

Edith Cowan University  
**Research Online**

---

ECU Publications Post 2013

---

2016

## Prostate-based biofluids for the detection of prostate cancer: A comparative study of the diagnostic performance of cell-sourced RNA biomarkers

Matthew J. Roberts  
*Princess Alexandra Hospital Brisbane*

Renée S. Richards  
*University of Queensland*

Clement W. K. Chow  
*University of Queensland*

Suhail A. Doi  
*University of Southern Queensland*

Horst J. Schirra  
*University of Queensland*

*See next page for additional authors*

Follow this and additional works at: <https://ro.ecu.edu.au/ecuworkspost2013>

 Part of the [Urology Commons](#)

---

10.1016/j.pnil.2016.04.002

Roberts, M.J., Richards, R.S., Chow, C.W.K., Doi, S.A.R., Schirra, H.J., Buck, M., Samaratunga, H., ... Gardiner, R.A. (2016). Prostate-based biofluids for the detection of prostate cancer: A comparative study of the diagnostic performance of cell-sourced RNA biomarkers. *Prostate International*, 4(3), 97-102. Available [here](#).

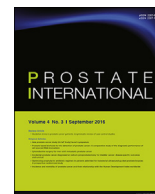
This Journal Article is posted at Research Online.

<https://ro.ecu.edu.au/ecuworkspost2013/2275>

---

**Authors**

Matthew J. Roberts, Renée S. Richards, Clement W. K. Chow, Suhail A. Doi, Horst J. Schirra, Marion A. Buck, Hemamali Samaratunga, Joanna L. Perry-Keene, Diane J. Payton, John W. Yaxley, Martin F. Lavin, and Robert A. Gardiner



## Research Article

# Prostate-based biofluids for the detection of prostate cancer: A comparative study of the diagnostic performance of cell-sourced RNA biomarkers



Matthew J. Roberts<sup>1, 2, 3, 4</sup>, Renee S. Richards<sup>2</sup>, Clement W.K. Chow<sup>2</sup>, Suhail A.R. Doi<sup>5, 6, 7</sup>, Horst Joachim Schirra<sup>3</sup>, Marion Buck<sup>8</sup>, Hemamali Samaratunga<sup>9</sup>, Joanna Perry-Keene<sup>10</sup>, Diane Payton<sup>10</sup>, John Yaxley<sup>4</sup>, Martin F. Lavin<sup>2</sup>, Robert A. Gardiner<sup>2, 4, 11, \*</sup>

<sup>1</sup> The University of Queensland, Division of Surgery, School of Medicine, Brisbane, QLD, Australia

<sup>2</sup> The University of Queensland, Centre for Clinical Research, Brisbane, QLD, Australia

<sup>3</sup> The University of Queensland, Centre for Advanced Imaging, Brisbane, QLD, Australia

<sup>4</sup> Department of Urology, Royal Brisbane and Women's Hospital, Brisbane, QLD, Australia

<sup>5</sup> Research School of Population Health, The Australian National University, Canberra, Australia

<sup>6</sup> School of Agricultural, Computational and Environmental Sciences, University of Southern Queensland, Toowoomba, Australia

<sup>7</sup> College of Medicine, Qatar University, Doha, Qatar

<sup>8</sup> Department of Environmental Health Sciences, University Medical Centre Freiburg, Freiburg, Germany

<sup>9</sup> Aquesta Pathology, Brisbane, QLD, Australia

<sup>10</sup> Anatomical Pathology, Pathology Queensland, Brisbane, QLD, Australia

<sup>11</sup> Edith Cowan University, Joondalup, Western Australia, Australia

## ARTICLE INFO

## Article history:

Received 19 January 2016

Received in revised form

2 April 2016

Accepted 28 April 2016

Available online 5 May 2016

## Keywords:

Biomarker

Ejaculate

mRNA

Prostate Cancer

Prostate Cancer Antigen 3

Prostate Specific Antigen

## ABSTRACT

**Background:** Prostate cancer (PCa) diagnosis requires improvement with the aid of more accurate biomarkers. Postejaculate urethral washings (PEUW) could be a physiological equivalent to urine obtained following rectal prostatic massage, the current basis for the prostate cancer antigen 3 (PCA3) test. The aim of this study was to investigate if PEUW contained prostate-based material, evidenced by the presence of prostate specific antigen (PSA), and to evaluate the diagnostic performance of PEUW-based biomarkers.

**Methods:** Male patients referred for elevated serum PSA or abnormal digital rectal examination provided ejaculate and PEUW samples. PSA, PCA3, and  $\beta$ 2-microglobulin ( $\beta$ 2M) were quantified in ejaculate and PEUW and compared with absolute and clinically significant (according to D'Amico criteria) PCA presence, as determined by biopsies. Diagnostic performance was determined and compared with serum PSA using receiver operating characteristic analysis.

**Results:** From 83 patients who provided PEUW samples, paired analysis with ejaculate samples was possible for 38 patients, while analysis in an unpaired, extended cohort was possible for 62 patients. PSA and PCA3 were detected in PEUW, normalized to  $\beta$ 2M, and PCA3:PSA was calculated. In predicting absolute PCa status, PCA3: $\beta$ 2M in ejaculate [area under the curve (AUC) 0.717] and PEUW (AUC 0.569) were insignificantly better than PCA3:PSA (AUC 0.668 and 0.431, respectively) and comparable with serum PSA (AUC 0.617) with similar trends observed for the extended cohort. When considering clinically significant PCa presence, serum PSA in the comparison (AUC 0.640) and extended cohorts (AUC 0.665) was comparable with PCA3:  $\beta$ 2M (AUC 0.667) and PCA3:PSA (AUC 0.605) in ejaculate, with lower estimates for PEUW in the comparison (PCA3:  $\beta$ 2M AUC 0.496; PCA3:PSA AUC 0.342) and extended (PCA3:  $\beta$ 2M AUC 0.497; PCA3:PSA AUC 0.469) cohorts. The statistical analysis was limited by sample size.

**Conclusion:** PEUW contains prostatic material, but has limited diagnostic accuracy when considering cell-derived DNA analysis. PCA3-based markers in ejaculate are comparable to serum PSA and digital rectal examination—urine markers.

Copyright © 2016 Asian Pacific Prostate Society, Published by Elsevier. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

\* Corresponding author. UQ Centre for Clinical Research (UQCCR), Level 6, Building 71/918 Royal Brisbane Hospital, Herston, QLD 4006, Australia.  
E-mail address: [f.gardiner@uq.edu.au](mailto:f.gardiner@uq.edu.au) (R.A. Gardiner).

## 1. Introduction

The detection of prostate cancer (PCa) is fraught with difficulties that include limitations of currently available biomarkers, access to imaging and tissue sampling. Total serum prostatic specific antigen (PSA) is currently the single most widely used marker clinically for identifying men at risk of PCa, but it is a nonspecific indicator of prostatic pathology that includes PCa among others so that population and opportunistic screening is discouraged due to over-investigation and over-detection, resulting in overtreatment.<sup>1</sup> Contemporary PCa detection approaches have included, amongst others, advocacy for a biomarker panel, the Prostate Health Index and the 4-kallikrein protein test.<sup>2,3</sup>

Although imaging modalities, such as multiparametric magnetic resonance imaging and even prostate specific membrane antigen (PSMA) positron emission tomography, are being integrated into the detection strategy for triaging patients with an elevated PSA and may improve detection of clinically significant PCa,<sup>4</sup> there are limitations that detract from their widespread use. The false negative rate (~15–20%) for multiparametric magnetic resonance imaging suggests a significant proportion of clinically significant tumors may be missed,<sup>4</sup> which is also observed with PSMA-positron emission tomography imaging of tumors that do not express PSMA.<sup>5</sup> That imaging is establishing a niche in detection strategies is undeniable; however, it is the cost of such imaging methods that really limits application to mainstream clinical practice. Improved patient selection for imaging with accurate biomarkers is likely to optimize their practical application clinically.

Prostate-specific biofluids are an ongoing source for investigation using new analytical platforms.<sup>6</sup> Prostate cancer antigen 3 (PCA3), a long noncoding RNA, collected in the first void of urine following a vigorous digital rectal examination (DRE) or prostatic massage, has been examined over the past 20 years,<sup>7</sup> with studies reporting to improve detection in men undergoing repeat biopsy,<sup>8</sup> but its role clinically remains uncertain. Modifications of PCA3,<sup>9</sup> as well as a combination with TMPRSS2-ERG fusion gene have been described to improve detection<sup>10</sup> but have not been accepted as a useful addition in routine patient testing.<sup>11</sup> Other approaches utilizing exosomes, proteomics, and metabolomics have the potential to improve early diagnosis of localized disease.<sup>12–14</sup> Indeed molecular and metabolomic markers in ejaculate or seminal fluid have been reported to improve diagnosis compared to serum PSA.<sup>15–17</sup> Paralleling the postmassage urine concept, collection of urine following ejaculation, or postejaculate urethral washings (PEUW) potentially represents a new source of prostate-specific biomarkers for PCa detection and characterization, providing a physiologically produced alternative to serum or digital prostatic massage effluent for further biomarker discovery and development.

The aim of this study was to investigate the diagnostic potential of selected molecular markers in PEUW in detecting PCa and comparing diagnostic accuracy with the same markers in ejaculate, as well as serum PSA. The hypothesis tested was that the diagnostic performance of molecular markers in postejaculate urine would be comparable with those in ejaculate or serum PSA.

## 2. Materials and methods

### 2.1. Patients

A clinical cohort of men ( $n = 83$ ) being investigated for PCa on the basis of an abnormal DRE and/or elevated serum PSA provided specimens of ejaculate and PEUW into sterile micro-urine jars, containing 20 mL Hanks' Balanced Salt Solution (Gibco, Life Technologies Australia Pty Ltd, Scoresby, Victoria, Australia) and empty urine jars, respectively, between January 2007 and

December 2009. As previously reported,<sup>15</sup> all specimens were processed within 2 hours of production after being delivered to the hospital campus without cooling. All specimens were collected prior to or at least 1 month following transrectal ultrasound guided biopsy (TRUSbx) or transperineal template biopsy (TPBx).

Ethical approval to conduct this study was obtained from the University of Queensland Medical Research Ethics Committee, Brisbane, Australia (Project No. 2006000262) and the Royal Brisbane and Women's Hospital, Human Research Ethics Committee, Brisbane, Australia (HREC/09/QRBW/320, HREC/09/QRBW/305 together with 1995/088B).

### 2.2. Clinical data

All patient data were prospectively collected following recruitment and included clinical details such as age, family history, and serum PSA. Initial and updated TRUSbx/TPBx/radical prostatectomy (RP) histology specimens were reviewed by D.P., J.P.-K., and H.S. and reported according to the 2005 International Society of Urological Pathology classification,<sup>18</sup> including standard biopsy (number of cores taken, number and percentage of positive cores, Gleason score) and RP (gland size, Gleason score, pathological stage, extracapsular status, and margin status) parameters.

In order to identify the patients for whom active treatment would be recommended, risk stratification for biopsies in determining clinically significant PCa presence was performed using the D'Amico criteria recommended in the American Urological Association Guidelines.<sup>19</sup> The clinically significant PCa category included patients defined as intermediate- and high-risk according to the D'Amico criteria, while the absence of clinically significant PCa was defined as low risk patients according to the D'Amico criteria or those without PCa.<sup>19</sup> The most accurate classification of clinically significant PCa, based on histopathology from TRUSbx, TPBx, and/or RP, was used given established disparity between TRUSbx and RP histopathology. As previously reported, patients were subsequently placed in one of two clinical groups based on classification scheme used. Because of the imprecise nature of TRUSbx in particular, patient follow-up was pursued for up to 7 years to ensure that those designated as negative for prostate cancer really were negative.

### 2.3. Specimen processing and cellular isolation

Ejaculate specimens combined with 20 mL Hanks' Balanced Salt Solution were layered over 10 mL isotonic Percoll (GE Healthcare—Pharmacia) and centrifuged at 974 g for 30–60 minutes at 4°C, with supernatants subsequently collected in 1 mL aliquots, snap-frozen on dry ice and stored at –80°C. The epithelial cell layer at the Percoll interface, present as a discrete band suspended between supernatant above and sperm and noncellular components below, was then pipetted, washed with 25 mL phosphate-buffered saline or Hanks' and centrifuged at 1,258 g for 10 minutes at 4°C.

### 2.4. RNA preparation

Total RNA from collected cells was isolated using TRIzol reagent (Invitrogen) then subjected to on-column DNase treatment and clean-up with the RNeasy kit (Qiagen). Low yield samples were amplified using the SenseAmp kit (Gensisphere).

### 2.5. cDNA synthesis and quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (qPCR) was undertaken using the QIAGEN Quantitect SYBR green

qPCR Mastermix (QIAGEN, Germany) on a Corbett Rotorgene machine 3000/6000 (Corbett Research, Australia). cDNA synthesis was performed with 200–500 ng of total RNA reverse transcribed using Superscript III (Invitrogen) and random hexamer primers (Promega). The synthesized cDNA was diluted 10-fold and 5  $\mu$ L was used for the assay in the presence of 7.5  $\mu$ L Quantitect SYBR green mastermix (Qiagen) and 5 pmol gene specific forward and reverse primers. Each reaction was performed in triplicate for both patient samples and calibrator. Reaction conditions were 95°C for 15 minutes followed by 45 cycles of 20 seconds at 95°C, 20 seconds at 58°C, and 20 seconds at 72°C. Data for each cycle were acquired at the 72°C step.

The genes that were characterized were  $\beta$ 2-microglobulin ( $\beta$ 2M), PCA3, and PSA using the following primers (Sigma–Aldrich, Australia) for qPCR:  $\beta$ 2M (forward: 5'-AGCAGAGAATGGAAAGTCAAAA-3', reverse: 5'-TGCTGCTTACATGTCTCG-3'); PCA3 (forward: 5'-GGAAGGACCTGATGATACAGAGGTGAG-3', reverse: 5'-CACAGGGC-GAGGCTCATCG-3'); PSA (forward: 5'-GCATCAGGAACAAAAGCGTG-3', reverse: 5'-CCTGAGGAATCGATTCTCA-3').

Standardized processing (including standard curve fitting, dynamic tube, slope correct) was performed for all runs using RotorGene 6000 Series Software version 1.7 (Corbett Research, Australia). To maintain quality control, specimens with atypical melt curves or quantitation curves below threshold for any single target (with normal melt curves) were excluded. A standard threshold value was manually set for each individual gene across all samples and used to calculate cycle threshold ( $C_t$ ) values, which were exported to Excel (Microsoft Corporation, Redmond, WA, USA). Based on average  $C_t$  ( $C_tAv$ ) and standard deviation ( $C_tSD$ ) values of triplicates observed across all genes (see Fig. S1), cutoff points defined for exclusion were  $C_tAv > 35$  and  $C_tSD > 1.5$  for PEUW and ejaculate, as previously described.<sup>15</sup> Analysis of PSA, PCA3, and  $\beta$ 2M in ejaculate and PEUW specimens was possible for 38 patients, with these markers detected in PEUW only from 62 patients.

## 2.6. Relative gene expression determination

Relative gene expression was calculated using the method previously described by Pfaffl,<sup>20</sup> which uses the following equation:

$$\text{Expression (R)} = E^{\Delta C_t(\text{Calibrator-sample})} / E^{\Delta C_t(\text{Calibrator-sample})} \quad (1)$$

$\Delta C_t$  (Calibrator-sample) estimates the amplification ( $C_t$  values) difference between the calibrator reaction (uniform template quantity to standardize all runs) and the target gene transcription of the sample reaction (unknown).<sup>20</sup> The reaction efficiency ( $E$ ) of the gene of interest (GOI) and endogenous reference gene (RG) are considered without the required use of a standard curve in every run, based on the assumption that reaction efficiency between different runs was consistent and normalized by the calibrator used.

## 2.7. Reference gene variation

$\beta$ 2M, a known housekeeping gene,<sup>7,21</sup> was used as the endogenous reference gene with subsequent relative gene expression calculated for PSA and PCA3. The commercial use of PCA3, which, unlike PSA, is highly overexpressed in prostate cancer,<sup>22</sup> requires PSA to be used as the reference gene on the basis of reports that PSA expression is relatively constant and considers only cells of prostatic origin so the expression of PCA3 relative to PSA was also calculated.<sup>7</sup>

## 2.8. Data analysis

Relative gene expression results were analyzed considering two clinical classifications, absolute PCa status (cancer vs. no cancer) and clinically significant (present vs. absent) PCa status, as determined using the D'Amico classification.<sup>19</sup> Univariate analysis was conducted with the nonparametric Mann–Whitney  $U$  test.

Receiver operating characteristic (ROC) analysis was performed for each marker and compared to each other and serum PSA, with binomial exact confidence interval and optimal cut points for each marker calculated in Stata Statistical Software 13 (StataCorp. College Station, TX, USA) using the Liu<sup>23</sup> method. Significance thresholds were Bonferroni corrected ( $< 0.05/4 = < 0.0125$ ) to adjust for multiple comparisons ( $n = 4$ ), and all reported  $P$  values  $< 0.0125$  were considered statistically significant.

## 3. Results

### 3.1. Clinical characteristics

From 83 potential patients with adequate clinical data who donated PEUW samples between January 2007 and December 2009, relative gene expression determination for PSA, PCA3, and  $\beta$ 2M was performed. Strict exclusion criteria (see Fig. S2) were applied, resulting in sample exclusion due to unsatisfactory qPCR analyses (atypical melt or quantitation curves,  $n = 1$ ), insufficient cDNA to reach detection threshold ( $n = 5$ ),  $C_tAv$  or  $C_tSD$  outside determined cutoffs ( $n = 14$ ) or coupled with an ejaculate sample excluded for a similar reason ( $n = 25$ ). Of the remaining 38 patients, the relationships observed for the entire cohort with respect to median (interquartile range) age [62 (57–68) years] and serum PSA [6.7 (4.75–9.15) ng/mL] were preserved in this group, with a median age 62 (57–69) years ( $P > 0.0125$ ) and serum PSA 6.3 (4.9–8.9) ng/mL ( $P > 0.0125$ ). Within the included 38 patients were 25 participants having a prostate biopsy positive for cancer, with 21 deemed to have clinically significant PCa.

The demographic information of the cohort including median (interquartile range) age, serum PSA, and relative expression ratios of RNA markers for each group, absolute and clinically significant PCa presence, are presented in Tables 1 and 2, respectively. Men were of comparable ages in each classification group. Serum PSA was similar for men diagnosed with PCa compared with men in the no PCa group. This relationship was preserved for serum PSA when comparing men with clinically significant PCa against those without clinically significant PCa. Similar trends were observed in the expanded cohort, which comprised 62 patients with 36 participants with a positive prostate biopsy and 30 deemed to have clinically significant PCa (Tables 1 and 2).

### 3.2. Biomarker performance

#### 3.2.1. Absolute PCa status

When considering absolute PCa status (Table 1) and compared to a chance area under the curve (AUC) of 0.500, serum PSA (AUC 0.617;  $P = 0.217$ ) and PSA: $\beta$ 2M (AUC = 0.600;  $P = 0.353$ ) in PEUW samples provided similar diagnostic performance. PCA3: $\beta$ 2M (AUC = 0.569;  $P = 0.522$ ) and. PCA3:PSA (AUC = 0.431;  $P = 0.528$ ) in PEUW demonstrated inferior predictive ability. Similar results were not observed in the expanded cohort, with serum PSA (AUC = 0.610,  $P = 0.128$ ) performing better than PSA: $\beta$ 2M (AUC = 0.506;  $P = 0.935$ ), PCA3: $\beta$ 2M (AUC = 0.550;  $P = 0.531$ ), and PCA3:PSA (AUC = 0.529;  $P = 0.728$ ).

**Table 1**  
Demographic information for patients based on absolute cancer status (positive vs. negative biopsy).

Absolute cancer status	Comparison cohort No PCa (n = 13); PCa (n = 25)						Extended PEUW cohort No PCa (n = 26); PCa (n = 36)					
	Demographic information		ROC analysis				Demographic information		ROC analysis			
	No PCa, median (IQR)	PCa, median (IQR)	AUC (95% CI)	Cutpoint	Sn	Sp	No PCa, median (IQR)	PCa, median (IQR)	AUC (95% CI)			
Age	65 (58.5–71)	63 (57–68.25)	–	–	–	–	64.5 (60–69)	63.5 (58–69)	–			
Serum PSA	6.20 (4.13–7.30)	6.50 (5.13–10.4)	0.617 (0.445–0.770)	9.05	36	100	6.30 (4.70–8.2)	7.00 (5.25–11.00)	0.610 (0.477–0.731)			
PEUW PSA:β2M	0.07 (0.003–0.36)	0.10 (0.02–1.24)	0.600 (0.429–0.755)	0.006	84	46	0.32 (0.005–1.18)	0.15 (0.14–0.97)	0.506 (0.376–0.636)			
PEUW PCA3:β2M	0.23 (0.01–0.60)	0.21 (0.06–1.24)	0.569 (0.399–0.728)	0.652	44	77	0.19 (0.02–0.48)	0.18 (0.07–0.99)	0.550 (0.419–0.677)			
PEUW PCA3:PSA	2.72 (0.52–61.97)	1.45 (0.51–9.66)	0.431 (0.272–0.601)	0.764	72	38	1.24 (0.14–20.80)	1.19 (0.55–9.32)	0.529 (0.398–0.657)			
Ejaculate PSA:β2M	0.07 (0.003–0.36)	0.03 (0.003–0.19)	0.514 (0.347–0.679)	0.003	80	38	–	–	–			
Ejaculate PCA3:β2M	0.01 (0.004–0.10)	0.04 (0.02–0.28)*	0.717 (0.548–0.851)	0.023	72	77	–	–	–			
Ejaculate PCA3:PSA	1.55 (0.12–2.80)	4.62 (0.77–11.84)	0.668 (0.496–0.812)	1.77	64	77	–	–	–			

Median and interquartile range are shown for age and each marker in postejaculate urethral washing and ejaculate samples. All comparisons using the Mann-Whitney *U* test (2-tailed; using a Bonferroni corrected threshold) were non-significant ( $P > 0.0125$ ).

\*  $P < 0.05$  for area under curve with 95% confidence interval (AUC 95% CI) comparisons of markers with chance (AUC 0.5) as determined by the DeLong method. All comparisons between AUC estimates were nonsignificant ( $P > 0.0125$ ).

β2M, β2-microglobulin; IQR, interquartile range; PCA3, prostate cancer antigen 3; PEUW, postejaculate urethral washings; PSA, prostate specific antigen; ROC, receiver operating characteristic; Sn, sensitivity; Sp, specificity.

**Table 2**  
Demographic information for patients based on clinically significant PCa classification.

Clinically significant PCa	Comparison cohort Absent (n = 17); Present (n = 21)						Extended PEUW cohort Absent (n = 32); Present (n = 30)					
	Demographic information		ROC analysis				Demographic information		ROC analysis			
	Absent, median (IQR)	Present, median (IQR)	AUC (95% CI)	Cutpoint	Sn	Sp	Absent, median (IQR)	Present, median (IQR)	AUC (95% CI)			
Age	61 (56.25–69.5)	63 (57.75–69.25)	–	–	–	–	63.5 (58.5–68)	64 (58–70)	–			
Serum PSA	5.80 (4.38–7.30)	6.50 (5.28–13.43)	0.640 (0.468–0.789)	6.500	57	65	6.00 (4.55–8.20)	7.60 (5.50–11.90)	0.665 (0.534–0.780)*			
PEUW PSA:β2M	0.04 (0.002–0.94)	0.10 (0.01–1.24)	0.608 (0.436–0.762)	0.006	86	41	0.21 (0.006–1.17)	0.18 (0.01–1.17)	0.525 (0.394–0.653)			
PEUW PCA3:β2M	0.23 (0.02–1.13)	0.17 (0.04–1.08)	0.496 (0.330–0.662)	0.652	43	71	0.20 (0.02–0.72)	0.16 (0.04–0.93)	0.497 (0.367–0.627)			
PEUW PCA3:PSA	2.77 (0.71–61.97)	1.11 (0.29–7.79)	0.342 (0.196–0.513)	0.764	67	29	1.58 (0.18–16.02)	1.03 (0.52–7.30)	0.469 (0.341–0.600)			
Ejaculate PSA:β2M	0.03 (0.003–0.23)	0.03 (0.003–0.31)	0.521 (0.353–0.685)	0.014	67	47	–	–	–			
Ejaculate PCA3:β2M	0.02 (0.004–0.25)	0.04 (0.02–0.28)	0.667 (0.495–0.811)	0.023	71	65	–	–	–			
Ejaculate PCA3:PSA	1.56 (0.44–6.58)	3.42 (0.66–16.1)	0.605 (0.434–0.759)	1.77	62	65	–	–	–			

Median and interquartile range are shown for age and each marker in postejaculate urethral washing and ejaculate samples. All comparisons using the Mann-Whitney *U* test (2-tailed; using a Bonferroni corrected threshold) were non-significant ( $P > 0.0125$ ).

\*  $P < 0.05$  for area under curve with 95% confidence interval (AUC 95% CI) comparisons of markers with chance (AUC 0.5) as determined by the DeLong method. All comparisons between AUC estimates were nonsignificant ( $P > 0.0125$ ).

β2M, β2-microglobulin; IQR, interquartile range; PCA3, prostate cancer antigen 3; PEUW, postejaculate urethral washings; PSA, prostate specific antigen; ROC, receiver operating characteristic; Sn, sensitivity; Sp, specificity.

In ejaculate, best diagnostic performance was observed for PCA3:β2M (AUC = 0.717;  $P = 0.033$ ), followed by PCA3:PSA (AUC = 0.668;  $P = 0.078$ ), followed by less impressive performance for serum PSA (AUC = 0.617;  $P = 0.217$ ), and poor performance of PSA:β2M (AUC = 0.486;  $P = 0.895$ ).

### 3.2.2. Clinically significant PCa

When considering clinically significant PCa (Table 2), similar performance was observed for serum PSA (AUC = 0.640;  $P = 0.124$ ), PSA:β2M (AUC = 0.608;  $P = 0.269$ ), and PCA3:PSA (AUC = 0.342;  $P = 0.093$ ). The performance of PCA3:β2M (AUC = 0.496;  $P = 0.966$ ) was poorer and less predictive of clinically significant PCa. Within the expanded cohort, only serum PSA (AUC = 0.665;  $P = 0.018$ ) performed as well, with poorer performance for the PEUW-based PSA:β2M (AUC = 0.525;  $P = 0.740$ ), PCA3:β2M (AUC = 0.503;  $P = 0.967$ ), and PCA3:PSA (AUC = 0.469;  $P = 0.681$ ).

In ejaculate samples, PCA3:β2M (AUC = 0.667,  $P = 0.083$ ) was similar to serum PSA, and PCA3:PSA (AUC = 0.605,  $P = 0.263$ ). The performance of PSA:β2M (AUC = 0.521,  $P = 0.828$ ) was less impressive. For graphical purposes, comparison ROC curves are available in Figs. S3 and S4 including the comparison *P* values against the serum PSA AUC.

## 4. Discussion

PEUW could potentially be a new source of prostate-specific biomarkers for PCa detection and characterization, providing an alternative to serum and urine for further biomarker discovery and development. Thus, we investigated in this study the utility of prostatic cells in PEUW as a physiological source of PCa biomarkers. We have shown that the diagnostic performance of the mRNA-based marker PCA3, normalized to PSA or β2M, in PEUW is likely to be inferior to these markers in ejaculate, which were comparable to serum PSA. Overall, the performance is similar to PCA3 in post-massage urine in isolation (AUC 0.62),<sup>24</sup> as well as in ejaculate in an expanded cohort (AUC 0.625).<sup>15</sup> PCA3 performed best in this cohort in detecting absolute PCa status compared with clinically significant PCa, in accordance with previous reports.<sup>10</sup> This preliminary investigation helps to build on current PCa biomarker research literature.

The use of PEUW as a prostate-based biofluid is advantageous for a number of reasons. First, it contains prostatic effluent following ejaculation, indicated here by the presence of PSA. In addition, there is no requirement for patient discomfort, in contrast to prostatic massage. Furthermore, there is the potential for tumor disruption and dissemination of malignant cells,<sup>25</sup>

given known elevations in serum PSA after TRUSBx and DRE.<sup>26</sup> Reports regarding serum PSA elevation after ejaculation are mixed, with levels reported to return to normal after 48 hours,<sup>27</sup> thus PEUW following global contraction of the prostate gland with ejaculation can be considered a physiological equivalent of nonphysiological postmassage urine. PEUW sampling enables postcoital donation, which may be more acceptable for some men and has been used to investigate infertility. The combination of urine and ejaculatory components in PEUW allows for assessment of markers reflecting local (ejaculate) and systemic (urine) pathology. While this enables use in clinical scenarios where systemic biological alterations are important to monitor, such as active surveillance and metastatic disease, it is also a potential drawback of PEUW, because the local pathology markers in the ejaculate component can be confounded by the systemic contributions from the urine component. These may contribute to the lower diagnostic performance of PEUW compared to ejaculate described here.

The benefits that we have previously outlined for the use of ejaculate as a prostate-based biofluid also apply to PEUW. Specifically, ejaculate contains malignant prostatic epithelial cells,<sup>28</sup> with cell-derived molecular markers PCA3 and hepsin shown to be comparable diagnostically with PCA3 in post-massage urine.<sup>15</sup> Analysis of microRNAs in cell-derived mRNA in ejaculate has been reported to improve PCa detection, with miR-200b combined with serum PSA (AUC = 0.751) significantly better than serum PSA alone (AUC = 0.555).<sup>16</sup> We have previously reported the use of a composite score, created using contributions from serum PSA, and ejaculatory micro RNAs-125b, and -200c, to significantly improve PCa detection (AUC = 0.869) compared with serum PSA alone (AUC = 0.672;  $P < 0.05$ ).<sup>15</sup> The ability to provide an ejaculate sample may also indicate a favorable performance status and consequent survival benefit, with a high and significantly better overall and PCa-specific survival benefit observed for these patients at 10 years, 15 years, and 20 years.<sup>29</sup> Incorporation of exosome and metabolome analysis may improve predictive accuracy using these non-invasively obtained biofluids,<sup>12,30</sup> reducing anxiety and uncertainty for clinicians and patients managed by active surveillance, in addition to assisting with PCA testing. Use of PEUW may be more favorable than ejaculate as PEUW samples can be provided in the comfort of the home environment and postintercourse, a strategy more likely to be used by men than the sterile surrounds of the clinic setting. Similar to urine cytology, the sample could be stored overnight in a refrigerator and brought to the clinic the morning after, assuming RNA integrity is maintained.

The aim of this comparative study was to investigate the diagnostic potential of selected molecular markers in PEUWs in detecting PCa on the basis of the D'Amico classification benchmark,<sup>19</sup> widely used to stratify in the past. However, the goal posts are in the process of being changed as it is being realized increasingly that intermediate risk PCa is not one condition but a spectrum of conditions. Recently, the management strategy of active surveillance has been extended to include some Gleason 3 + 4 (ISUP 2) tumors regarded as *favorable* by the National Comprehensive Cancer Network (NCCN) for men with a life-expectancy < 10 years<sup>31</sup> with this change supported by the American Society of Clinical Oncology.<sup>32</sup> By inference, this means that not all intermediate-risk tumors can be considered as clinically significant. However, for the purpose of comparison, risk classifications such as that proposed by D'Amico remain relevant until the entity of clinically significant can be defined better.

The limitations of this preliminary, exploratory study include the small sample size resulting in large confidence intervals and low statistical power. Biologically, the potential for low ejaculatory contribution or dilution reducing the prostate-specific RNA yield

may impair results. PCA3:PSA levels in PEUW were lower in men with PCa, both in the comparison and expanded cohorts, resulting in ROC estimates below 0.5, which was the inverse of that observed for ejaculate samples and other published reports. Although the sample drop-out rates were similar for PEUW and ejaculate, with positive PSA signals suggesting the presence of prostatic material in both sample sets, these results suggest malignant cells may dominate in the ejaculate. A potential explanation for this are changes in cell adhesion molecules, which have been recognized for some time in PCa,<sup>33</sup> with most attention focused on E-cadherin. Loss of E-cadherin is particularly evident in more aggressive tumors with cadherin switching also recently described.<sup>34</sup> Thus, disaggregated cells or cell clusters from aggressive tumors first appear in ejaculate as a result of global contraction of prostatic smooth muscle following accumulation in acini prior to ejaculation. This in turn may cause relatively fewer cancerous cells to be present in the urethra from the latter part of the ejaculate, which would be dislodged with subsequent voiding. As a result, PEUW may contain fewer cancerous cells but does contain prostate epithelial cells, evidenced here by the presence of PSA. Thus, in patients suspected to harbor nonaggressive PCa, the presence of prostatic cells in PEUW with low expression of PCA3 may help determine which patients have nonaggressive PCa amenable to active surveillance or watchful waiting.

In conclusion, we introduced and investigated PEUW as a physiological source of PCa biomarkers. We found that PEUW contains prostatic cells, as evidenced by PSA signal. However, significantly upregulated PCA3 levels, consistent with those reported for malignant tissue, were only observed in ejaculate specimens. PEUW may yet prove to be a useful source of cell-free secreted markers, as opposed to cell-derived markers. Further biomarker development using these prostate-specific biofluids may result in improved diagnosis and monitoring of PCa, reducing anxiety and doubt for the benefit of clinicians and patients.

### Conflicts of interest

All authors have no conflict of interest to declare.

### Acknowledgments

This research received financial support from Royal Brisbane and Women's Hospital Foundation (Ref: 2014002678), the Prostate Cancer Foundation of Australia and Cancer Council Queensland. M.J.R. is supported by a Doctor in Training Research Scholarship from Avant Mutual Group Ltd, Cancer Council Queensland PhD Scholarship and Professor William Burnett Research Fellowship from the Discipline of Surgery, School of Medicine, The University of Queensland. H.J.S. is supported by DVCR Strategic Initiative Funding from the University of Queensland (Grant No DVCR4480). The authors wish to thank the patients and clinical staff involved in facilitating this research.

### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.pnrl.2016.04.002>.

### References

- Gardiner RA, Chambers SK, Williams SG, Yaxley J, Samaratunga H, Frydenberg M. Prostate cancer—part one: detection. In: De Groot LJ, Beck-Peccoz P, Chrousos G, Dungan K, Grossman A, Hershman JM, et al., eds. *Endotext*. South Dartmouth (MA): MDText.com, Inc.; 2000.
- Hori S, Blanchet JS, McLoughlin J. From prostate-specific antigen (PSA) to precursor PSA (proPSA) isoforms: a review of the emerging role of proPSAs in the detection and management of early prostate cancer. *BJU Int* 2013;112:717–28.

3. Parekh DJ, Punnen S, Sjoberg DD, Asroff SW, Bailen JL, Cochran JS, et al. A multi-institutional prospective trial in the USA confirms that the 4Kscore accurately identifies men with high-grade prostate cancer. *Eur Urol* 2015;68:464–70.
4. Hamoen EH, de Rooij M, Witjes JA, Barentsz JO, Rovers MM. Use of the prostate imaging reporting and data system (PI-RADS) for prostate cancer detection with multiparametric magnetic resonance imaging: a diagnostic meta-analysis. *Eur Urol* 2015;67:1112–21.
5. Ceci F, Uprimny C, Nilica B, Geraldo L, Kandler D, Kroiss A, et al. (68)Ga-PSMA PET/CT for restaging recurrent prostate cancer: which factors are associated with PET/CT detection rate? *Eur J Nucl Med Mol Imaging* 2015;42:1284–94.
6. Roberts MJ, Richards RS, Gardiner RA, Selth LA. Seminal fluid: a useful source of prostate cancer biomarkers? *Biomark Med* 2015;9:77–80.
7. Hessels D, Smit FP, Verhaegh GW, Witjes JA, Cornel EB, Schalken JA. Detection of TMPRSS2-ERG fusion transcripts and prostate cancer antigen 3 in urinary sediments may improve diagnosis of prostate cancer. *Clin Cancer Res* 2007;13:5103–8.
8. Wei JT, Feng Z, Partin AW, Brown E, Thompson I, Sokoll L, et al. Can Urinary PCA3 Supplement PSA in the Early Detection of Prostate Cancer? *J Clin Oncol* 2014;32:4066–72.
9. Drayton RM, Rehman I, Clarke R, Zhao Z, Pang K, Miah S, et al. Identification and diagnostic performance of a small RNA within the PCA3 and BMCC1 gene locus that potentially targets mRNA. *Cancer Epidemiol Biomarkers Prev* 2015;24:268–75.
10. Leyten GH, Hessels D, Jannink SA, Smit FP, de Jong H, Cornel EB, et al. Prospective multicentre evaluation of PCA3 and TMPRSS2-ERG gene fusions as diagnostic and prognostic urinary biomarkers for prostate cancer. *Eur Urol* 2014;65:534–42.
11. National Institute for Health and Care Excellence (NICE). *Diagnosing prostate cancer: PROGENSA PCA3 assay and Prostate Health Index*. London (UK): National Institute for Health and Care Excellence (NICE); 2015:50.
12. Roberts MJ, Schirra HJ, Lavin MF, Gardiner RA. Metabolomics: a novel approach to early and noninvasive prostate cancer detection. *Korean J Urol* 2011;52:79–89.
13. Donovan MJ, Noerholm M, Bentink S, Belzer S, Skog J, O'Neill V, et al. A molecular signature of PCA3 and ERG exosomal RNA from non-DRE urine is predictive of initial prostate biopsy result. *Prostate Cancer Prostatic Dis* 2015;18:370–5.
14. Clarke RA, Schirra HJ, Catto JW, Lavin MF, Gardiner RA. Markers for detection of prostate cancer. *Cancers (Basel)* 2010;2:1125–54.
15. Roberts MJ, Chow CW, Schirra HJ, Richards R, Buck M, Selth LA, et al. Diagnostic performance of expression of PCA3, Hepsin and miR biomarkers in ejaculate in combination with serum PSA for the detection of prostate cancer. *Prostate* 2015;75:539–49.
16. Selth LA, Roberts MJ, Chow CW, Marshall VR, Doi SA, Vincent AD, et al. Human seminal fluid as a source of prostate cancer-specific microRNA biomarkers. *Endocr Relat Cancer* 2014;21:L17–21.
17. Neuhaus J, Schiffer E, von Wilcke P, Bauer HW, Leung H, Siwy J, et al. Seminal plasma as a source of prostate cancer peptide biomarker candidates for detection of indolent and advanced disease. *PLoS One* 2013;8:e67514.
18. Epstein JI, Allsbrook Jr WC, Amin MB, Egevad LL. The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. *Am J Surg Pathol* 2005;29:1228–42.
19. D'Amico AV, Whittington R, Malkowicz S, Schultz D, Blank K, Broderick GA, et al. Biochemical outcome after radical prostatectomy, external beam radiation therapy, or interstitial radiation therapy for clinically localized prostate cancer. *JAMA* 1998;280:969–74.
20. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45.
21. Landers KA, Samaratunga H, Teng L, Buck M, Burger MJ, Scells B, et al. Identification of claudin-4 as a marker highly overexpressed in both primary and metastatic prostate cancer. *Br J Cancer* 2008;99:491–501.
22. Clarke RA, Zhao Z, Guo AY, Roper K, Teng L, Fang ZM, et al. New genomic structure for prostate cancer specific gene PCA3 within BMCC1: implications for prostate cancer detection and progression. *PLoS One* 2009;4:e4995.
23. Liu X. Classification accuracy and cut point selection. *Stat Med* 2012;31:2676–86.
24. Vedder MM, de Bekker-Grob EW, Lijja HG, Vickers AJ, van Leenders GJ, Steyerberg EW, et al. The added value of percentage of free to total prostate-specific antigen, PCA3, and a kallikrein panel to the ERSPC risk calculator for prostate cancer in prescreened men. *Eur Urol* 2014;66:1109–15.
25. Seiden MV, Kantoff PW, Krithivas K, Propert K, Bryant M, Haltom E, et al. Detection of circulating tumor cells in men with localized prostate cancer. *J Clin Oncol* 1994;12:2634–9.
26. Klein LT, Lowe FC. The effects of prostatic manipulation on prostate-specific antigen levels. *Urol Clin North Am* 1997;24:293–7.
27. Tchetgen MB, Oesterling JE. The effect of prostatitis, urinary retention, ejaculation, and ambulation on the serum prostate-specific antigen concentration. *Urol Clin North Am* 1997;24:283–91.
28. Gardiner RA, Samaratunga ML, Gwynne RA, Clague A, Seymour GJ, Lavin MF. Abnormal prostatic cells in ejaculates from men with prostatic cancer—a preliminary report. *Br J Urol* 1996;78:414–8.
29. Ashrafi D, Baade P, Yaxley J, Roberts MJ, Williams S, Gardiner RA. Long-term survival outcomes for men who provided ejaculate specimens for prostate cancer research: implications for patient management. *Eur Urol Focus* 2015;1:200–6.
30. Nawaz M, Camussi G, Valadi H, Nazarenko I, Ekstrom K, Wang X, et al. The emerging role of extracellular vesicles as biomarkers for urogenital cancers. *Nat reverse Urol* 2014;11:688–701.
31. Newcomb LF, Thompson Jr IM, Boyer HD, Brooks JD, Carroll PR, Cooperberg MR, et al. Outcomes of active surveillance for clinically localized prostate cancer in the prospective, multi-institutional canary PASS cohort. *J Urol* 2016;195:313–20.
32. Chen RC, Rumble RB, Loblaw DA, Finelli A, Ehdai B, Cooperberg MR, et al. Active surveillance for the management of localized prostate cancer (Cancer Care Ontario Guideline): American Society of Clinical Oncology Clinical Practice Guideline Endorsement. *J Clin Oncol* 2016. in press.
33. Kourtidis A, Ngok SP, Pulimeno P, Feathers RW, Carpio LR, Baker TR, et al. Distinct E-cadherin-based complexes regulate cell behaviour through miRNA processing or Src and p120 catenin activity. *Nat Cell Biol* 2015;17:1145–57.
34. Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ, et al. Cadherin switching in human prostate cancer progression. *Cancer Res* 2000;60:3650–4.