Non-invasive detection of clinically significant prostate cancer using circulating tumor cells

Lei Xu[1,2*], Xueying Mao[1*], Alistair Grey[3,4,5], Glenda Scandura[1], Tianyu Guo[1], Edwina Burke[1], Jacek Marzec[1], Semah Abdu[1], Elzbieta Stankiewicz[1], Caitlin R Davies[1], Prabhakar Rajan[1,3,4,6], Karen Tipples[3], John Hines[3,6], Pui Ying Chan[7], Diane Campbell[3], Karen Wilkinson[3,6], Sakunthala Kudahetti[1], Jonathan Shamash[7], Tim Oliver[1], Daniel Berney[1], Greg Shaw[3,4,6], Yong-Jie Lu[1,8]

[1]Centre for Molecular Oncology, Barts Cancer Institute, Queen Mary University of London, London, UK.

[2]Department of Urology, Zhongshan Hospital, Fudan University, Shanghai, China.

[3]Department of Urology, Barts Health NHS, London, UK.

[4]Division of Surgery and Interventional Sciences, University College London, UK

[5]Department of Surgery and Cancer, Imperial College London, UK

[6]Department of Urology, University College London NHS Foundation Trust, UK

[7]Department of Medical Oncology, Barts Health NHS, London, UK.

[8]First Affiliated Hospital & Academy of Medical Sciences, Zhengzhou University, Zhengzhou, China

[*]These authors contributed equally to this work.

Corresponding Author: Yong-Jie Lu, Centre for Molecular Oncology, Barts Cancer Institute, Queen Mary University of London, John Vane Science Centre, Charterhouse Square, London, EC1M 6BQ, UK. Phone: +44 (0)20 7882 3597; Fax: +44 (0)20 7882 3884; Email: y.j.lu@qmul.ac.uk

Word account: 2490

Running title: CTCs in prostate cancer diagnosis

Abstract

Purpose: PSA testing results in unnecessary biopsy and over-diagnosis with consequent over-treatment. Tissue biopsy is an invasive procedure, associated with significant morbidity. More accurate non- or minimum-invasive diagnostic approaches should be developed to avoid unnecessary prostate biopsy and over-diagnosis. We investigated the potential of using circulating tumor cell analysis in cancer diagnosis, particularly in predicting clinically significant prostate cancer in pre-biopsy patients.

Material and methods: We enrolled 155 treatment naïve prostate cancer patients and 98 pre-biopsy patients for circulating tumor cell numeration. RNA was extracted from circulating tumor cells from 184 patients for gene expression analysis. Kruskal-Wallis, Spearman's rank, multivariate logistic regression and random forest were applied to assess the association of circulating tumor cells with aggressive prostate cancer.

Results: In localized prostate cancer patients, 54% were scored as circulating tumor cell positive, which was associated with higher Gleason score (p=0.0003), risk group (p<0.0001) and clinically significant prostate cancer (p<0.0001). In pre-biopsy group, positive circulating tumor cell score in combination with PSA predicted clinically significant prostate cancer with AUC=0.869. A 12-gene panel prognostic for clinically significant prostate cancer was also identified. Combining PSA level, circulating tumor cell-score and the 12-gene panel, AUC for clinically significant prostate cancer prediction was 0.927 and in cases with multi-parametric MRI data, adding these to multi-parametric MRI significantly increased the prediction accuracy (AUC 0.936 vs 0.629).

Conclusions: Circulating tumor cell analysis has the potential to significantly improve patient stratification by PSA and/or multi-parametric MRI for biopsy and treatment.

Key words: circulating tumor cell, gene expression, non-invasive diagnosis, clinically significant prostate cancer, pre-biopsy triage

1. Introduction

PSA testing lacks specificity **in the detection of prostate cancer (PCa), the most common cancer in Western men[1, 2] and over half** patients with elevated PSA levels do not have cancer on biopsy**, an invasive procedure** with significant risks of urinary retention, bleeding and infection. In addition more than50% of the patients diagnosed with early stage PCa will not die of the disease[3-6] **suggesting** PSA may lead to unnecessary biopsies, over-diagnosis, and overtreatment[7]. Histological grading by Gleason score (GS) from biopsy specimens is currently needed for risk stratification, allowing the offer of appropriate therapeutic options[7, 8]. An accurate, non-invasive test for clinically significant PCa (csPCs) might provide a safer, more efficient means of diagnosis.

Multiparametric MRI (mpMRI) has recently shown value in the detection of csPCA, with specificities of 23-87% and sensitivities of 58-96% reported[9-11]. **The PROMIS trial of** 576 men demonstrated a 93% sensitivity and 41% specificity of mpMRI, compared to 48% and 96% respectively for untargeted transrectal biopsy[9] **suggesting 27% could avoid biopsy using mpMRI as triage but with an accurate pre-biopsy biomarker a further 50% might do so**.

Circulating tumor cell (CTC) analysis using CellSearch system has been approved by FDA for prognostics in advanced, metastatic PCa[12]. The study of CTCs in non-metastatic PCa has been predominantly in locally advanced disease[13-18]. Most studies used CellSearch, concluding that CTCs are rare in patients with non-metastatic PCa[13-15]. Recent studies using new CTC isolation systems demonstrate greater CTC capture efficiency than CellSearch in locally advanced PCa[16-18]. Most methods detect CTCs with epithelial cell features, missing CTCs undergoing epithelial-mesenchymal transition (EMT), an important process in metastasis development.

We demonstrated that the Parsortix system, which uses cell size and deformability to capture CTCs, harvested different subtypes in greater numbers than CellSearch[19, 20]. Here we investigate its efficiency in capturing CTCs from patients with localized PCa and in PCa diagnosis and risk stratification.

2. Materials and methods

2.1 Study patient cohorts

155 treatment-naïve, histologically confirmed localized PCa patients and 98 biopsy-naïve patients with concerning PSA levels and/or abnormal digital rectal examination were enrolled at St Bartholomew's Hospital. MpMRI was performed before biopsy. Ultrasound guided transrectal or transperineal biopsy was performed with targeted biopsy on **suspicious (Likert 3+) mpMRI lesions**. Two pre-biopsy patients had bone metastases demonstrated by bone scintigraphy. Control samples were collected from 12 healthy volunteers.

Clinical data including age, PSA, radiological results, biopsy results and TNM stage were collected (**Supplementary Table 1**). Patients were classified into low-, intermediate-, and high-risk tumor following EAU guidelines[7] and favorable disease or csPCa were defined based on previous publications[21, 22] shown in **Supplementary Table 2**. The primary outcome was men diagnosed with PCa, including risk stratification into favorable/clinically significant disease.

2.2 Cell lines

Three PCa cell lines, PC3, LNCaP and VCaP from ATCC were used with authentication by short tandem repeat testing.

2.3 CTC isolation, enumeration and RNA extraction

7.5 mL of whole blood was used for CTC isolation and enumeration as described previously[19,20]. Positive CTC-score was defined as any epithelial CTC (CK+/VIM-/CD45-), any EMTing CTC (CK+/VIM+/CD45-), and/or >3 mesenchymal CTCs (CK-/VIM+/CD45-) based on our previous analysis of 24 age-matched male healthy control samples and the confirmation of the malignant nature of CTCs in PCa cases by fluorescence *in situ* hybridization analysis of multiple genomic regions commonly altered in PCa cells[20]. 97/155 PCa patients and 87/98 pre-biopsy patients had an extra 7.5 mL blood for CTC mRNA analysis **harvested from cassette**. Total RNA was extracted using miRNeasy micro kit (Qiagen) following manufacturer's instructions but eluted with a final volume of 11.5 μL. cDNA synthesis was performed using SuperScript™ II Reverse Transcriptase (ThermoFisher Scientific).

2.4 Quantitative RT-PCR (qRT-PCR) for analytical validation

Gene expression was determined either using ABI 7500 Real-Time PCR system (Life technologies) or Fluidigm multiplex PCR.

2.5 Statistical analyses

Kruskal-Wallis test was applied to assess the equality of CTCs between subgroups based on CTC-score and different clinical features, such as mpMRI data, primary GS, and risk classification. Data was shown as median (interquartile range [IQR]). Spearman's rank correlation was used to assess the association between CTC counts and concurrent PSA level. Receiver operating characteristic (ROC) curve analysis was performed to test the ability of MRI, PSA, CTCs and different combined risk scores (CRSs) to predict patients with PCa and

csPCa. Regression coefficients for individual variables in CRSs were computed by multivariate logistic regression. Optimal cut-off point was calculated to provide best available sensitivity and specificity. Random forest classification algorithm[23] was applied to **rank prediction abilities of** CTC expression genes and the final gene set selection was conducted by comparing out-of-bag error rates of random forest models composed of decreasing number of genes. Bonferroni correction method was applied to adjust *p* values (p_{adj}) for multiple testing. Statistical analyses were performed using Stata 13.0 and R3.3.1.

3. Results

3.1 Detection of CTCs in patients with localized PCa and their correlations with risk groups

We first investigated the ability of CTCs, analyzed in three categories: epithelial (CK+/VIM-/CD45-), EMTing (CK+/VIM+/CD45-) and mesenchymal (CK-/VIM+/CD45-) CTCs (**Fig. 1A**) for a CTC score, in distinguishing clinically insignificant and significant cancers in diagnosed localized PCa patients. In 155 patients with localized PCa, at least one traditional epithelial CTC (all CK+ CTCs) were detected in 30% (46/155) of patients, at least one of any subtypes of our defined CTCs in 78% (121/155) of patients and 54% (84/155) of patients were CTC-score positive. In the 64 GS 3+3 and 40 low-risk cancer patients, CTCs were scored positive in 34% (22/64) and 25% (10/40) of cases respectively, indicating that cancer cells are released into the circulation at an early development stage.

Considering subtypes of CTCs, epithelial, EMTing and mesenchymal CTCs all showed trends of correlations (Spearman's ϱ=0.15, 0.24 and 0.11, respectively) with serum PSA levels (*p*=0.07, 0.0029 and 0.17 respectively), although only EMTing CTCs are significant. Epithelial, EMTing and mesenchymal CTC counts generally increased from low to high GS groups (3+3, 3+4, 4+3,

and ≥4+4) but without statistical significance (p_{adj}=0.16, 0.06 and 0.24 respectively, **Supplementary Fig. 1**). Positive CTC-score was significantly associated with high GS (p_{adj}=0.0012, **Table 1**).

If the 155 patients were divided into low-, intermediate- and high-risk groups, EMTing and mesenchymal CTCs significantly increased with higher risk (p_{adj}=0.0136 and 0.016 respectively) but not epithelial CTCs (p_{adj}=0.44, **Table 1, Fig. 1B**). CTC-score positivity associated more significantly (p_{adj}<0.0001) with high-risk disease. Dividing into clinically significant and favorable disease, high PSA level (p=0.0001), positive CTC-score (p_{adj}<0.0001), epithelial (p_{adj}=0.0264), EMTing (p_{adj}=0.01) and mesenchymal (p_{adj}=0.0384) CTC counts were all significantly correlated to csPCa (**Table 1, Fig. 1C**). Combining CTC-score with PSA, we generated the combined risk score (CRS-PC) by 0.233xPSA + 1.548xCTC-score, which discriminated csPCa better than PSA alone (AUC: 0.826 vs 0.764, ***p*=0.03, Fig. 2A**). In the 115 patients with mpMRI data at diagnosis, a significantly higher MRI positive (using Likert=3 as threshold) rate was found in csPCa (P=0.0001) than favorable patients (**Table 1**). The AUC using Likert 1-5 was 0.753 (95%CI: 0.663-0.842, with a cut-off point ≥3 to reach sensitivity of 98.59% and specificity of 47.73%, or a cut-off point ≥5 to reach sensitivity of 7.04% and specificity of 100%).

3.2 Predicting csPCa in pre-biopsy patients using serum PSA and CTC positivity

We then assessed the potential of using CTCs to predict csPCa in 98 pre-biopsy patients. Positive CTC-score was significantly associated with a positive biopsy results (p_{adj}<0.0001) and csPCa (p_{adj}<0.0001, **Table 2**). Positive MRI (Likert≥3) had similar distribution in benign and malignant patients (p=0.52), but was significantly more frequent in csPCa than in favorable

disease (*p*=0.0002, **Table 2**) and favourable combined with benign patients (*p*=0.0017, **Table 2**). The AUC to identify csPCa by PSA level was 0.733 (95%CI: 0.630-0.835, with an optimal cut-off point ≥15 ng/mL to reach 44.19% sensitivity and 96.36% specificity), by CTC-score was 0.811 (95%CI: 0.732-0.890 with 76.74% sensitivity and 85.45% specificity) and by CRS-PC was 0.869 (95%CI: 0.792-0.945, with an optimal cut-off point ≥2.87 to reach 87.27% sensitivity and 83.67% specificity)(**Fig. 2B**), using the model developed previously in localized patients, significantly (*p*=0.0008) better than PSA alone. In the 87 pre-biopsy patients with pre-biopsy MRI data, the AUC to predict csPCa using Likert 1-5 was 0.698 (95%CI: 0.588-0.808, with a cut-off point ≥3 to reach sensitivity of 97.2% and specificity of 29.4%, or a cut-off point ≥5 to reach sensitivity of 47.2% and specificity of 90.2%), PSA 0.739 and CTC-score 0.783 (**Fig. 2C**). Various combinations of these three factors were produced; CRS-PM (combining PSA and MRI Likert as 0.201xPSA + 0.550xMRI Likert), CRS-PC (combining PSA and CTC-score as 0.179xPSA + 2.798xCTC-score), CRS-MC (combining MRI Likert and CTC-score as 0.593xMRI Likert + 2.528xCTC-score) and CRS-PMC (combining PSA, MRI Likert and CTC-score as 0.207xPSA + 2.477xCTC-score + 0.551xMRI Likert), in predicting csPCa. Each combination increased the prediction value **(*p*<0.01 for all combinations including CTC score compared to PBS or MRI)**. AUC for the combination of all three factors (CRS-PMC) reached 0.891 (**Fig. 2C**).

3.3 Using CTC gene expressions to improve the prediction of csPCa

We identified 74 PCa prognostic genes through extensive bioinformatic analysis of **all available** transcriptome sequencing data and 50 reported PCa-specific and/or prognostic genes by **literature search** (**Supplementary Table 3**). 38 **of the 124 genes** were selected based on their relatively high expression in prostate and low/zero expression in WBC/whole blood using Genecards data (http://www.genecards.org/, **Supplementary Table 3**) for experimental

validation by qRT-PCR in PC3, LNCaP, VCaP and PBMC samples (**Supplementary Fig. 2**). Out of the 38 genes, 30 with low expression in PBMC (minimum median Ct of 33.9, **Table 3, Supplementary Fig. 2**) were finally selected for further analysis together with housekeeping genes *GAPDH* and *MRFAP1*. Good qRT-PCR amplification efficiency was achieved both for the ABI 7500 and Fluidigm systems using *FOLH1* (*PSMA*) assay in 1, 5 10, and 20 spiked LNCaP samples (**Supplementary Fig. 3**). Minimum detectability of spiked cells for each gene using the Fluidigm system were shown in **Supplementary Table 4**. All the 30 genes were negative in PBMC controls. *CDH12, CHGA, CSMD3, GRHL2, KLK2,* and *PART1* were only positive in cancer patients and csPCa cases were more frequently (17/108, 15.7%) with >6 gene positive than the remaining patients (6/76, 8%)(*p*=0.049).

Using random forest classifier, we identified a 12-gene panel (**Table 3**, Supplementary **Fig. 4)** to distinguish csPCa from favorable disease with an AUC of 0.707 (95%CI: 0.634-0.779, with an optimal cut-off point ≥0.442, sensitivity 51.85%, specificity 80.26%). When we combined PSA level, CTC-score and 12-gene panel score as CRS-PCG (0.200xPSA + 2.082xCTC-score + 1.035x12-gene panel score) the AUC increased from CRS-PC AUC=0.844 to 0.881 (95%CI: 0.832-0.929 with an optimal cut-off point ≥3.154 to reach 83.33% sensitivity and 80.26% specificity, **Fig. 2D**)**(*p*=**0.024) in above 184 samples and it increased to 0.927 (95%CI: 0.870-0.985, with an optimal cut-off point ≥3.095 to reach 87.5% sensitivity and 89.36% specificity) from a CRS-PC of 0.899 in the 87 pre-biopsy patients with CTC gene expression data (**Fig. 2E**)**(*p*=0.23)**. In the 78 samples with both MRI results and RNA samples, adding PSA and CTC data to mpMRI (valued as 1 if Likert≥3 and 0 otherwise) as CRS-PCGM=3.127xMRI likert + 0.276xPSA + 3.014xCTC-score + 1.174x12-gene panel) dramatically increased AUC from 0.629 to 0.936 **(*p*<0.0001)**(**Fig. 2F**).

3.4 Clinical implications

Modelling CTC score use in the 98 pre-biopsy men, 85% of biopsies were avoided, but 23% of csPCas were missed, reflecting a high specificity but low sensitivity (**Table 4**). Combining PSA and CTC score increased biopsies avoided to 87% while missing 23% of csPCa (**Table 4**). With the additional 12-gene panel, 91% biopsies were avoided with 18% csPCa missed. mpMRI predicted csPCa at a high sensitivity (94% negative predictive value) but lower specificity compared to CTC-score, avoiding 27% vs 85% biopsies **(Table 4**). Adding PSA and CTC data to mpMRI (CRS-PMC), 89% biopsies were avoided with only 15% csPCa missed **(Table 4**). With an alternative cut-off point, CRS-PCGM could avoid 42% biopsies without missing csPCa, doubling that by MRI alone.

4. Discussion

The recent development of efficient CTC capture systems permits study of CTCs in non-metastatic PCa, but its value in PCa detection is yet to be evaluated[16-18]. Using a cell size and deformability-based CTC isolation system in a large cohort of localized PCa, we detected CTCs at a high frequency and in low GS and low-risk cancer patients. Most importantly, we showed that CTC analysis in combination with serum PSA can efficiently detect csPCa, potentially avoiding prostate biopsy, and bringing major benefits to the PCa diagnostics. Cancer can invade the blood circulation at early development stages, including cancer precursor conditions[24]. However, due to their rarity and challenges in capturing CTCs, their potential for cancer detection has only been explored in lung malignancy[12, 25, 26]. Our study further supports the application of CTCs to early cancer detection.

High (>50%) negative biopsy rates in abnormal PSA (>4ng/ml) highlight its limitation as a biopsy trigger[9]. Additionally, many early-stage PCas are indolent, do not affect mortality[3]. A non-invasive biomarker, which can be used to avoid unnecessary biopsies, over-diagnosis, and over-treatment, would be a useful addition to the diagnostic pathway, allowing resources to be focused on patients with csPCa[9]. mpMRI shows promise in triaging patients with suspected PCa for prostate biopsy and play an increasing role[9-11]. Here, we show that CTCs may efficiently predict biopsy results, particularly for csPCa, and improve csPCa prediction value of mpMRI. Further study in large cohorts is warranted to establish the roles of CTCs in csPCa prediction alongside mpMRI, to improve patient biopsy triage and cancer prognosis.

The prognostic value of cancer RNA expression has been demonstrated[27, 28] and AR-V7 expression in CTCs has been used to predict the response to androgen deprivation therapy. Here we demonstrate that, in addition to CTC enumeration, CTC gene expression analysis may provide further prognostic information and bypass the problem of tumor heterogeneity which occurs when analysing prostate biopsy samples[29]. Future CTC analysis in combination of both CTC enumeration and gene expression level may significantly increase the potential of using CTCs for cancer diagnosis and prognosis.

Including mesenchymal CTCs, our study significantly increased the CTC positive cases in both the localized PCa and pre-biopsy cohorts of cancer cases. Mesenchymal cancer cells show invasive growth properties and may cause spread at early stage of cancer development[30]**.** In our localized disease cohort, only EMTing and mesenchymal CTCs were significantly associated with GS.

There are limitations to this study. Firstly, our CTC analysis may miss small CTCs. Secondly, **The CTC gene expression panel is yet to be validated. Finally,** this is a single centre study, which requires validation by independent research centres.

5. Conclusion

In a large series of localized PCa, we detected using our novel CTC analysis method, a high CTC positive rate which was correlated with higher GS and aggressive cancer. Importantly, positive CTC-score was associated with csPCa. In the pre-biopsy cohort, CTCs in combination with PSA efficiently predict csPCa. A CTC 12-gene prognostic panel was also identified to further increase the prediction accuracy of csPCa, which can be used to improve mpMRI prediction value. Therefore, we demonstrate the value of CTCs in PCa detection and prognostication.

Financial support

This work was supported by Orchid Cancer Appeal, Cancer Research UK [grant number: C16420/A18066] and ANGLE plc. The grant numbers of Orchid Cancer Appeal and AGNLE plc are not applicable.

Conflict of interest

This study is partially supported by ANGLE plc, which holds the marketing rights of Parsortix system, by providing research funds and reagents to Y-J.L.. G.S. works as a medical consultant for ANGLE plc. The remaining authors declare no competing interests. The funding source had no role in the design of the study; the collection, analysis, or interpretation of the data; or the writing of the manuscript.

295

296 **References**

297 1. Siegel R, Ma J, Zou Z*, et al.* Cancer statistics, 2014. CA Cancer J Clin 2014; **64**:9-29.

298 2. Society AC. Cancer Facts & Figures 2016. Atlanta: American Cancer Society: 2016.

299 3. Wolf AM, Wender RC, Etzioni RB*, et al.* American Cancer Society guideline for the early
300 detection of prostate cancer: update 2010. CA Cancer J Clin; **60**:70-98.

301 4. Wilt TJ, Brawer MK, Jones KM*, et al.* Radical prostatectomy versus observation for localized
302 prostate cancer. N Engl J Med 2012; **367**:203-13.

303 5. Stattin P, Holmberg E, Johansson JE*, et al.* Outcomes in localized prostate cancer: National
304 Prostate Cancer Register of Sweden follow-up study. J Natl Cancer Inst 2010; **102**:950-8.

305 6. Draisma G, Etzioni R, Tsodikov A*, et al.* Lead time and overdiagnosis in prostate-specific
306 antigen screening: importance of methods and context. J Natl Cancer Inst 2009; **101**:374-83.

307 7. Mottet N, Bellmunt J, Bolla M*, et al.* EAU-ESTRO-SIOG Guidelines on Prostate Cancer. Part
308 1: Screening, Diagnosis, and Local Treatment with Curative Intent. Eur Urol 2017; **71**:618-29.

309 8. Hurley P, Dhir A, Gao Y*, et al.* A Statewide Intervention Improves Appropriate Imaging in
310 Localized Prostate Cancer. J Urol 2017; **197**:1222-8.

311 9. Ahmed HU, El-Shater Bosaily A, Brown LC*, et al.* Diagnostic accuracy of multi-parametric
312 MRI and TRUS biopsy in prostate cancer (PROMIS): a paired validating confirmatory study.
313 Lancet 2017; **389**:815-22.

314 10. Futterer JJ, Briganti A, De Visschere P*, et al.* Can Clinically Significant Prostate Cancer Be
315 Detected with Multiparametric Magnetic Resonance Imaging? A Systematic Review of the
316 Literature. Eur Urol 2015; **68**:1045-53.

317 11. Pokorny MR, de Rooij M, Duncan E*, et al.* Prospective study of diagnostic accuracy
318 comparing prostate cancer detection by transrectal ultrasound-guided biopsy versus

magnetic resonance (MR) imaging with subsequent MR-guided biopsy in men without previous prostate biopsies. Eur Urol 2014; **66**:22-29.

12. Moon DH, Lindsay DP, Hong S*, et al.* Clinical indications for, and the future of, circulating tumor cells. Adv Drug Deliv Rev 2018; **125**:143-50.

13. Davis JW, Nakanishi H, Kumar VS*, et al.* Circulating tumor cells in peripheral blood samples from patients with increased serum prostate specific antigen: initial results in early prostate cancer. J Urol 2008; **179**:2187-91.

14. Loh J, Jovanovic L, Lehman M*, et al.* Circulating tumor cell detection in high-risk non-metastatic prostate cancer. J Cancer Res Clin Oncol 2014; **140**:2157-62.

15. Khurana KK, Grane R, Borden EC*, et al.* Prevalence of circulating tumor cells in localized prostate cancer. Curr Urol 2013; **7**:65-69.

16. Kuske A, Gorges TM, Tennstedt P*, et al.* Improved detection of circulating tumor cells in non-metastatic high-risk prostate cancer patients. Sci Rep 2016; **6**:39736.

17. Todenhofer T, Park ES, Duffy S*, et al.* Microfluidic enrichment of circulating tumor cells in patients with clinically localized prostate cancer. Urol Oncol 2016; **34**:483.

18. Theil G, Fischer K, Weber E*, et al.* The Use of a New CellCollector to Isolate Circulating Tumor Cells from the Blood of Patients with Different Stages of Prostate Cancer and Clinical Outcomes - A Proof-of-Concept Study. Plos One 2016; **11**:e0158354.

19. Xu L, Mao X, Imrali A*, et al.* Optimization and Evaluation of a Novel Size Based Circulating Tumor Cell Isolation System. PLoS One 2015; **10**:e0138032.

20. Xu L, Mao X, Guo T*, et al.* The Novel Association of Circulating Tumor Cells and Circulating Megakaryocytes with Prostate Cancer Prognosis. Clin Cancer Res 2017; **23**:5112-22.

21. Zumsteg ZS, Spratt DE, Pei I*, et al.* A new risk classification system for therapeutic decision making with intermediate-risk prostate cancer patients undergoing dose-escalated external-beam radiation therapy. Eur Urol 2013; **64**:895-902.

22. Zumsteg ZS, Zelefsky MJ. Short-term androgen deprivation therapy for patients with intermediate-risk prostate cancer undergoing dose-escalated radiotherapy: the standard of care? Lancet Oncol 2012; **13**:e259-69.

23. Breiman L. Random Forests. Machine Learning. Kluwer Academic Publishers 2001; **45**:5-32.

24. Kang Y, Pantel K. Tumor cell dissemination: emerging biological insights from animal models and cancer patients. Cancer Cell 2013; **23**:573-81.

25. Fiorelli A, Accardo M, Carelli E*, et al.* Circulating Tumor Cells in Diagnosing Lung Cancer: Clinical and Morphologic Analysis. Ann Thorac Surg 2015; **99**:1899-905.

26. Ilie M, Hofman V, Long-Mira E*, et al.* "Sentinel" circulating tumor cells allow early diagnosis of lung cancer in patients with chronic obstructive pulmonary disease. PLoS One 2014; **9**:e111597.

27. 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**:245-55.

28. Bostrom PJ, Bjartell AS, Catto JW*, et al.* Genomic Predictors of Outcome in Prostate Cancer. Eur Urol 2015; **68**:1033-44.

29. Boyd LK, Mao X, Lu YJ. The complexity of prostate cancer: genomic alterations and heterogeneity. Nat Rev Urol 2012; **9**:652-64.

30. Peng Z, Wang CX, Fang EH*, et al.* Role of epithelial-mesenchymal transition in gastric cancer initiation and progression. World J Gastroenterol 2014; **20**:5403-10.

365

Table 1. Summary of CTC count in 155 treatment-naïve prostate cancer patients by risk groups

	Spearman's ϱ with PSA (*p*)	GS = 3+3 (n = 64)	GS = 3+4 (n = 51)	GS = 4+3 (n = 22)	GS ≥ 4+4 (n = 18)	*p* (p_{adj}[a])	Low-risk (n = 40)	Intermediate-risk (n = 78)	High-risk (n = 37)	*p* (p_{adj}[a])	FD (n = 63)	csPCa (n = 92)	*p* (p_{adj}[a])
Age, y						0.0078				0.0021			0.0076
Median (IQR)		61 (56-68)	66 (56-71)	63.5 (57-71)	69 (62-76.5)		60 (55-67)	65 (56-71)	68 (61.5-73)		61 (55-68)	66 (58-72)	
PSA, ng/mL						0.0001				0.0001			0.0001
Median (IQR)		6.7 (5-9.9)	8.3 (5.6-12)	12 (7.7-18.3)	18.8 (8.9-26)		5.5 (4.7-7.4)	9.1 (6.2-12)	17.6 (8.1-26)		6.5 (5-9.0)	10.5 (7.0-17.5)	
mpMRI, n (%)						0.0001				0.0001			0.0001
1,2		22 (34)	0 (0)	0 (0)	0 (0)		16 (40)	6 (8)	0 (0)		21 (33)	1 (1)	
3,4,5		21 (33)	43 (84)	17 (77)	12 (67)		10 (25)	60 (77)	23 (62)		23 (37)	70 (76)	
n/a		21 (33)	8 (16)	5 (23)	6 (33)		14 (35)	12 (15)	14 (38)		19 (30)	21 (23)	
Epithelial CTC	0.15 (0.07)					0.0425 (0.17)				0.11 (0.44)			0.0066 (0.0264)
Median (IQR) (% detected)		0 (0-0) (14%)	0 (0-1) (29.4%)	0 (0-0.5) (23%)	0 (0-2) (44%)		0 (0-0) (15%)	0 (0-0) (23%)	0 (0 -1) (35%)		0 (0-0) (13%)	0 (0-1) (32%)	
EMTing CTC	0.24 (0.0029)					0.0155 (0.06)				0.0034 (0.0136)			0.0025 (0.01)
Median (IQR) (%)		0 (0-0) (6%)	0 (0-0) (12%)	0 (0-0) (18%)	0 (0-1.3) (67%)		0 (0-0) (2.5%)	0 (0-0) (12%)	0 (0-1) (27%)		0 (0-0) (3%)	0 (0-0) (20%)	
mesenchymal CTC	0.11 (0.17)					0.0608 (0.25)				0.0040 (0.016)			0.0096 (0.0384)
Median (IQR) (%)		1 (0-2) (61%)	1 (0-4) (66%)	3 (0-10.5) (68%)	3 (0.75-7) (94%)		1 (0-2) (55%)	2 (0-4.3) (64%)	4 (0-7.5) (73%)		1 (0-2) (57%)	2 (0-5) (68%)	
CTC-score, n (%)						0.0003[b] (0.0012)				<0.0001[b] (<0.0001)			<0.0001[b] (<0.0001)
Negative		42 (66)	19 (37)	5 (23)	5 (28)		30 (75)	33 (42)	8 (22)		44 (70)	27 (29)	
Positive		22 (34)	32 (63)	17 (77)	13 (72)		10 (25)	45 (58)	29 (78)		19 (30)	65 (71)	

[a] *p* value adjusted for multiple testing using Bonferroni correction method; [b] Fisher's exact test.

PSA: prostate specific antigen; GS: Gleason score; n: number; FD: favorable disease; csPCa: clinically significant prostate cancer; IQR: interquartile range; mpMRI: Multi-Parametric magnetic resonance imaging; n/a: data not available; CTC: circulating tumor cell; EMTing: during epithelial-mesenchymal transition.

Table 2. Summary of CTC count in 98 pre-biopsy patients by biopsy results

	Benign biopsy (n = 33)	Malignant biopsy (n = 65)	*p* (p_{adj}[a])	FD (n = 22)	csPCa (n = 43)	*p* (p_{adj}[a])	FD+Benign biopsy (n=55)	csPCa (n=43)	*p* (p_{adj}[a])
Age, y			0.06			0.021			0.0097
Median (IQR)	65 (56-69)	65 (59.5-70)		63 (56-66)	68 (63-71)		63 (57-68)	68 (63-71)	
PSA, ng/mL			0.0173			0.0017			0.0001
Median (IQR)	6.5 (5.2-10.2)	9.3 (6.4-17)		7.2 (6.0-9.4)	11 (7.0-23)		7.2 (5.4-10)	11 (7.0-23)	
Abnormal PSA, n (%)			1.0[b]			0.0108[b]			0.0333
> 4 ng/mL	31 (94)	61(94)		18 (82)	43 (100)		49 (89)	43 (100)	
≤ 4 ng/mL	2 (6)	4 (6)		4 (18)	0 (0)		6 (11)	0 (0)	
mpMRI, n (%)			0.52			0.0002			0.0017
1,2	7 (21)	9 (14)		8 (36)	1 (2)		15 (27)	1 (2)	
3,4,5	25 (76)	46 (71)		11 (50)	35 (82)		36 (66)	35 (82)	
n/a	1 (3)	10 (15)		3 (14)	7 (16)		4 (7)	7 (16)	
Epithelial CTC			0.0146 (0.06)			0.0147 (0.06)			0.0002 (0.0008)
Median (IQR)(%)	0 (0-0)**(3%)**	0 (0-0)**(22%)**		0 (0-0)**(5%)**	0 (0-1)(30%)		0 (0-0)(4%)	0 (0-1)(30%)	
EMTing CTC			0.0181 (0.07)			0.0806 (0.32)			0.0019 (0.0076)
Median (IQR)(%)	0 (0-0)**(0%)**	0 (0-0)**(15%)**		0 (0-0)(5%)	0 (0-0)(21%)		0 (0-0)(2%)	0 (0-0)(21%)	
Mesenchymal CTC			0.0022 (0.0088)			0.0105 (0.042)			0.0001 (0.0004)
Median (IQR)(%)	0 (0-1.5)**(36%)**	2 (0-6)**(63%)**		0 (0-2.25)(45%)	3 (0-7)(72%)		0 (0-2)(40%)	3 (0-7)(72%)	
CTC-score, n (%)			<0.0001[b] (<0.0001)			<0.0001[b] (0.0002)			<0.0001[b] (<0.0001)
Negative	30 (91)	27 (41.5)		17 (77)	10 (23)		47 (85)	10 (23)	
Positive	3 (9)	38 (58.5)		5 (23)	33 (77)		8 (15)	33 (77)	

[a] *p* value adjusted for multiple test; [b] Fisher's exact test.

N: number; FD: favorable disease; csPCa: clinically significant prostate cancer; IQR: interquartile range; PSA: prostate specific antigen; mpMRI: Multi-Parametric magnetic resonance imaging; n/a: data not available; CTC: circulating tumor cell; EMTing: during epithelial-mesenchymal transition.

Table 3. Threshold cycle of candidate genes in PBMC and regression coefficients of genes in 12-gene panel

Genes in 12-gene panel	C_T in PBMC, median (range)	Regression coefficient in panel	Rest genes in test	Ct in PBMC, median (range)	Genes not included in test	Ct in PBMC, median (range)
AOX1	35.2 (34.9-36.1)	0.854	*AR-V7*	undetermined	***CPLX1***	33.7 (33.0-35.1)
ACOX2	34.8 (33.4-36.9)	-1.89	*CDH12*	Undetermined	***COL5A2***	33.0 (32.7-34.1)
EYA4	36.3 (34.5-undetermined)	1.25	*CHGA*	Undetermined	***ACTG2***	33.0 (32.4-33.9)
FAT1	34.8 (34.1-36.5)	0.265	*CSMD3*	undetermined	***WNT5A***	33.3 (32.6-36.0)
FOXA1	34.9 (32.8-36.2)	-0.389	*CYP3A5*	Undetermined	***FRMD6***	32.4 (32.3-33.1)
GRHL2	Undetermined	0.934	*LCE2B*	undetermined	***SYP***	32.3 (31.8-32.7)
HOXB13	36.1 (35.8-36.5)	-0.146	*MSMB*	Undetermined	***AR***	31.8 (29.9-32.6)
KLK2	35.8(35.4-37.2)	0.71	*PART1*	Undetermined	***CDH1***	31.5 (30.2-33.1)
MNX1	35.6 (34.6-37.1)	-7.8	*ROBO2*	undetermined		
FOLH1(PSMA)	36.6 (35.4-37.2)	0.078	*TMPRSS2:ERG*	undetermined		
RAB3B	34.5 (34.0-36.5)	0.693	*KLK3 (PSA)*	37.3 (37.2-37.9)		
SRD5A2	Undetermined	-16.708	*TWIST2*	36.3 (35.3-37.8)		
			SPOCK3	35.9 (35.0-36.4)		
			FAM107A	35.3(35.0-undetermined)		
			HSPB8	37.0 (35.6-undetermined)		
			PCDH18	34.7 (32.2-37.1)		
			PCA3	34.5 (32.7-35.0)		
			TBX3	33.9 (30.8-37.1)		

PBMC: peripheral blood mononuclear cell.
Genes in bold were those not selected due to relative lower Ct value.

Table 4. Clinical implications of CTC enumeration and gene expression in 98 pre-biopsy patients

	Benign biopsies avoided (n = 33) (%)	prostate cancers missed (n = 65) (%)	PPV	NPV	FD+benign biopsy diagnosis avoided (n = 55) (%)	csPCa missed (n = 43) (%)	PPV	NPV
PSA > 4 ng/mL	2 (6)	4 (6)	66%	33%	6 (11)	0 (0)	47%	100%
CTC-score (positive)	30 (91)	27 (42)	93%	53%	47 (85)	10 (23)	80%	82%
CRS-PC ≥ 2.87	29 (88)	29 (45)	90%	50%	48 (87)	10 (23)	83%	83%
	n = 28	n = 59			n = 47	n = 40		
CRS-PCG ≥ 3.154	25 (89)	25 (38)	92%	50%	43 (91)	7 (18)	89%	86%
CRS-PCG ≥ 1.072	-	-	-	-	11 (23)	0 (0)	53%	100%
	n = 32	n = 55			n = 47	n = 40		
MRI positive (likert ≥ 3)	7 (22)	9 (16)	65%	44%	15 (27)	1 (3)	49%	94%
	n = 28	n = 50			n = 45	n = 33		
CRS-PCGM ≥ 7.327	25 (89)	20 (40)	91%	56%	40 (89)	5 (15)	85%	89%
CRS-PCGM ≥ 4.582	-	-	-	-	19 (42)	0 (0)	56%	100%

N: number; PPV: positive predictive value; NPV: negative predictive value; FD: favorable disease; csPCa: clinically significant prostate cancer; CRS: combined risk score; CRS-PC: combining PSA and CTC-score; CRS-PCG: combining PSA, CTC-score, and 12-gene panel score; CRS-PCGM: combining MRI, PSA, CTC-score and 12-gene panel score.

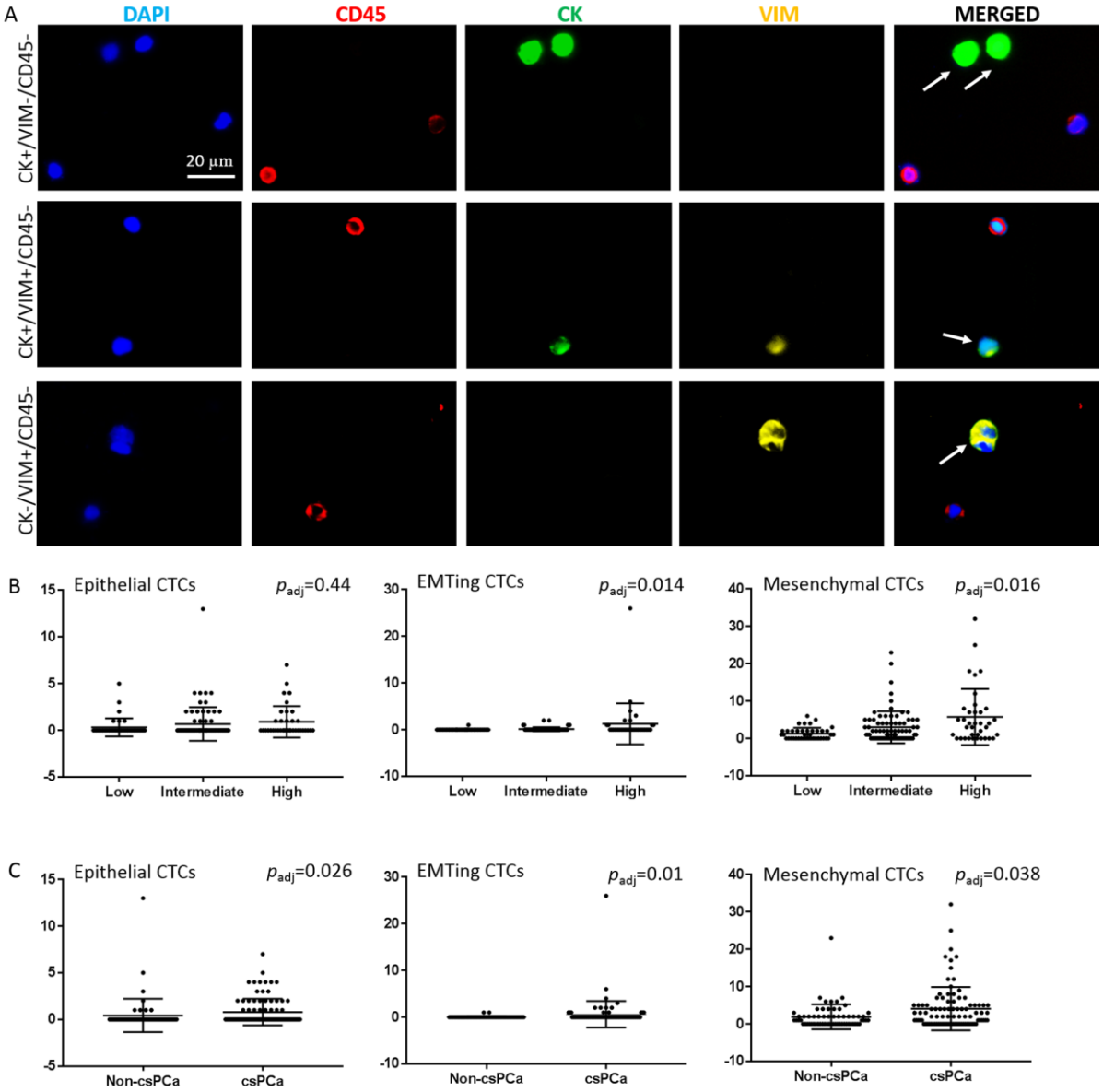
Figure legend

Figure 1. Representative CTC images and the distribution of subtypes of CTCs in PCa patient groups with different progression risk. (A) Representative CTC images identified by immunofluorescence. (B) The distribution of epithelial, EMTing and mesenchymal CTCs in patient groups with low, intermedium and high progression risk PCa. (C) The distribution of epithelial, EMTing and mesenchymal CTCs in patient groups with favorable cancer and csPCa. In B and C, data are expressed as mean (middle horizontal bar) ± SD (top and bottoms). X-axis: Gleason score groups; Y-axis: CTC numbers in each patient.

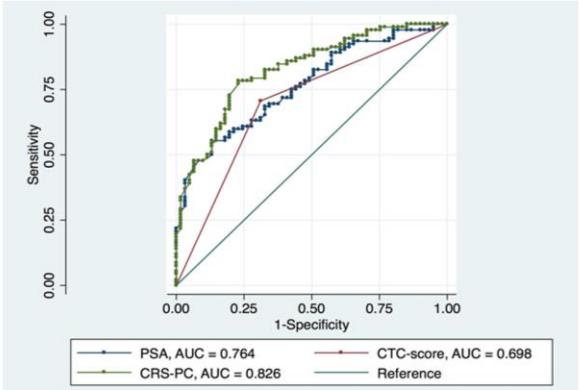
Figure 2. AUCs of CTCs and in combinations with other parameters for csPCa prediction in treatment-naïve prostate cancer and pre-biopsy patients. AUCs in predicting csPCa in 155 localized PCa patients (A), 98 pre-biopsy patients (B), 87 patients with MRI, PSA and CTC-score data(C), 184 patients with CTC gene expression data(D), 87 pre-biopsy patients(E) and the 78 samples with MRI, PSA, CTC-score and gene expression data (F). CRS-PC: PSA combined with CTC; CRS-PM: PSA combined with MRI likert; CRS-MC: MRI likert combined with CTC-score; CRS-PMC: PSA combined with MRI likert and CTC-score; CRS-PCG: PSA combined with CTC count and 12-gene panel score; CRS-PCGM: MRI combined with PSA, CTC-score and 12-gene panel score.

Key of Definitions for Abbreviations

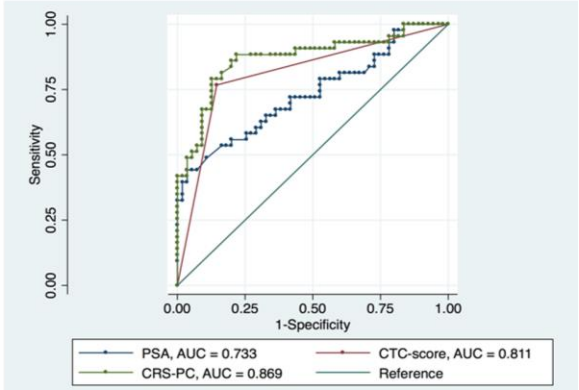
AUC	Area under the ROC curve
CRS	combined risk score
csPCa	clinically significant prostate cancer
CTC	circulating tumor cell
EMT	epithelial-mesenchymal transition
GS	Gleason score
IQR	interquartile range
mpMRI	Multi-Parametric MRI
PBMC	peripheral blood mononuclear cells
PCa	prostate cancer
QRT-PCR	quantitative RT-PCR
ROC	receiver operating characteristic


A
DAPI
CD45
CK
VIM
MERGED
CK+/VIM-/CD45-
CK+/VIM+/CD45-
CK-/VIM+/CD45-
20 µm
B
Epithelial CTCs
p_{adj}=0.44
EMTing CTCs
p_{adj}=0.014
Mesenchymal CTCs
p_{adj}=0.016
Low
Intermediate
High
C
Epithelial CTCs
p_{adj}=0.026
EMTing CTCs
p_{adj}=0.01
Mesenchymal CTCs
p_{adj}=0.038
Non-csPCa
csPCa

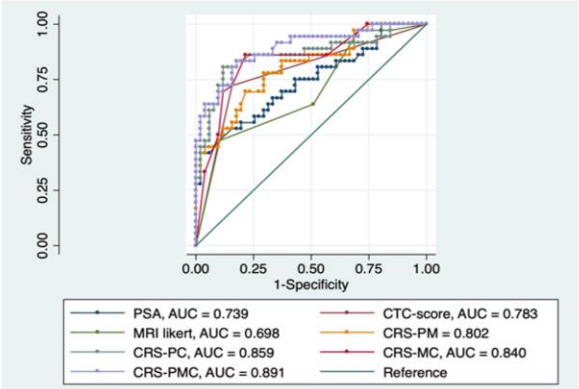
A


Sensitivity
0.00
0.25
0.50
0.75
1.00
1-Specificity
PSA, AUC = 0.764
CTC-score, AUC = 0.698
CRS-PC, AUC = 0.826
Reference

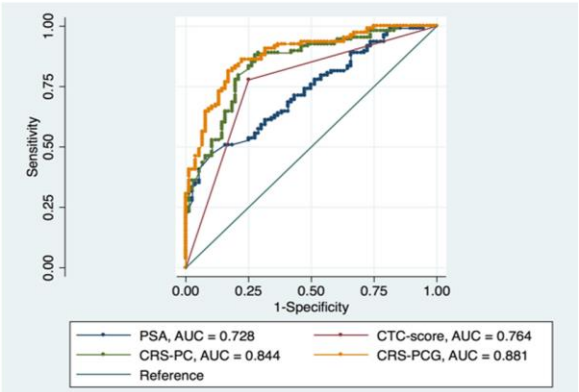
B


Sensitivity
0.00
0.25
0.50
0.75
1.00
1-Specificity
PSA, AUC = 0.733
CTC-score, AUC = 0.811
CRS-PC, AUC = 0.869
Reference

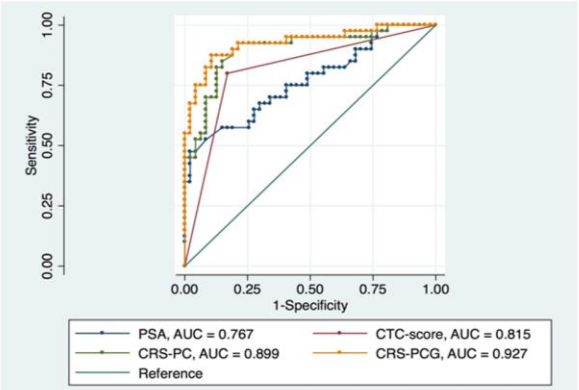
C


Sensitivity
0.00
0.25
0.50
0.75
1.00
1-Specificity
PSA, AUC = 0.739
CTC-score, AUC = 0.783
MRI likert, AUC = 0.698
CRS-PM = 0.802
CRS-PC, AUC = 0.859
CRS-MC, AUC = 0.840
CRS-PMC, AUC = 0.891
Reference

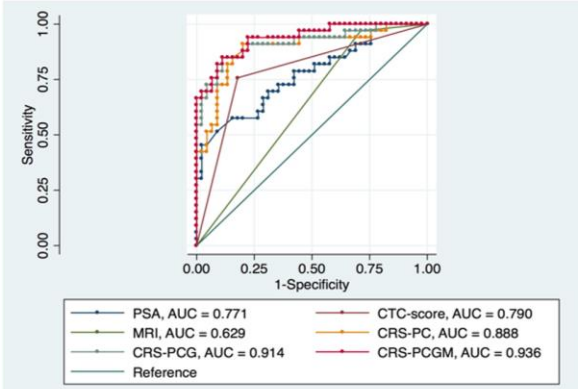
D


Sensitivity
0.00
0.25
0.50
0.75
1.00
1-Specificity
PSA, AUC = 0.728
CTC-score, AUC = 0.764
CRS-PC, AUC = 0.844
CRS-PCG, AUC = 0.881
Reference

E


Sensitivity
0.00
0.25
0.50
0.75
1.00
1-Specificity
PSA, AUC = 0.767
CTC-score, AUC = 0.815
CRS-PC, AUC = 0.899
CRS-PCG, AUC = 0.927
Reference

F


Sensitivity
0.00
0.25
0.50
0.75
1.00
1-Specificity
PSA, AUC = 0.771
CTC-score, AUC = 0.790
MRI, AUC = 0.629
CRS-PC, AUC = 0.888
CRS-PCG, AUC = 0.914
CRS-PCGM, AUC = 0.936
Reference