Combined microfluidic isolation and immunofluorescence staining of circulating tumour cells for the assessment of Androgen Receptor expression in metastatic prostate cancer blood samples.

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Introduction

Most diagnosed cases of metastatic Prostate Cancer (mPCa) are classified as Androgen dependent[1]. Measurement of Androgen receptor (AR) expression can therefore be integral to determining treatment response of mPCa patients. AR activity is typically measured indirectly through downstream biomarkers such as prostate-specific antigen[2].

The ANGLE Portrait® AR assay allows direct interrogation of AR expression without necessitating solid tissue biopsy. By combining identification and phenotyping of circulating tumour cells (CTCs), isolated through the epitope-independent Parsortix® instrument, with assessment of AR expression and intracellular localisation, this workflow may facilitate minimally invasive longitudinal monitoring of mPCa patients throughout treatment.

NB. ***Following abstract submission, assay optimization identified an AR antibody clone with an improved dynamic range in patient samples. Subsequent cohorts were processed using this updated workflow. As a result, the data presented in this poster differs from that in the original abstract submission.***

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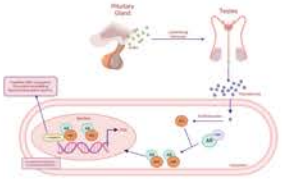

Figure 1. Androgen signaling pathway. Androgens regulate cell cycle progression and differentiation. Dysregulation of AR expression in mPCa is associated with tumour progression[3] (Image adapted from He, Y., *et al* (2022))[4]

Workflow

- For analytical investigation, peripheral blood from Healthy Volunteers (HVs) was drawn into Streck Cell-Free DNA Blood Collection tubes (BCTs) (7.5mL per BCT), spiked with LNCaP cells pretreated with ascending concentrations of an AR inhibitor, or untreated PC-3 cells as an AR negative control. These samples were used to assess sensitivity and specificity of the assay and establish percentage AR positivity of each condition.
- For clinical investigation, 7.5mL of peripheral blood was drawn into Streck BCT from 20 castration-resistant mPCa patients (unspiked). All patients included in the study had stage IV disease and had failed at least one line of AR pathway inhibitor treatment (e.g. Abiraterone, Enzalutamide etc).
- All samples were processed as per the workflow described in Figure 2 below.

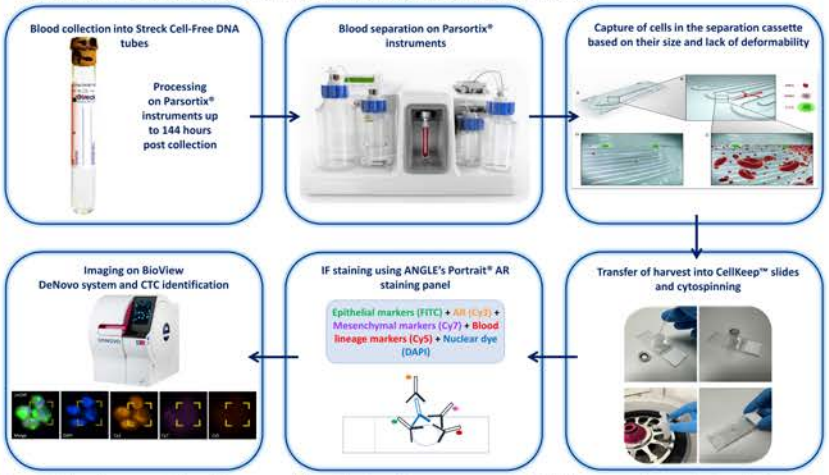

Figure 2. Schematic representation of the assay workflow. Blood samples were processed at 72-144 hours post draw on the Parsortix® instrument (RUO), a microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability. Harvests were cytocentrifuged onto ANGLE CellKeep™ slides then stained using ANGLE's Portrait® AR IF assay, an antibody panel comprising a nuclear dye (Hoechst), antibodies against epithelial markers (FITC), AR (Cy3), mesenchymal markers (Cy7), and blood lineage markers (Cy5), including antigens expressed by blood cells (lymphocytes, macrophages, granulocytes, monocytes, fibroblasts, and cells of megakaryoblastic potential). Stained slides were imaged using BioView DeNovo system, a platform equipped with artificial intelligence for automated imaging, CTC candidate identification and reporting.

Analytical results

- A statistically significant negative correlation ($p<0.0001$) was observed between AR inhibitor treatment concentration and AR expression detected based on mean fluorescence intensity (MFI) in the Cy3 channel (**Figure 3A**). Decrease in AR signal correlated with results observed in Western blot analysis (not shown).
- 80.7% of vehicle-treated LNCaP cells were positive for AR, whilst in LNCaP treated with 10nM or 1000nM inhibitor concentration AR positivity was 64.3% and 19.6%, respectively (**Figures 3B, 3D**).
- The dynamic range of mean signal intensity between vehicle-treated LNCaP and PC-3 was 3.9.
- Comparable MFI results between conditions in each channel show that inhibitor treatment did not impact expression of CTC phenotyping markers; LNCaP cells expected to be epithelial (FITC+,CY7-,CY5-) (**Figure 3C**).

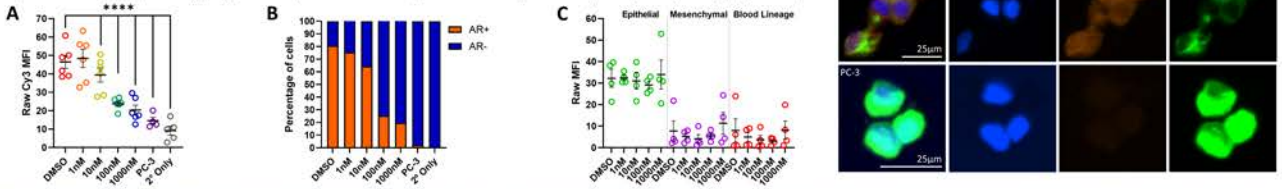

Figure 3. Analytical Performance Data. (A) Dot plot showing mean fluorescence intensity of AR in the Cy3 channel for LNCaP cells at each inhibitor treatment concentration, PC-3 (AR- control cell line), and secondary antibody only (autofluorescence control). Error bars represent Mean ±1 SEM (**** ps0.00001; 2-way ANOVA with Dunnet test). **(B)** The percentage of cells showing positive AR signal for each condition using a CY3 MFI threshold of 27. **(C)** Dot plot showing mean fluorescence intensity in the FITC, Cy5, and Cy7 channels (±1 SEM) for LNCaP cells at all inhibitor concentrations. **(D)** Representative images of spiked cells (Top= LNCaP (DMSO); Middle= LNCaP (1000nM); Bottom= PC-3) after IF staining with the Portrait AR assay. From left to right: Merge, DNA in blue (DAPI), Androgen receptor in orange (Cy3), epithelial markers in green (FITC), mesenchymal markers in purple (Cy7), blood lineage markers in white (Cy5). 10X magnification; Scale bar = 25 µm.

Patients' results

- ≥1 CTCs were identified in 10 (50%) patients, with a median of 46 and mean of 68 CTCs identified per patient (**Figures 4A, 4B**).
- CTC clusters harvested by the Parsortix® instrument were observed in 80% of the CTC+ patients. Cluster size ranged from 2-63 CTCs per cluster, and the number of clusters per patient ranged from 2-37 (**Figures 4A, 4C**).
- Of the CTC+ patients, 100% presented AR+ CTCs. The number of AR+ CTCs detected ranged from 1 – 116, with a median of 29 and mean of 37 AR+ CTCs identified per AR+ patient. One patient showed CTCs with cytoplasmic AR positivity only, and three showed nuclear positivity only. The remaining 6/10 showed a mix of nuclear and cytoplasmic AR positivity across detected CTCs (**Figures 4D, 4E, 4F, 4G**).
- 57% (339/592) of detected mesenchymal CTCs were AR+, compared to 29% (34/85) of EMT CTCs; no epithelial CTCs were detected in any donor (**Figure 4A**).

A	mPCa
N donors	20
N donors with ≥1 CTC (CTC+)	10 (50%)
N donors with ≥5 CTC	8 (40%)
Median CTCs (CTC+ donors)	46
Mean CTCs (CTC+ donors)	68
CTCs per CTC+ donor (range)	1-226
N CTC+ donors with ≥1 CTC Cluster	8 (80%)
N CTCs per Cluster (range)	2-63
N Clusters per donor (range)	2-37
Total CTCs	677
Mesenchymal CTCs	592 (88%)
EMT CTCs	85 (13%)
Epithelial CTCs	0
CTC+ donors with ≥1 AR+ CTC (AR+)	10 (100%)
CTC+ donors with ≥5 AR+ CTC	7 (70%)
AR+ Mesenchymal CTCs	339/592 (57%)
AR+ EMT CTCs	29/85 (34%)
AR+ Epithelial CTCs	0
Median AR+ cells (of AR+ donors)	29
Mean AR+ cells (of AR+ donors)	37
AR+ CTCs per AR+ donor (range)	1-116

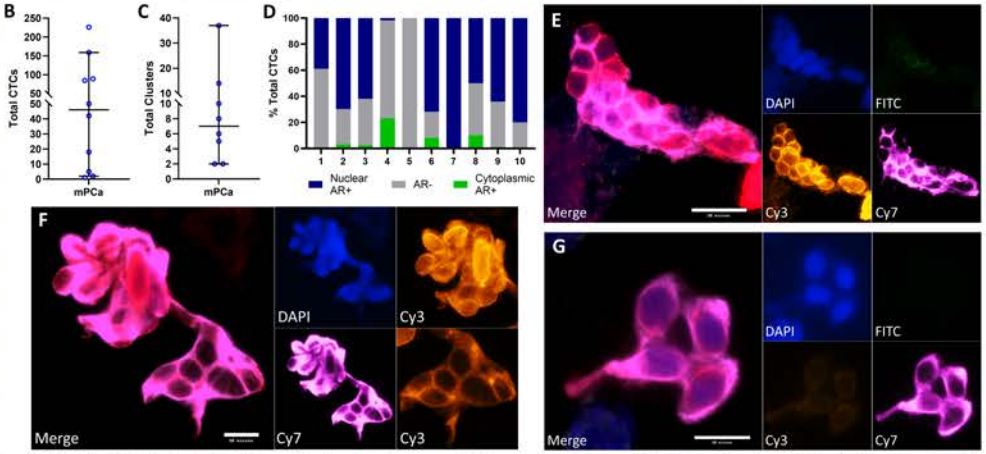

Figure 4. CTC positivity and AR expression status of mPCa patients' samples. (A) Table showing descriptive statistics for all samples processed. **(B)** Dot plot showing mean ±1 SEM of the total number of CTCs isolated in mPCa patients. **(C)** Dot plot showing mean ±1 SEM of the total number of CTC clusters isolated in mPCa patients. **(D)** Percentage distribution of AR positivity status for all CTCs detected in each CTC+ patient. **(E)** Representative image of an EMT CTC cluster showing nuclear AR positivity (40X magnification; Scale bar = 20 µm) **(F)** Image of mesenchymal CTC cluster showing nuclear (Cy3:Top), and cytoplasmic (Cy3:Bottom) AR positivity (40X magnification; Scale bar = 10 µm). **(G)** Representative image of an AR negative mesenchymal CTC cluster (40X magnification; Scale bar = 10 µm) For all images: DNA in blue (DAPI), epithelial markers in green (FITC), AR in orange (red in merge) (Cy3), mesenchymal markers in purple (Cy7).

References

1. Aurilio, G. *et al.* (2020) 'Androgen receptor signalling pathway in prostate cancer: From genetics to clinical applications', *Cells*, 9(12), p. 2653. doi:10.3390/cells9122653.
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3. McEwan, I.J. and Brinkmann, A.O. (2021) *Androgen physiology: Receptor and metabolic disorders*, *Endotext*. Available at: https://www.ncbi.nlm.nih.gov/books/NBK279028/.
4. He, Y. et al. (2022). Targeting signaling pathways in prostate cancer: mechanisms and clinical trials. Signal Transduction and Targeted Therapy, 7(1). doi:10.1038/s41392-022-01042-7

Conclusions

The Portrait AR assay was able to accurately assess AR expression and localisation in CTCs isolated from mPCa patients. This demonstrates that combining epitope-independent CTC isolation with immunofluorescence staining for detection of AR in CTCs has the capability to enable dynamic, easy monitoring of AR status, with potential utility for longitudinal monitoring of patient outcomes.