Validation of a Metastatic Assay using biopsies to improve risk stratification in patients with prostate cancer treated with radical radiation therapy

Jain, S., Lyons, C. A., Walker, S. M., McQuaid, S., Hynes, S., Mitchell, D., ... Waugh, D. J. (2017). Validation of a Metastatic Assay using biopsies to improve risk stratification in patients with prostate cancer treated with radical radiation therapy. Annals of Oncology. https://doi.org/10.1093/annonc/mdx637

Published in:
Annals of Oncology

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Publisher rights
Copyright 2017 Oxford University Press. This work is made available online in accordance with the publisher's policies. Please refer to any applicable terms of use of the publisher.

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Download date:05. Mar. 2020
Validation of a Metastatic Assay using biopsies to improve risk stratification in patients with prostate cancer treated with radical radiation therapy

S. Jain1, C.A. Lyons1, S.M. Walker1,2, S. McQuaid1, S. Hynes3, D. Mitchell4, B. Pang5, G. E. Logan2, A. M. McCavigan2, D. O‘Rourke6, D. McArt1, S. McDade1, I. Mills1, K.M. Prise1, L.A. Knight1,2, C.J. Steele2, P.W. Medlow2, V. Berge7, B. Katz7, D.A. Loblaw8, D.P. Harkin1,2, J. James1, J. M. O’Sullivan1, R.D. Kennedy1,2, D.J. Waugh1

1 Centre for Cancer Research & Cell Biology, Queen’s University Belfast, Belfast, United Kingdom
2 Almac Diagnostics, Seagoe Industrial Estate, Craigavon, United Kingdom
3 Department of Pathology, University Hospital Galway, Galway, Ireland
4 Northern Ireland Cancer Centre, Belfast City Hospital, Belfast, United Kingdom
5 Department of Pathology, National University Cancer Institute, Singapore
6 Department of Pathology, Belfast City Hospital, Belfast, United Kingdom
7 Department of Urology, Oslo University Hospital, Oslo, Norway
8 Sunnybrook Health Sciences Centre, University of Toronto, Toronto, Canada

Corresponding author
Dr. Suneil Jain
Honorary Consultant Clinical Oncologist
The Northern Ireland Cancer Centre
Clinical Senior Lecturer in Clinical Oncology
Centre for Cancer Research & Cell Biology
Queen’s University Belfast
Belfast
UK
BT9 7BL
+44 2890972180
(s.jain@qub.ac.uk)

Word Count (Abstract) = 296
Word Count (Main Text excluding References/Figures) = 2237
Word Count (Main Text including References/Figures) = 3774

© The Author 2017. Published by Oxford University Press on behalf of the European Society for Medical Oncology. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com
Abstract

Background

Radiotherapy is an effective treatment for intermediate/high-risk locally-advanced prostate cancer, however, >30% of patients relapse within five years. Clinicopathological parameters currently fail to identify patients prone to systemic relapse and those whom treatment intensification may be beneficial. The purpose of this study was to independently validate the performance of a 70-gene Metastatic Assay in a cohort of diagnostic biopsies from patients treated with radical radiotherapy and androgen deprivation therapy (ADT).

Patients & Methods

A bridging cohort of prostate cancer diagnostic biopsy specimens was profiled to enable optimization of the Metastatic Assay threshold prior to further independent clinical validation in a cohort of diagnostic biopsies from patients treated with radical radiotherapy and ADT. Multivariable Cox proportional hazard regression analysis was used to assess assay performance in predicting biochemical failure-free survival (BFFS) and metastasis-free survival (MFS).

Results

Gene expression analysis was performed in 248 patients from the independent validation cohort and the Metastatic Assay applied. Ten year MFS was 72% for Metastatic Assay positive patients and 94% for Metastatic Assay negative patients (HR=3.21, [1.35-7.67]; p=0.003). On multivariable analysis the Metastatic Assay remained predictive for development of distant metastases (HR=2.71, [1.11-6.63]; p=0.030). The assay retained independent prognostic performance for MFS when assessed with the Cancer of the Prostate Assessment Score (CAPRA) (HR=3.23 [1.22-8.59]; p=0.019) whilst CAPRA itself was not significant (HR=1.88, [0.52-6.77]; p=0.332). A high
concordance (100% [61.5-100]) for the assay result was noted between two separate foci taken from 11 tumours, whilst Gleason score had low concordance.

Conclusions

The Metastatic Assay demonstrated significant prognostic performance in patients treated with radical radiotherapy both alone and independent of standard clinical and pathological variables. The Metastatic Assay could have clinical utility when deciding upon treatment intensification in high-risk patients. Genomic and clinical data are available as a public resource.

Keywords: Prostate Cancer, Risk stratification, Radiation Therapy, Prognostic, Metastatic, Biomarker
Introduction

In developed countries, prostate cancer is the most commonly diagnosed male cancer [1]. Clinicopathologic parameters are used for risk stratification prior to therapy decisions. Recently, treatment options for localized prostate cancer have increased. Patients may be managed with active surveillance, while locally-advanced and intermediate/high risk patients are offered radical surgery or radiotherapy. Upfront docetaxel chemotherapy and abiraterone-acetate have recently been shown to improve failure-free survival in hormone-sensitive locally-advanced prostate cancer [2, 3].

There is robust evidence supporting conventional, moderately hypofractionated or stereotactic radiotherapy, recognized in national treatment guidelines [4-7]. Furthermore, when combined with short (≤6 months) or long (>6-36 months) course ADT, overall survival is prolonged [8-10]. However, approximately 30% of patients will relapse within five years potentially due to undetectable metastatic disease, radio-resistant clones or insufficient treatment. Identifying patients at high-risk of relapse post-radiotherapy may inform treatment intensification used to reduce life-threatening disease. Routinely, there are no diagnostic tests used to identify these patients.

Gene expression (GE) biomarkers have shown promise for prostate cancer risk stratification, particularly in men undergoing radical prostatectomy [11, 12]. Few studies have assessed GE biomarkers in formalin fixed paraffin embedded (FFPE) diagnostic biopsies in men treated with primary radiotherapy, likely due to small tissue samples that may have degraded [13, 14].

A novel 70-gene Metastatic Assay was recently reported which identifies localized prostate cancer harboring tumour gene expression patterns similar to metastatic disease. The Metastatic Assay was validated in a cohort of radical prostatectomy samples for biochemical and metastatic recurrence [15]. The objective of this study was to further independently validate the prognostic utility of the Metastatic Assay in FFPE diagnostic biopsies from
Original Article

patients receiving radical radiotherapy for localized or locally-advanced prostate cancer.
Patients and Methods

Study Design
This study was designed and conducted in accordance with the Reporting Recommendations for tumour marker prognostic studies (REMARK) guidelines (Figure.1/Appendix A) [16]. Ethical approval was obtained from the Office for Research Ethics Committees for Northern Ireland via the NI Biobank (16/NI/0030).

Patients
A bridging cohort of 75 FFPE diagnostic biopsies from patients with localized prostate cancer were collected to optimize the Metastatic Assay threshold in biopsy material (Suppl.Table.1).

For the independent validation cohort, 473 localized/locally-advanced prostate cancer patients commencing radical radiotherapy (with/without ADT) at the NI Cancer Centre, Belfast Health and Social Care Trust (BHSCT), between 01/012005-31/12/2009 were considered for inclusion (Figure.1 & Suppl.Table.2). Patients were treated with 70-74Gy external beam radiation therapy (EBRT) in 2Gy fractions with 3D conformal or intensity modulated techniques over 7-7.5 weeks. Node-negative patients received elective pelvic nodal irradiation at the physician’s discretion; node-positive patients had radiotherapy to pelvic nodal regions. Short (≤6 months) or long (6-36 months) course ADT commenced at least 3 months prior to radiation with LHRH agonists or anti-androgens.

A cohort of 22 FFPE diagnostic biopsies representing 11 localized prostate cancer patients were identified from The Prostate Biobank, Oslo University Hospital and used to assess intra-tumour heterogeneity.

Gene Expression Profiling
As previously described [15] microarray profiling was performed in a Clinical Laboratory Improvement Amendments (CLIA) certified laboratory (Almac Diagnostics, UK). Stratagene UHR samples and ES-2 cell lines were used as
process control measures, monitored using statistical process control (SPC) charts. Quality control (QC) analysis was performed in each cohort (Supplementary Methods).

**Generation of Metastatic Assay Scores**

Samples were pre-processed independently including RMA background correction; median summarization of probes to probesets; median summarization of probesets to Entrez Gene ID and quantile normalization using a pre-defined model. Scores were calculated on a per sample basis using the Metastatic Assay parameters and algorithm [15]. Metastatic assay calls were derived by applying the optimized biopsy threshold (0.5505), where scores >0.5505 were classified as ‘Assay Positive’, otherwise ‘Assay Negative’.

**Outcomes**

Primary outcome measures were time to biochemical failure (BFFS) and time to metastatic recurrence (MFS). BFFS was defined as a PSA rise >2.0 ng/mL above nadir PSA followed by a subsequent rise [17]. MFS was defined as radiologic evidence of metastatic disease, including non-pelvic lymph nodes, bone and visceral metastases using radiological imaging. Follow-up times started on the date of commencement of EBRT and were censored on the date of last follow-up or recurrence.

**Statistical Methods**

Cox proportional hazards (PH) regression was used to investigate prognostic effects of the Metastatic Assay on time to BFFS/MFS. The estimated effect of the Metastatic Assay was adjusted for clinical covariates by fitting a multivariable analysis (MVA) model, including Gleason, age, PSA, T-stage and ADT; and secondly by evaluating adjustment for CAPRA. The Cox PH assumption was verified using statistical tests based on the Schoenfeld residuals [18].
Original Article

Intra-tumour heterogeneity was assessed by calculating overall percentage agreement of the dichotomous assay call between different biopsies from the same patient.

Samples with missing clinical information were excluded. All statistical tests were two-sided at a 5% significance level.
Results

Threshold optimization and application of the Metastatic Assay in diagnostic biopsies

A bridging cohort of 75 diagnostic specimens from localized prostate cancer patients was used to optimize the Metastatic Assay biopsy threshold (Suppl.Table.1). Semi-supervised hierarchical clustering identified four sample clusters. Metastatic Assay scores were highest within sample cluster S1, characterized predominantly by down-regulation of GE, indicative of our pre-defined Metastatic biology subgroup (Suppl.Figure.1). A suitable biopsy threshold was derived where all performance metrics (sensitivity, specificity, NPV, PPV and accuracy) were deemed optimal (y=0.93) (Suppl.Figure.2). A threshold of 0.5505 was selected, tested initially within this bridging cohort and predicted BFFS (HR=3.31 [0.93–11.82]; p=0.003) and MFS (HR=8.05 [0.96-67.12]; p=0.001) (Suppl.Figure.3).

Metastatic Assay performance in an independent primary prostate cancer radiation therapy cohort

We identified 473 patients for possible inclusion in the study. In 35%, residual tissue was absent or insufficient due to previous diagnostic procedures (Figure.1). In total, 248 patients (52% of original cohort, 93% of profiled cohort) had successful GE profiling and generation of a Metastatic Assay result (Table.1) (Original cohort, Suppl.Table.2). Median follow-up of the analyzable cohort was 99 months with one patient lost to follow-up. Median age at diagnosis was 68 years, 107 (43%) patients had Gleason 8-10 disease, 9 (4%) were node-positive and 78% had NCCN high-risk disease. Median radiation dose was 74Gy with 27% of patients receiving pelvic nodal irradiation. 141 patients (57%) received long-course ADT, 184 patients (74%) with LHRH agonists.

In total, 31.5% of patients were Assay Positive with confirmed clinical failure occurring in 65/248 (26.2%) patients. At 10 years, BFFS was 50% for Assay
Positive patients compared to 71% for Assay Negative (HR=1.96, [1.15-3.34]; p=0.006) (Figure.2A). In total, 24 patients (9.7%) developed distant metastases. Assay Positive patients were more likely to develop distant metastases than Assay Negative patients, with 10-year MFS of 72% compared to 94% (HR=3.21, [1.35-7.67]; p=0.003) (Figure.2B). Similar results were observed in patients with stratification for Gleason≥4+3, (BFFS: HR=2.59 [1.43-4.71]; p=0.001 and MFS: HR=3.59 [1.46-8.80]; p=0.005) (Figure.3). Low numbers of events precluded subset analysis within Gleason≤4+3 patients.

**Correlation of standard clinical variables with the Metastatic Assay**

In univariate analysis, aside from the Metastatic Assay, PSA and T-stage were significantly associated with BFFS, (HR=1.01 [1.00-1.02]; p=0.029 and HR=1.00 [1.14-3.09]; p=0.023 respectively) (Figure.2C). However, PSA (HR=1.01 [1.00-1.02]; p<0.007), T-stage (HR=3.20 [1.41-7.26]; p=0.025) and CAPRA (HR=1.30 [1.03-1.63]; p=0.027) were also significantly associated with MFS (Figure.2D). Importantly, the Metastatic Assay performance was better than clinical variables at predicting BFFS (HR=1.96 [1.15-3.34]; p=0.006) and MFS (HR=3.21 [1.35-7.67]; p=0.003). Proportions of clinical factors within Metastatic Assay groupings indicated that Assay Positive patients had higher Gleason score (67% Gleason 8-10), PSA levels (Median 21.4 ng/ml) and CAPRA scores (86% high-risk) than Assay Negative patients. All clinical factors with the exception of age were significantly correlated with the Metastatic Assay result (Gleason p<0.0001, CAPRA p=0.0007, ADT p<0.0001, PSA p=0.0047 and T-stage p=0.0001) (Suppl.Table.3).

**Comparison of the Metastatic Assay with clinical risk stratification**

On MVA the Metastatic Assay significantly predicted for BFFS and MFS (HR=1.94, [1.13-3.31]; p=0.016, HR=2.71, [1.11-6.63]; p=0.030 respectively). For BFFS, age was significant in MVA whilst for MFS, ADT was significant. All other factors were not significant following adjustment for other prognostic factors and the Metastatic Assay.
When standard clinicopathological variables were combined using the CAPRA tool, the CAPRA score did not significantly predict for BFFS (HR=1.24 [0.61-2.55]; \( p=0.550 \)) or MFS (HR=1.88 [0.52-6.77]; \( p=0.332 \)) (Table.2). The Metastatic Assay remained prognostic independent of CAPRA for BFFS and MFS (HR=2.46 [1.31-4.62]; \( p=0.005 \) and HR=3.23 [1.22-8.59]; \( p=0.019 \) respectively).

**Application of the Metastatic Assay as a continuous predictor of BFFS and MFS**

When considered as a continuous variable the Metastatic Assay demonstrated improved AUCs, HRs and concordance-index (CI) performance than CAPRA alone for both BFFS (AUC=0.62, HR=1.25 [1.09-1.43]; \( p=0.001 \), CI=0.62 and AUC=0.58, HR=1.13 [0.99-1.30]; \( p=0.080 \), CI=0.52 for Metastatic Assay and CAPRA respectively) and MFS (AUC=0.69, HR=1.44 [1.16-1.78]; \( p=0.001 \), CI=0.65 and AUC=0.66, HR=1.30 [1.03-1.63]; \( p=0.028 \), CI=0.57 for Metastatic Assay and CAPRA respectively) (Suppl.Table.4). Furthermore, the performance improved from AUC=0.66, HR=1.30 [1.03-1.63]; \( p=0.028 \), CI=0.57 using CAPRA alone to AUC=0.72, HR=2.20 [1.28-3.79]; \( p=0.005 \), CI=0.70) when combining CAPRA and the Metastatic Assay in a continuous model to assess metastatic recurrence (Suppl.Table.4).

**Assessment of Intra-patient Heterogeneity**

The Metastatic Assay was applied to 22 diagnostic biopsy samples representing 2 tumour foci from 11 patients to assess intra-tumour heterogeneity both as a dichotomous (Figure.4B) and continuous result (Figure.4A). The overall agreement across tumour foci of the cohort was calculated as 100% (95% CI: 61.5%-100%), indicating a low level of heterogeneity for the Metastatic Assay, albeit in a small cohort.

**Gene expression and pathway analysis of the Metastatic subgroup**
Differential gene analysis between Assay Positive and Assay Negative samples identified 1039 genes, encompassing 138 that were significantly overexpressed and 901 that were significantly underexpressed (Suppl.Table.5a). Functional analysis using GO and DAVID identified 7 key pathways defined by negatively regulated genes in the Metastatic Assay positive subgroup following Bonferroni multiple-test-correction (Suppl.Table.5b). Of significant relevance were PI3K-AKT signaling ($p=0.04$), protein digestion and absorption ($p<0.001$) and focal adhesion ($p<0.001$).
Original Article

Discussion

Molecular diagnostic tests have potential to tailor therapeutic decision-making, including treatment intensification in high-risk patients. In this study, we have demonstrated that the Metastatic Assay, is independently predictive of recurrence in men treated with EBRT whereby Assay Positive patients were more likely to develop BFFS and MFS in this dataset. Importantly, the Metastatic Assay was superior to conventional clinical parameters in predicting outcomes. Additionally, when combined with CAPRA performance was superior to either alone when assessed as continuous variables. Key strengths of this study include successful application of the assay to diagnostic FFPE biopsies with low levels of heterogeneity, large cohort (n=248), follow-up (median 99 months) and generation of high-quality GE data (93% pass-rate).

Currently, three tissue-based prognostic assays are commercially available: Cell cycle progression (CCP) score (Prolaris® Myriad Genetics), Genomic Prostate Score (Oncotype Dx®, Genomic Health Inc.) and Genomic Classifier (GC) (Decipher™, Genome DX Biosciences). Most studies evaluating these panels have been in the context of radical prostatectomy. Two studies evaluated patients treated with primary radiotherapy.

In 141 men treated with radical radiotherapy, with a median follow-up of 4.8 years, the CCP assay predicted for BFFS (HR=2.55 one-unit increase in CCP score; \( p=0.03 \)) [13]. In 100 intermediate/high-risk men treated with radiotherapy and ADT, with a median follow-up of 5.1 years, the Genomic Classifier predicted MFS (HR=1.36 per 10% increase in score) [14]. The Metastatic Assay compares favorably to these assays and increases confidence in applying genomic biomarkers to prostate cancer clinical management.

Additional value of the Metastatic Assay is also supported by the observed failure of Gleason grade to demonstrate independent prognostic utility. Given the nature of its derivation, identification of a molecular subgroup of primary
prostate cancer similar to metastatic disease [15], the Metastatic Assay has superior predictive value for MFS compared to BFFS. This may be explained by BFFS being a non-specific endpoint which cannot discriminate between local and distant failure. A proposed further utility of the Metastatic Assay may be identification of M0 patients who already have occult metastatic disease at presentation. The provision of localized therapy alone to Assay Positive patients will likely be insufficient, therefore we propose treatment intensification using of systemic therapy, including brachytherapy [19], stereotactic radiotherapy [5] with ADT, adjuvant chemotherapy [5] or extended adjuvant ADT [9, 10]. Importantly, the Metastatic assay also retained prognostic performance in high-risk prostate cancer (Gleason≥4+3), consistent with its application to support treatment intensification.

A previously reported feature of Metastatic Assay positivity was significant loss of gene expression post-surgery, predominantly related to epigenetic silencing [15]. Supporting this findings, we detected a similar proportion of underexpressed (n=901) to overexpressed genes (n=138) in this study. Functional analysis identified PI3K-AKT and FAK signaling enrichment which provide a biological foundation to support the Metastatic Assay and the emergence of clinical relapse.

Limitations of this study include the retrospective validation within a single-centre cohort. Prostate cancer studies are restricted by timescales required to accurately quantify clinical endpoints. Another consideration is the pathology attrition-rate prior to GE profiling in archived biopsy samples [20, 21]. However, routinely clinical samples will be a few weeks old and better quality. In addition, the small sample size of the intra-tumour heterogeneity cohort and the differences of these samples compared to the validation cohort are considered to be limitations. Finally, there was an increased proportion of higher-risk disease in the final cohort when compared to the original. This was expected as there was a higherattrition rate of low-volume Gleason 6 disease.
In summary, the Metastatic Assay provides independent prognostic information for localized or locally-advanced prostate cancer patients treated with radical radiotherapy and ADT who might benefit from treatment intensification. Future prospective studies could be designed to demonstrate potential benefit in patients including upstaging and intensification of treatment.
**Key Message**

The Metastatic Assay has demonstrated significant prognostic performance in patients with prostate cancer treated with radical radiotherapy both alone and independent of standard clinicopathological variables, including CAPRA. Therefore, the Metastatic Assay could have potential clinical utility to guide which patients may benefit from treatment intensification.
Acknowledgements
The authors gratefully acknowledge the contribution of the Scientific Advisory Board Members, Professor Kenneth J. Pienta (Johns Hopkins) and Professor Robert G. Bristow (Princess Margaret Hospital) for their advice and discussion of this work.

Funding
The study was funded by the Movember Prostate Cancer UK Centre of Excellence (CEO13_2-004), the Research and Development Division of the Public Health Agency of NI (COM/4965/14), the Simms Family Bequest (to CAL, JOS and SJ) and an investigator award from Friends of the Cancer Centre to SJ (NIC101345).

Disclosures

Suneil Jain – consultancy for Almac Diagnostics.

Steven Walker—employment at Almac Diagnostics, patent or IP “Molecular Test for Prostate Cancer”.

Gemma Logan—employment at Almac Diagnostics.

Andrena McCavigan —employment at Almac Diagnostics, patent or IP “Molecular Test for Prostate Cancer”.

Laura Knight—employment at Almac Diagnostics, patent or IP “Molecular Test for Prostate Cancer”.

Christopher Steele—employment at Almac Diagnostics, patent or IP “Molecular Test for Prostate Cancer”.

Paul Medlow—employment at Almac Diagnostics, patent or IP “Molecular Test for Prostate Cancer”.

Paul Harkin - employment at Almac Diagnostics, patent or IP “Molecular Test for Prostate Cancer”.

Richard Kennedy - employment at Almac Diagnostics, patent or IP “Molecular Test for Prostate Cancer”.

David Waugh – consultancy for Almac Diagnostics.
Bibliography
1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7-30.
2. James ND, Sydes MR, Clarke NW et al. Addition of docetaxel, zoledronic acid, or both to first-line long-term hormone therapy in prostate cancer (STAMPEDE): survival results from an adaptive, multiarm, multistage, platform randomised controlled trial. Lancet 2016; 387: 1163-1177.
3. James ND, de Bono JS, Spears MR et al. Abiraterone for Prostate Cancer Not Previously Treated with Hormone Therapy. N Engl J Med 2017.
4. De Puma ET, Syndikus I, Sumo G et al. Conventional versus hypofractionated high-dose intensity-modulated radiotherapy for prostate cancer: preliminary safety results from the CHHiP randomised controlled trial. Lancet Oncol 2012; 13: 43-54.
5. King CR, Brooks JD, Gill H, Presti JC, Jr. Long-term outcomes from a prospective trial of stereotactic body radiotherapy for low-risk prostate cancer. Int J Radiat Oncol Biol Phys 2012; 82: 877-882.
6. Kuban DA, Levy LB, Cheung MR et al. Long-term failure patterns and survival in a randomized dose-escalation trial for prostate cancer. Who dies of disease? Int J Radiat Oncol Biol Phys 2011; 79: 1310-1317.
7. Network. NCC. Prostate Cancer. https://www.nccn.org/professionals/physician_gls/pdf/prostate.pdf. 2017.
8. Bolla M, Maingon P, Carrie C et al. Short Androgen Suppression and Radiation Dose Escalation for Intermediate- and High-Risk Localized Prostate Cancer: Results of EORTC Trial 22991. J Clin Oncol 2016; 34: 1748-1756.
9. Bolla M, Van Tienhoven G, Warde P et al. External irradiation with or without long-term androgen suppression for prostate cancer with high metastatic risk: 10-year results of an EORTC randomised study. Lancet Oncol 2010; 11: 1066-1073.
10. Horwitz EM, Bae K, Hanks GE et al. Ten-year follow-up of radiation therapy oncology group protocol 92-02: a phase III trial of the duration of elective androgen deprivation in locally advanced prostate cancer. J Clin Oncol 2008; 26: 2497-2504.
11. Cuzick J, Swanson GP, Fisher G et al. Prognostic value of an RNA expression signature derived from cell cycle proliferation genes in patients with prostate cancer: a retrospective study. Lancet Oncol 2011; 12: 245-255.
12. Erho N, Crisan A, Vergara IA et al. Discovery and validation of a prostate cancer genomic classifier that predicts early metastasis following radical prostatectomy. PLoS One 2013; 8: e66855.
13. Freedland SJ, Gerber L, Reid J et al. Prognostic utility of cell cycle progression score in men with prostate cancer after primary external beam radiation therapy. Int J Radiat Oncol Biol Phys 2013; 86: 848-853.
14. Nguyen PL, Martin NE, Choeurng V et al. Utilization of biopsy-based genomic classifier to predict distant metastasis after definitive radiation and short-course ADT for intermediate and high-risk prostate cancer. Prostate Cancer Prostatic Dis 2017; 20: 186-192.
15. Walker SM, Knight LA, McCavigan AM et al. Molecular Subgroup of Primary Prostate Cancer Presenting with Metastatic Biology. Eur Urol 2017.
16. McShane LM, Altman DG, Sauerbrei W et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005; 97: 1180-1184.
17. Roach M, 3rd, Hanks G, Thames H, Jr. et al. Defining biochemical failure following radiotherapy with or without hormonal therapy in men with clinically localized prostate cancer: recommendations of the RTOG-ASTRO Phoenix Consensus Conference. Int J Radiat Oncol Biol Phys 2006; 65: 965-974.

18. Grambsch PM. Goodness-of-fit and diagnostics for proportional hazards regression models. Cancer Treat Res 1995; 75: 95-112.

19. Morton GC, Loblaw DA, Sankreacha R et al. Single-fraction high-dose-rate brachytherapy and hypofractionated external beam radiotherapy for men with intermediate-risk prostate cancer: analysis of short- and medium-term toxicity and quality of life. Int J Radiat Oncol Biol Phys 2010; 77: 811-817.

20. Gnanapragasam VJ. Unlocking the molecular archive: the emerging use of formalin-fixed paraffin-embedded tissue for biomarker research in urological cancer. BJU Int 2010; 105: 274-278.

21. Xie R, Chung JY, Ylaya K et al. Factors influencing the degradation of archival formalin-fixed paraffin-embedded tissue sections. J Histochem Cytochem 2011; 59: 356-365.
**Figure & Table Legends**

**Figure.1:** REMARK study design flow diagram and resultant cohort for validation of the Metastatic Assay. Inclusion/exclusion criteria for the independent validation cohort is outlined. Critical steps within the design are highlighted in blue and sample failures at each step are highlighted in yellow.

**Figure.2:** Metastatic Assay validation in the radiation biopsy cohort. Kaplan-Meier survival analysis for the Metastatic Assay predicting biochemical failure free survival (A) in Metastatic Assay Positive patients (yellow) compared to Metastatic Assay Negative patients (blue) (HR 1.96 [1.15-3.34]; \( p = 0.006 \)) and metastasis free survival (B) in Metastatic Assay Positive patients (yellow) compared to Metastatic Assay Negative patients (blue) (HR 3.21 [1.35-7.67]; \( p = 0.003 \)). Forest plot representing the UVA performance of standard clinical factors for biochemical failure free survival (C) and metastasis free survival (D). Factors included are Gleason grade, age at diagnosis, PSA at diagnosis, T-stage, ADT treatment group and CAPRA.

**Figure.3:** Metastatic Assay validation in the radiation biopsy cohort in higher-risk patients with Gleason \( \geq 4+3 \). Kaplan-Meier survival analysis for the Metastatic Assay predicting biochemical failure free survival (A) in Metastatic Assay Positive patients (yellow) compared to Metastatic Assay Negative patients (blue) (HR 2.59 [1.43-4.71]; \( p = 0.001 \)) and metastasis free survival (B) in Metastatic Assay Positive patients (yellow) compared to Metastatic Assay Negative patients (blue) (HR 3.59 [1.46-8.80]; \( p = 0.005 \)).

**Figure.4:** Impact of intra-patient tumour heterogeneity on the Metastatic Assay. Bar chart depicting Metastatic Assay continuous signature scores for each of the 11 patients in both the primary and secondary tumour foci (A) and table outlining the Metastatic Assay dichotomous calls of 11 patients in both primary and secondary tumour foci representative of different Gleason grades (B).
Table.1: Summary of clinical characteristics for the independent radiation cohort.

Table.2: Multivariable analysis for the Metastatic Assay validation in the radiation biopsy cohort for biochemical failure free survival (left) and metastasis-free survival (right). MVA Model 1 includes adjustment for the following clinical factors, age, PSA, Gleason, T-stage and ADT (dichotomized by treatment group). MVA Model 2 includes adjustment for the CAPRA tool. In all models, p-values, hazard ratios and 95% confidence intervals are indicated.
**Supplementary Figure & Table Legends**

**Suppl.Figure.1**: Semi-supervised hierarchical clustering analysis for the 75 unique prostate cancer patient samples using the 907 probeset list to identify the metastatic biology subgroup. Clustering identified four key sample clusters and 3 key gene clusters. Down-regulated genes are shown in green and up-regulated genes shown in red.

**Suppl.Figure.2**: Plot showing sensitivity, specificity, NPV, PPV and accuracy across a number of thresholds for the Metastatic Assay in the technical biopsy cohort. The optimal threshold (where all parameters intersect) is highlighted.

**Suppl.Figure.3**: Testing the optimized biopsy threshold for the Metastatic Assay in the technical diagnostic biopsy cohort. Kaplan-Meier survival analysis for the Metastatic Assay predicting biochemical failure free survival (A) in Metastatic Assay Positive patients (yellow) compared to Metastatic Assay Negative patients (blue) (HR = 3.31 [0.93-11.82]; p=0.003) and metastasis free survival (B) in Metastatic Assay Positive patients (yellow) compared to Metastatic Assay Negative patients (blue) (HR = 8.05 [0.96-67.12]; p=0.001).

**Suppl.Table.1**: Summary of clinical characteristics for the technical biopsy cohort utilized to optimize a suitable biopsy threshold.

**Suppl.Table.2**: Summary of clinical characteristics for the full original independent radiation cohort, prior to pathology review.

**Suppl.Table.3**: Distribution of the clinical prognostic factors across Metastatic Assay positive and negative samples. Correlation of clinical factors to the Metastatic Assay is indicated by p-values.

**Suppl.Table.4**: Assessment of the Metastatic Assay as a continuous predictor in the radiation biopsy cohort (AUC, HR and C-index) in comparison to
Original Article

CAPRA alone and in a combined model with continuous Metastatic Assay and CAPRA.

Suppl.Table.5a; Supplemental file; Differentially expressed genes between Metastatic Assay positive and negative subgroups, Suppl.Table.5b; Supplemental file; Functional analysis results of identified differentially expressed genes highlighting key pathways of significance which are altered in the Metastatic Assay positive subgroup.
REMARK study design flow diagram

338x190mm (96 x 96 DPI)
Metastatic Assay validation in the radiation biopsy cohort

338x190mm (96 x 96 DPI)
Metastatic Assay validation in the radiation biopsy cohort in higher-risk patients with Gleason ≥4+3.

338x190mm (96 x 96 DPI)
Impact of intra-patient tumour heterogeneity on the Metastatic Assay

338x190mm (96 x 96 DPI)
| Case included in final analysis | No. of patients (n) |
|-------------------------------|---------------------|
| Age at diagnosis, median (IQR) | 68 63 – 72 |
| Age-adjusted Charlson comorbidity index, median (IQR) | |
| ECOG performance status (%) | 212 86% |
| Baseline PSA (ng/mL), median (IQR) | 18 11.1 – 27.4 |
| Clinical T stage | |
| T1 | 51 20% |
| T2 | 76 31% |
| T3 | 92 37% |
| T4 | 4 2% |
| Unknown | 25 10% |
| N0 | 239 96% |
| N1 | 9 4% |
| N | 0 0% |
| Gleason score | |
| < 7 | 41 17% |
| 7 | 100 40% |
| 3+4 | 60 24% |
| 4+3 | 40 16% |
| > 7 | 107 43% |
| Percent positive cores | |
| Median (IQR) | 56% 38% - 83% |
| Modified D’Amico risk group (%) | |
| Low | 4 2% |
| Intermediate | 47 19% |
| High | 197 79% |
| NCCN risk group (%) | |
| Low | 4 2% |
| Intermediate | 51 20% |
| High | 193 78% |
| 0 – 2 | 3 1% |
| CAPRA score (%) | |
| 3 – 5 | 57 23% |
| 6 – 10 | 120 49% |
| Unknown | 68 27% |
| MB score (%) | |
| Positive | 78 31% |
| Radiation dose (Gy2), median | 74 |
| Treatment site (%) | |
| Prostate alone | 182 74% |
| Prostate and pelvic lymph nodes | 66 27% |
| ADT duration (%) | |
| None | 1 < 1% |
| Short-term | 106 43% |
| Long-term | 141 57% |
| ADT subtype (%) | |
| Anti-androgen | 63 26% |
| LHRH agonist | 184 | 74% |
| Multivariate Model 1 | Biochemical Failure Free Survival (BFFS) | Metastatic Free Survival (MFS) |
|---------------------|----------------------------------------|--------------------------------|
| Covariate           | HR          | 95% CI      | p     | Covariate           | HR          | 95% CI      | p     |
| Metastatic Assay    | 1.94        | 1.13 to 3.31| 0.0163 | Metastatic Assay    | 2.71        | 1.11 to 6.63| 0.0300 |
| (Negative* v Positive) |            |              |       | (Negative* v Positive) |            |              |       |
| Gleason: ("3+4")   |             |              |       | Gleason: ("3+4")   |             |              |       |
| 6                   | 0.66        | 0.25 to 1.74| 0.3506 | 6                   | 0.57        | 0.06 to 5.46| 0.6240 |
| 4+3                 | 1.71        | 0.79 to 3.71| 0.1280 | 4+3                 | 2.34        | 0.55 to 10.03| 0.2545 |
| 8-10                | 1.33        | 0.60 to 2.95| 0.4280 | 8-10                | 3.13        | 0.71 to 13.88| 0.1349 |
| Age                 | 0.96        | 0.93 to 1.00| 0.0505 | Age                 | 0.99        | 0.93 to 1.05| 0.7259 |
| PSA                 | 1.00        | 1.00 to 1.01| 0.2430 | PSA                 | 1.01        | 1.00 to 1.02| 0.0914 |
| ADT                 | 1.53        | 0.77 to 3.04| 0.2304 | ADT                 | 3.01        | 0.99 to 9.15| 0.0538 |
| (Long Course* v Short Course) | | | | (Long Course* v Short Course) | | | |
| T Stage             | 1.62        | 0.86 to 3.03| 0.1345 | T Stage             | 1.91        | 0.58 to 6.30| 0.2912 |
| (1&2* v 3&4)        |             |              |       | (1&2* v 3&4)        |             |              |       |
| **Multivariate Model 2** | | | | **Multivariate Model 2** | | | |
| Covariate           | HR          | 95% CI      | p     | Covariate           | HR          | 95% CI      | p     |
| Metastatic Assay    | 2.46        | 1.31 to 4.62| 0.0051 | Metastatic Assay    | 3.23        | 1.22 to 8.59| 0.0185 |
| (Negative* v Positive) |            |              |       | (Negative* v Positive) |            |              |       |
| CAPRA               | 1.24        | 0.61 to 2.55| 0.5499 | CAPRA               | 1.88        | 0.52 to 6.77| 0.3320 |
| (Low Risk* v High Risk) |            |              |       | (Low Risk* v High Risk) |            |              |       |

Abbreviations: HR, hazard ratio; CI, confidence intervals; PSA, prostate specific antigen; CAPRA, Cancer of the Prostate Risk Assessment; ADT, androgen deprivation therapy.
*Reference category.
| Covariate                          | No. | %  |
|-----------------------------------|-----|-----|
| **Age**                           | 58 (45-71) |     |
| **Recurrence Event**              |     |     |
| Recurrence                        | 26  | 35  |
| Non-Recurrence                    | 49  | 65  |
| **Time to Recurrence**            |     |     |
| Biochemical                       | 37 (7-102) | 64 (19-105) |
| **Metastatic**                    |     |     |
| **Pre-operative PSA**             |     |     |
| Median (Min - Max)                | 7.7 (2.6 – 61.4) |     |
| **Gleason Score**                 |     |     |
| <7                                | 5   | 7   |
| 7                                 | 58  | 77  |
| >7                                | 12  | 16  |
| **Surgical Margins (SM)**         |     |     |
| Positive                          | 33  | 56  |
| Negative                          | 42  | 44  |
| **Lymph Node Invasion (LNI)**     |     |     |
| Yes                               | 2   | 2   |
| No                                | 17  | 23  |
| Unknown                           | 56  | 75  |
| **T-stage**                       |     |     |
| T2                                | 36  | 48  |
| T3                                | 39  | 52  |