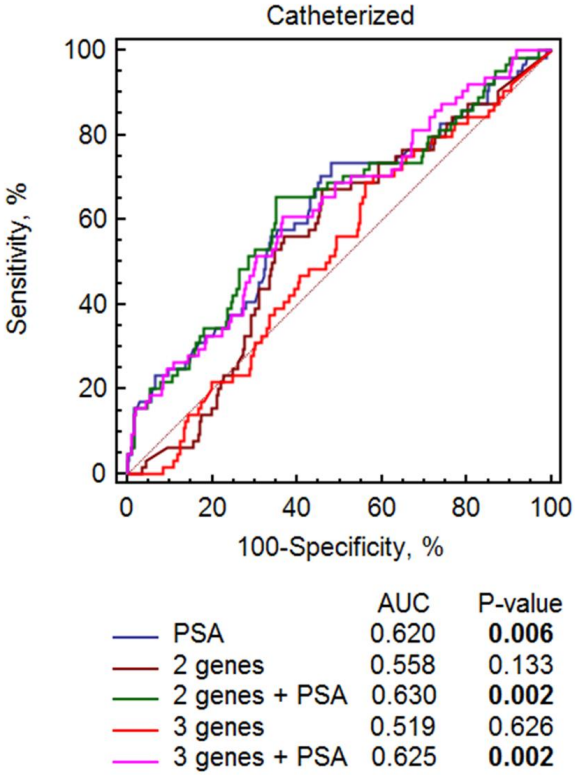

Fig. 22. ROC curve analysis of two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1* and *GSTP1*) panels as well as their combinations with PSA for prediction of PCa upstaging in catheterized urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

Abbreviations: AUC = area under the curve; ROC = receiver operating characteristic; PSA = prostate-specific antigen.

The *Partin* value was a significant predictor for upstaging in voided (Figure 23) and catheterized (Figure 24) urine. Though, the combination of the two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) set with *Partin* nomogram only slightly increased the AUC, with more apparent difference observed in voided cohort (Figure 23 and 24).

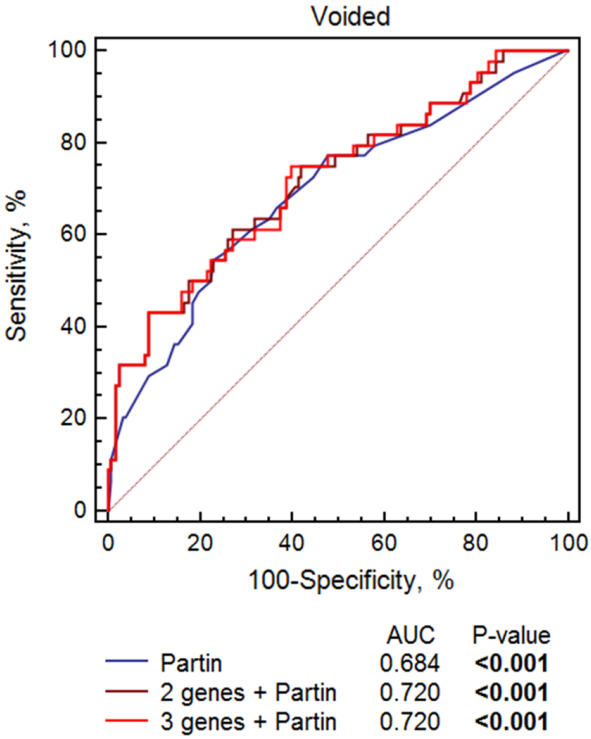

Fig. 23. ROC curve analysis of *Partin* value separately and in combination with two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1* and *GSTP1*) panels for prediction of PCa upstaging in voided urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.
Abbreviations: AUC = area under the curve; ROC = receiver operating characteristic.

NPV of two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) panels as well as their combinations with PSA was 85-87%, while PPV – 24-33%, sensitivity – 50-69% and specificity – 44-74% (Table 4).

In both cohorts, the addition of *Partin* value to two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) panels increased the test's specificity and PPV: NPV – 82-91%, PPV – 33-56%, sensitivity – 43-75% and specificity – 60-91% (Table 6).

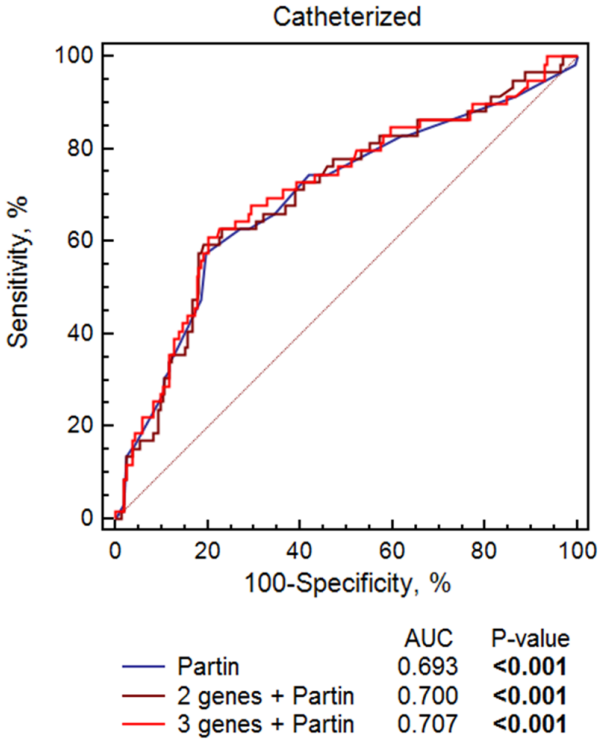

Fig. 24. ROC curve analysis of *Partin* value separately and in combination with two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1* and *GSTP1*) panels for prediction of PCa upstaging in catheterized urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.
Abbreviations: AUC = area under the curve; ROC = receiver operating characteristic.

During multivariate logistic regression analysis (Table 5) three-gene (*RARβ, RASSF1* and *GSTP1*) panel in combination with PSA showed the highest OR for upstaging in voided (OR = 122.2; 95% CI: 2.3-6556.6; $p = 0.008$) and catheterized (OR = 169.1; 95%CI: 8.6-3335.0; $p < 0.001$) urine.

Table 6. Sensitivity, specificity, PPV and NPV of *Partin* value separately and in combination with two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1*, *GSTP1*) panels for predicting PCa upstaging.

Parameter	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Voided urine (N = 188)				
Partin value	53.3	77.0	37.1	86.6
RASSF1, *GSTP1* + *Partin* value	43.2	91.3	55.7	86.3
RARβ, *RASSF1*, *GSTP1* + *Partin* value	75.0	60.3	32.5	90.5
Catheterized urine (N = 326)				
Partin value	56.5	79.8	41.6	82.4
RASSF1, *GSTP1* + *Partin* value	59.3	80.8	44.0	88.6
RARβ, *RASSF1*, *GSTP1* + *Partin* value	61.0	79.8	43.5	88.9

Abbreviations: N = number of patients; NPV = negative predictive value; PPV = positive predictive value; PCa = prostate cancer.

4.11 DNA methylation analysis in urine and risk change

In the catheterized urine, higher average methylation level of *GSTP1* was detected in the cases with increased risk after RP ($p = 0.012$; Figure 25), while no associations were detected in the voided urine samples (Figure 26).

During ROC curve analysis PSA separately in any cohort (Figure 27 and 28) and none of the genes separately in voided urine (Table 3) did not reveal any statistically significant result. In catheterized urine samples *GSTP1* alone ($p = 0.047$) and PSA combination with any single gene (all $p < 0.050$) significantly predicted PCa risk increase after RP (Table 3). Two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) panels in combination with PSA were predictive for PCa risk increase in the catheterized urine (all $p < 0.050$; Figure 28), while a weak tendency was observed in the voided urine samples (Figure 27).

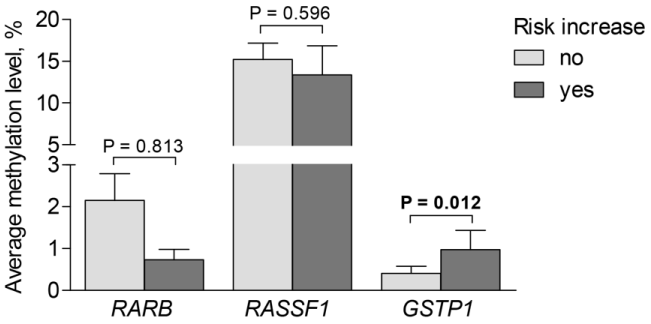

Fig. 25. Average methylation levels of *RARβ*, *RASSF1*, and *GSTP1* according to risk change in catheterized urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

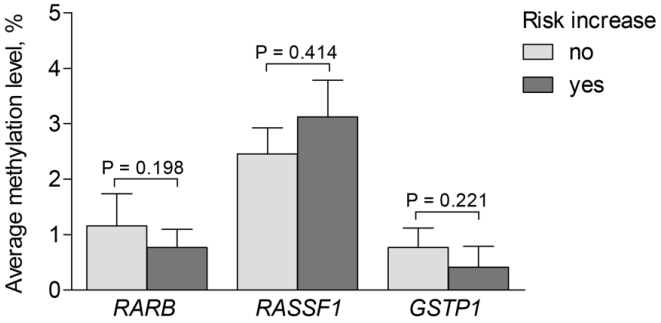

Fig. 26. Average methylation levels of *RARβ*, *RASSF1*, and *GSTP1* according to risk change in voided urine samples.

NPV of two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) panels as well as their combinations with PSA was 79-89%, while PPV – 27-35%, sensitivity – 36-90% and specificity – 27-82% (Table 4).

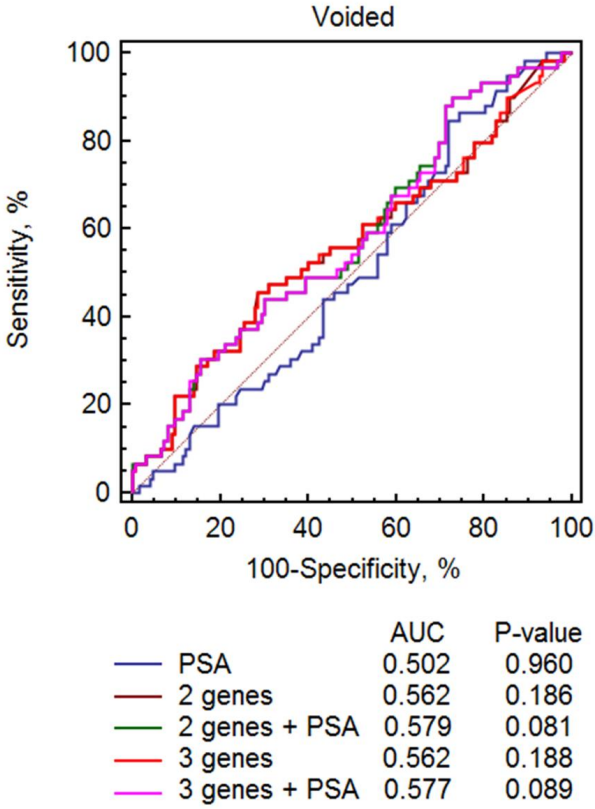

Fig. 27. ROC curve analysis of two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1* and *GSTP1*) panels as well as their combinations with PSA for prediction of PCa risk increase in voided urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

Abbreviations: AUC = area under the curve; ROC = receiver operating characteristic; PSA = prostate-specific antigen.

During multivariate logistic regression analysis (Table 5) two-gene (*RASSF1* and *GSTP1*) panel showed the highest OR for postoperative risk increase in voided urine (OR = 85.2; 95% CI: 0.8-9215.9; p = 0.045), while three-gene (*RARβ, RASSF1* and *GSTP1*) panel showed the highest OR for postoperative risk increase in catheterized urine (OR = 416.0; 95% CI: 1.4-10000.0; p = 0.037).

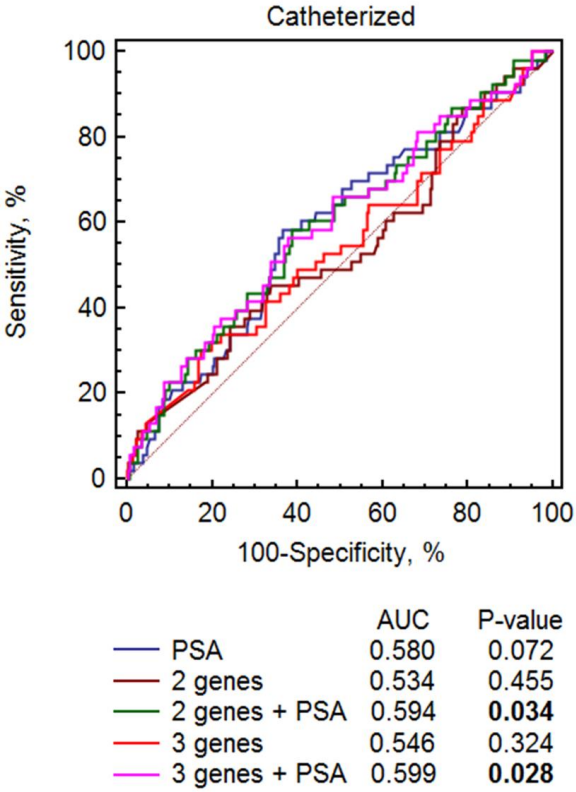

Fig. 28. ROC curve analysis of two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ*, *RASSF1* and *GSTP1*) panels as well as their combinations with PSA for prediction of PCa risk increase in catheterized urine samples. Statistically significant p-values ($p < 0.050$) are marked in bold.

Abbreviations: AUC = area under the curve; ROC = receiver operating characteristic; PSA = prostate-specific antigen.

5. DISCUSSION

PCa with high-levels of molecular and morphological diversity is a particularly heterogeneous disease ranging from clinically indolent localised tumour to metastatic and life-threatening disease. Thus, accurate assessment of disease's aggressiveness and optimal disease management are the main goals for clinicians. The modified D'Amico classification system is the most commonly used criteria for the definition of PCa risk, however 24-41% of PCa patients are upgrading and 29-34% – upstaging after RP. According to our study, upgrading was observed in 27% and upstaging in 20% of PCa patients after RP. Among the upgraded cases, 86% of the patients were initially diagnosed with cISUP 1 PCa, while 49 % of upstaged cases were clinically diagnosed with cT1c disease. According to the D'Amico criteria, the latter characteristics characterize low-risk PCa and are the main indications for active surveillance.

The discrepancy between initial PB and RP material are mainly attributable to sampling and analysis errors:

1. During PB just a limited amount of prostate tissue is collected, so a sampling error could occur when a higher Gleason grade is missed on the needle biopsy;
2. 20% of RP specimens have a tertiary Gleason grade which can be captured in biopsy but missed during histopathological examination of RP material, especially when partial embedded technique is considered;
3. Besides, borderline neoplastic changes can be interpreted differently by different pathologists, especially in nonreferral centres when high quality samples are unavailable.

The clinical and prognostic significance of PCa upgrading and upstaging remains controversial. According to our findings, patients undergoing upgrading or upstaging are 1.5 times more likely to have a PSM on pathological specimen. It is generally known that PSM is associated with RP for high-risk disease and surgical experience, especially in upgrading and upstaging settings when surgeons are facing the disease clinically suspected to have a low risk of progression.

According to literature, it has been shown that upgrading is associated with BCR. Ham W. S. et al. reported that downgrading is associated with better BCR-free survival, while upgrading increases the risk for BCR. According to our findings, upgrading increase the risk for BCR at 1.8 times and upstaging at 2.1 times, while patients with clinically diagnosed intermediate and high-risk PCa carry the highest risk. Different risk for BCR could be explained by different upgrading categories, while the vast majority of low-risk patients (cISUP 1) postoperatively upgraded to intermediate-risk disease (pISUP 2; in the present study 86.3% of patients) and intermediate-risk cases (cISUP 2) upgraded to an even higher-risk disease, i.e. pISUP 3 and higher.

The association between BCR and progression to metastatic disease and death of PCa is well documented in scientific literature. According to our findings, MFS was significantly shorter in patients with upgrading and upstaging, while grade and stage increase after RP did not reveal any impact on OS but was associated with inferior ten-year CSS: 89% vs. 98% for upgrading, and 87% vs. 98% for upstaging. Our findings are consistent with other investigators, where inferior CSS results have been reported for patients undergoing upstaging and upgrading.

The impact of PCa upgrading and upstaging on surgical and oncological outcomes are raising the issue about serious diagnostic problems. Several nomograms have been suggested to predict the probability of pathologic upgrading, however, most of them are designed for low-risk PCa and have limited value in counselling patients with intermediate and high-risk disease. Molecular markers containing information from all tumour foci and reflecting PCa genetic and epigenetic changes may provide valuable information for improved diagnosis and timely prediction of PCa aggressiveness.

In our study, promoter methylation of *RARβ*, *RASSF1* and *GSTP1* was evaluated in voided and catheterized urine for prediction of PCa aggressiveness, local stage and risk group. Methylation frequency in cancerous tissue was as follows: *RARβ* – 99%, *RASSF1* – 97% and *GSTP1* – 69% (Figure 12), while in voided and catheterized urine: *RARβ* – 35% and 32%, *RASSF1* – 76% and 79%, *GSTP1* – 17% and 9%

(Figure 13). According to other authors, *RARβ* was hypermethylated in 40-96% of PCa tissue specimens and in 35-62% of urine samples, while *RASSF1* – in 28-99% of PCa tissue specimens and in 38-78% of urine samples and *GSTP1* – in 17-95% of PCa tissue specimens and in 27-83% of urine samples. The differences between methylation frequencies and levels could be attributed to:

1. Different cohorts with different sample size, different familial and epidemiological history as well as different clinico-pathological characteristics;
2. Different methods for collecting cancerous tissue: RP, PB, transurethral resection of the prostate (TRUP) and even metastasectomy. Cancerous tissue obtained during PB or TURP may not properly reflect the aggressiveness and stage of the disease;
3. Different percentage of cancerous cells in cancerous tissue. According to our protocol, cancerous tissue contained ≥ 70% of cancer cells, while in the majority of other studies it was not reported;
4. Different methods for collecting urine samples, i.e. with and without prostate massage, voided or catheterized urine;
5. Different methods for detecting PCa (PB or RP), when urine was used for molecular analysis. PCa characteristics obtained during PB may not properly reflect the aggressiveness and stage of the disease;
6. Different control cohorts, while differences were observed not only in clinico-pathological characteristics or familial and epidemiological history, but also in sample size and noncancerous tissue. Some studies investigating biomarkers in urine used normal prostate tissue and benign prostate hyperplasia tissue as a control;
7. Different methods for collecting noncancerous prostate tissue: RP, PB, TURP and adenomectomy. Benign tissue obtained during PB, TURP or adenomectomy may not exclude the diagnosis of PCa. During TURP and adenomectomy the tissue for histological evaluation is obtained from the transition zone, while most of PCa are located in the peripheral zone. During all these procedures paraneoplastic tissue could be obtained, where hypermethylation could be identified, while no morphological changes are detected;

8. Other malignancies, which are also associated with hypermethylation of *RARβ, RASSF1* and *GSTP1*;
9. Different processing and storage of prostate tissues specimens. The quality of DNA extracted from snap frozen tissue specimens is better as compared to formalin-fixed and paraffin-embedded samples, which more often are fragmented. Poor quality and fragmented DNA can distort the methylation analysis because only shorter amplicons will be amplified;
10. Different processing of urine samples. Different methylation results could be obtained from different fractions of urine, i.e. sediments, exosomes or supernatant;
11. Different methods of DNA methylation analysis. Qualitative methylation analysis, such as MSP provides sensitive detection of methylated DNA, though low methylation levels could be undetected or classified as false positive. Quantitative methylation analysis, such as QMSP or pyrosequencing allows precise detection of DNA methylation, though control samples and other technical measures are needed to allow accurate interpretation of low methylation levels;
12. Different methylation level which is considered as the threshold for QMSP. According to our protocol, methylation level of 0.1% was considered as the threshold, while in majority of the studies it was not shown.

Despite differences in methylations frequencies and levels, all genes (*RARβ, RASSF1* and *GSTP1*) have shown significantly different methylation frequencies in normal and PCa tissues, when higher methylation frequencies were detected in cancerous tissue (Figure 12).

According to our findings, the general *GSTP1* methylation level, as well as frequency, was relatively low, although significantly different in cases with postoperative upgrading, upstaging and risk increase. More intense *GSTP1* methylation was observed in voided urine of patients who underwent upstaging, whereas it was associated with upgrading and risk increase in catheterized urine. All these findings are in agreement with other studies reporting associations of *GSTP1* with aggressiveness and pathological stage of PCa.

A slightly higher methylation level of *RASSF1* was detected in voided urine of the patients with upstaging, while the same tendency was observed in both cohorts of the patients with upgrading. *RARβ* hypermethylation did not show any potential in predicting change of clinical parameters.

Value of each gene individually to predict postoperative PCa upstaging and upgrading was limited, while single genes combination with PSA, as well as two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) panel combination with PSA revealed moderate test performance parameters in both voided and catheterized urine cohorts.

GSTP1 separately and PSA combination with all single genes, as well as two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) panels was also predictive for PCa risk change, but only in the catheterized urine. The discrepancies observed between the two cohorts could be explained by the different methylation levels detected in voided and catheterized urine, especially those of *RASSF1* and *GSTP1*, when this was most likely related to the different techniques employed for urine sample collection.

Nevertheless, I would like to mention several limitations of the present study. Firstly, some differences in clinico-pathological characteristics between the patients with voided and catheterized urine were identified, which could have influenced the differences observed in the methylation levels and frequencies. Secondly, although the biological samples were collected prospectively, some of clinical data were collected and analysed in a retrospective way. Furthermore, no pathological re-evaluation of biopsy specimens from outside institutions were performed so inter-observer variation might affect the results. Finally, multiparametric magnetic resonance imaging was not included in our protocol, which is widely used nowadays clinical practice and associated with less pathological upgrading at RP.

6. CONCLUSIONS

1. Clinico-pathological characteristics presently used in everyday clinical practice, such as cISUP, cTNM and PSA, are not sufficiently reliable diagnostic tools to characterize PCa aggressiveness, local stage and risk group. Postoperative upgrading was detected in 27.2% of PCa patients, while upstaging in 20.3% and risk increase in 23.9% of the patients, with the total misclassification rate, i.e. upgrading and/or upstaging, as high as 39.0%.
2. PCa upgrading and upstaging after RP is associated with:
   a. PSM, when each of them increases the risk 1.5 times;
   b. BCR, when upgrading increased the risk 1.8 times and upstaging – 2.1 times;
   c. Metastatic disease, when metastases 2.0 times more often were diagnosed to the patients undergoing upgrading and 3.2 times more often to the patients undergoing upstaging;
   d. MFS, while mean time to metastases was significantly shorter for the patients undergoing upstaging: 10.3 vs.12.1 years;
   e. 10-year CSS, while it was significantly shorter for the patients undergoing upgrading (89% vs. 98%) and upstaging (87% vs. 98%).
3. *RARβ*, *RASSF1* and *GSTP1* gene promoter methylation frequencies are significantly different in cancerous and noncancerous prostate tissues.
4. Higher methylation frequency and level of *GSTP1* was detected in voided urine, while higher methylation level of *RASSF1* was detected in catheterized urine.
5. Two-gene (*RASSF1*, *GSTP1*) and three-gene (*RARβ, RASSF1*, *GSTP1*) panels in combination with PSA revealed significant results predicting postoperative upgrading, upstaging and risk increase, consequently, they are suitable for PCa diagnostics.

7. RECOMMENDATIONS

- Taking into account that 39% of PCa stage and/or differentiation grade established during clinical diagnosis do not match RP histology, clinical evaluation of PCa patients should be improved by non-invasive molecular markers.
- While developing new PCa diagnostic and prognostic tests, we recommend to use combinations of epigenetic markers with each other and with PSA, as well as with clinical characteristics.
- Having diagnosed low-risk PCa and considering active surveillance, we recommend to additionally perform the three-gene (*RARβ*, *RASSF1* and *GSTP1*) urine test, which, in combination with PSA, could improve characterization of PCa aggressiveness and stage.
- During RP for low-risk disease, we recommend to carefully follow all principles of oncological safety, especially dissecting the apex of the prostate, where possibility for PSM is the highest.
- We recommend strict monitoring of PSA and considering the usage of adjuvant radiotherapy for low-risk PCa when upgrading or upstaging is detected after RP.
- We recommend adjuvant radiotherapy for intermediate-risk PCa when upgrading or upstaging is detected after RP.

8. PUBLICATIONS AND PRESENTATIONS

8.1 Publications

1. Bakavičius A, Daniūnaitė K, Žukauskaitė K, Barisienė M, Jarmalaitė S, Jankevičius F. Urinary DNA methylation biomarkers for prediction of prostate cancer upgrading and upstaging. Clin Epigenetics. 2019; 11(1): 115-125.
2. Bakavičius A, Drevinskaitė M, Daniūnaitė K, Barisienė M, Jarmalaitė S, Jankevičius F. The impact of prostate cancer upgrading and upstaging on biochemical recurrence and cancer-specific survival. Medicina (Kaunas). 2020; 56(2): E61.

Publications not directly related to the topic of dissertation:

1. Daniūnaitė K, Dubikaitytė M, Gibas P, Bakavičius A, Lazutka J R, Ulys A, Jankevičius F, Jarmalaitė S. Clinical significance of miRNA host gene promoter methylation in prostate cancer. Hum Mol Genet. 2017; 26(13): 2451-2461.
2. Stuopelytė K, Daniūnaitė K, Bakavičius A, Lazutka J R, Jankevičius F, Jarmalaitė S. The utility of urine-circulating miRNAs for detection of prostate cancer. Br J Cancer. 2016; 115(6): 707-715.
3. Damidenko R, Daniūnaitė K, Bakavičius A, Sabaliauskaitė R, Skeberdytė A, Petroška D, Laurinavičius A, Jankevičius F, Lazutka J R, Jarmalaitė S. Decreased expression of *MT1E* is a potential biomarker of prostate cancer progression. Oncotarget. 2017; 8(37): 61709-61718.

8.2 Presentations

1. Bakavičius A, Daniūnaitė K, Jarmalaitė S, Jankevičius F. Urinary 3-gene methylation test for prostate cancer risk assessment. 6th EAU Baltic Meeting 2019. 2019 May 24-25. Tallinn, Estonia.
2. Bakavičius A, Daniūnaitė K, Ulys A, Jarmalaitė S, Jankevičius F. *GSTP1* as promising diagnostic tool for more precise prostate

cancer diagnosis. 38th Congress of SIU. 2018 October 4-7. Seoul, South Korea.

3. Bakavičius A, Daniūnaitė K, Jarmalaitė S, Jankevičius F. Epigenetic markers to overcome limitations in prostate cancer diagnostics. 5th EAU Baltic Meeting 2018, 2018 May 25-26. Riga, Latvia. Nominated with Second Berlin Chemie Award for the Best Poster Presentation.
4. Bakavičius A, Daniūnaitė K, Jarmalaitė S, Jankevičius F. Epigenetic markers to overcome limitations in prostate cancer diagnostics. 34th Annual EAU Congress 2019. 2019 March 15-19. Barcelona, Spain.
5. Žukauskaitė K, Daniūnaitė K, Bakavičius A, Jarmalaitė S. Promoter methylation analysis of *RARβ*, *RASSF1* and *GSTP1* in prostate cancer. The Coins 2018. 2018 February 28-March 2. Vilnius, Lithuania.
6. Žukauskaitė K, Daniūnaitė K, Bakavičius A, Jarmalaitė S. Promoter methylation analysis of tumor suppressor genes in prostate cancer tissue and urine. 15th International conference of the Lithuanian Biochemical Society. 2018 June 26-29. Dubingiai, Lithuania.

9. FUNDING

The research was supported by Research Council of Lithuania (No. LIG-14/2012 and No. SEN-9/2016), Lithuanian State Science and Studies Foundation (No. C03/2007-09) and Vilnius University Faculty of Medicine. Fellowship at L'Institut Mutualiste Montsouris in Paris was funded by Erasmus+ programme and the French Government (No. 913108F). The author received a scholarship for academic activities from Research Council of Lithuania (2019; No. V-108).

10. *CURRICULUM VITAE*

Personal information:	
Name:	Arnas
Surname:	Bakavičius
Date of birth:	1986-06-10
Gender:	Male
Citizenship:	Lithuanian
Address:	M.Bukšos str. 14-3, 08431 Vilnius, Lithuania
Telephone:	+370 698 83048
Email address:	arnas.bakavicius@gmail.com
Education:	
2015 – 2019	Vilnius University, Faculty of Medicine, Doctoral (PhD) studies
2017 – 2018	Clinical Fellowship in Robotics & Minimally Invasive Urology; L'Institut Mutualiste Montsouris, Paris, France
2011 – 2016	Vilnius University, Residency in Urology
2005 – 2011	Vilnius University, Faculty of Medicine, Medicine
2001 – 2005	Kuršėnai Laurynas Ivinskis Gymnasium
Work experience:	
2018 – present	Teaching assistant at Faculty of Medicine of Vilnius University; M. K. Čiurlionio str. 21/27, 03101 Vilnius, Lithuania
2018 – present	Junior researcher at Research Centre of National Cancer Institute; P.Baublio str. 3b, 08406 Vilnius, Lithuania
2016 – present	Urologist at Department of Urology of Vilnius University Hospital Santaros Klinikos; Santariskiu 2, 08661 Vilnius, Lithuania

Professional qualifications:

15-16 January, 2020	Young Experts in Science (YES) Meeting 2020; Karolinska Institute, Stockholm, Sweden
14-17 November, 2019	11th European Multidisciplinary Congress on Urological Cancers (EMUC19); Vienna, Austria
25-26 May, 2019	6th EAU Baltic Meeting 2019; Tallinn, Estonia
15-19 March, 2019	34th Annual EAU Congress; Barcelona, Spain
25-26 May, 2018	5th EAU Baltic Meeting 2018; Riga, Latvia
18-21 May, 2018	AUA Annual Meeting 2018; San Francisco, USA
7-8 December, 2017	2nd ESU-ESUT-ESUI Masterclass on Focal therapy for localized prostate cancer; Paris, France
26-27 May, 2017	4th EAU Baltic Meeting 2017; Vilnius, Lithuania
27-28 January, 2017	Nordic Advanced Prostate Cancer (NAPC) Camp; Stockholm, Sweden
4-6 December, 2016	Advances in the Armamentarium of CRPC; Growing Radium-223 Dichloride Understanding and Treatment Expertise; Guy's and St Thomas' Hospital, London, United Kingdom
27-28 May, 2016	3rd EAU Baltic Meeting 2016; Tallinn, Estonia
21-23 March, 2016	EUREP Advanced HOT Follow-up; Olympus EndoClub Academy; Hamburg, Germany

8 September, 2015	The European Training in Basic Laparoscopic Urological Skills Exam (E-BLUS); 13th European Urology Residents Education Programme (EUREP); Prague, Czech Republic
4-9 September, 2015	13th European Urology Residents Education Programme (EUREP); Prague, Czech Republic
5-11 May, 2015	ESU-Weill Cornell Masterclass in Urology; Salzburg, Austria
1 March - 31 May, 2015	Clinical Observership in Uro-oncology; Fundació Puigvert, Barcelona, Spain.
1-6 February, 2015	ESU Hands-on training course on Endourology and Laparoscopy; Minimally Invasive Surgery Centre Jesús Usón, Caceres, Spain
30-31 October, 2014	1st ESU Masterclass on Lasers in urology, in collaboration with the EAU Section of Uro-Technology (ESUT); Barcelona, Spain
10-12 October, 2014	14th EAU Central European Meeting 2014; Cracow, Poland
23-25 May, 2014	1st EAU Baltic Meeting 2014; Vilnius, Lithuania
11-15 April, 2014	29th Annual EAU Congress; Stockholm, Sweden
15-19 March, 2013	28th Annual EAU Congress; Milan, Italy

Good clinical practice:

1. GCP Basic Course; Approved by State Medicines Control Agency; 18 June, 2017; Certificate No. e-627.
2. ASCLS P.A.C.E.® Certificate for Transporting Dangerous Goods; Mayo Clinic Laboratories; 22 December, 2019; Program No. 058/024/19, Provider No. 058.

Research activities:

2016 – 2018	Investigator in biomedical clinical study investigating diagnostic biomarkers of metastatic castration-resistant prostate cancer
2015 – 2018	Investigator in biomedical clinical study investigating diagnostic biomarkers of prostate cancer

Awards:

1. Young Urologists Competition Award (BALTIC16). Received the First Prize in the Young Urologists Competition for the presentation: Sentinel lymph node mapping as an option in prostate cancer. 3rd EAU Baltic Meeting; 27-28 May 2016, Tallinn, Estonia.
2. Second Berlin Chemie Award for Best Poster Presentation, entitled: Epigenetic Markers to Overcome Limitations in Prostate Cancer Diagnostics. 5th EAU Baltic Meeting 2018, 25-26 May 2018, Riga, Latvia.
3. First Berlin Chemie Award for Best Poster Presentation, entitled: Predicting Abiraterone Acetate Treatment Resistance from Blood-circulating Androgen Receptor Variants in Castration Resistant Prostate Cancer. 6th EAU Baltic Meeting 2019, 24-25 May 2019, Tallinn, Estonia.

Memberships:

2014 – present	Member of Lithuanian Urological Society (LUD)
2012 – present	Member of European Association of Urology (EAU)

Main areas of interest:

Uro-oncology, Robotics & Minimally Invasive Urology, Molecular Markers of Prostate Cancer.

Vilniaus universiteto leidykla
Saulėtekio al. 9, LT-10221 Vilnius
El. p. info@leidykla.vu.lt,
www.leidykla.vu.lt
Tiražas 30 egz.