Monitoring of DNA Damage Response Biomarkers using Circulating Tumour Cells captured with ANGLE's Parsortix® instrument from Ovarian, Prostate, and Breast cancer patients

Amina Mezni [1], Rachel Downes [1], Morgan Spode [1], Elisha Duhig [1], Aarabhi Varatharajah [1], Mariacristina Ciccioli [1], Anne-Sophie Pailhes-Jimenez [1]

[1] ANGLE Europe Limited, 10 Nugent Road, Surrey Research Park, Guildford, Surrey GU2 7AF United Kingdom

8th Annual DDR Inhibitors Summit — Making Strides in Targeting PARP, ATR, ATM, WEE1 & POLθ

Introduction

With increasing research and development into new DNA damage response (DDR) inhibitors, along with new combination therapies, it is critical to be able to rapidly and repeatedly assess a patient's therapy response. Monitoring of various DDR biomarkers in cancer cells can be valuable when evaluating effectiveness of DDR-targeting therapies. Assessing DDR biomarkers in solid tissue biopsies, for evaluating cancer treatment response, is generally not feasible as obtaining solid biopsies is invasive, challenging and often non-repeatable. ANGLE has developed a Research Use Only (RUO) workflow to evaluate DDR biomarker expression in Circulating Tumour Cells (CTCs), enriched from a blood sample (**Figure 1**), offering minimally invasive, repeatable and real-time assessments of treatment response. In this research study, we assessed the performance of ANGLE's immunofluorescence (IF) assays for the identification of epithelial, mesenchymal and transitioning CTCs, harvested using the Parsortix® instrument, and for the determination of DNA Damage status (targeting either pKAP1 or ɣ-H2AX) on the identified CTCs. We also demonstrate the feasibility of developing an CTC-detection IF assay with two customisable channels, allowing for the potential for detection of two DDR markers of interest.

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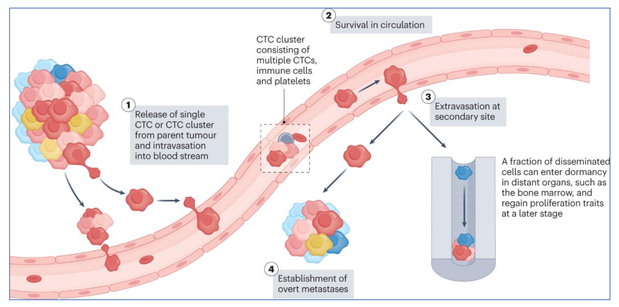
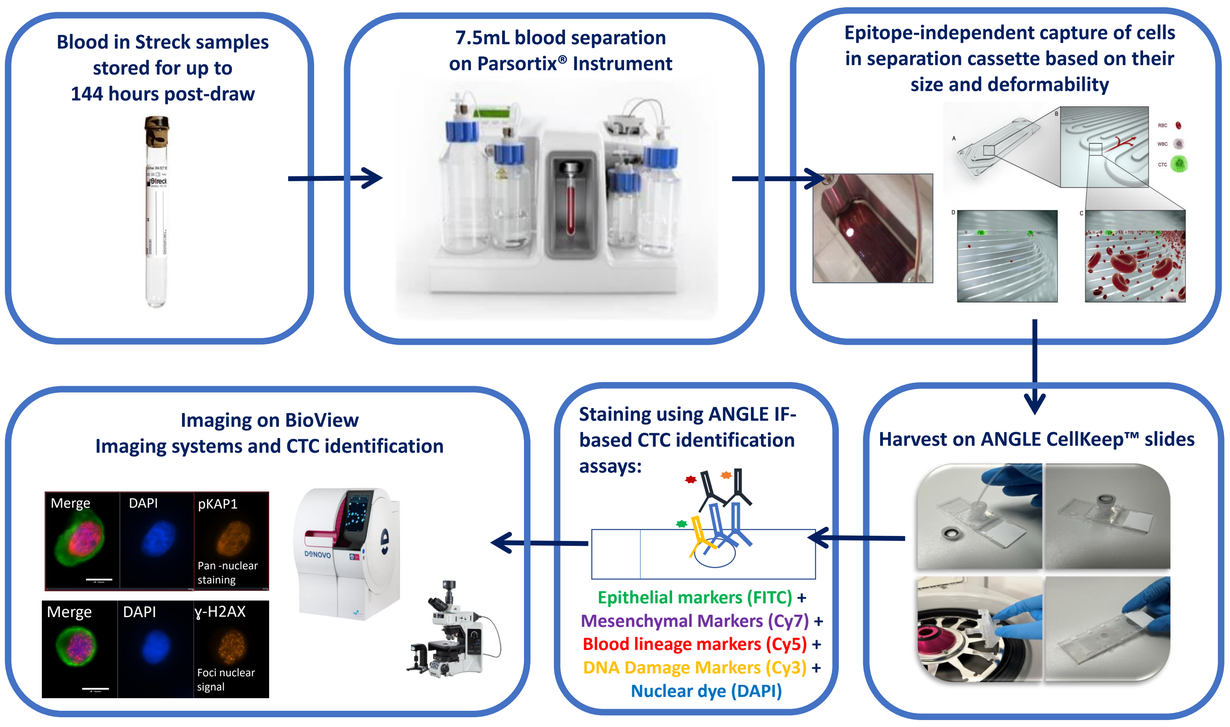


Figure 1. Schematic depicting circulating tumour cell formation. Figure adapted from Lawrence, R et al (2023). Circulating tumour cells for early detection of clinically relevant cancer. Nature Reviews Clinical Oncology.

Workflow

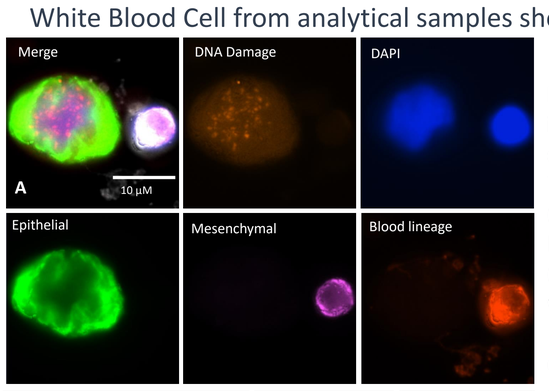


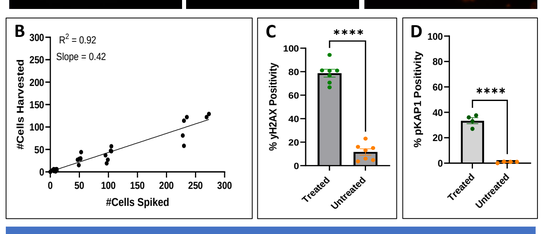
- For analytical verification, blood samples from healthy volunteers were collected into Streck Cell-Free DNA Blood Collection tubes (BCTs) and spiked with DNA Damage-induced H226 or MCF7 cancer cell lines (treated with Etoposide). Samples were processed between 96 and 144 hours post draw, as per workflow detailed in **Figure 2,** and used to assess analytical sensitivity, specificity, and linearity.
- Samples from 17 Breast, 21 Prostate and 16 Ovarian cancer patients, on a variety of treatment programmes, were collected into Streck Cell-Free DNA BCTs and processed following the same workflow (**Figure 2**). Blood was collected for up to six draws per donor, with two tubes collected per draw, and stained using ANGLE's IF-based CTC identification assay combined with DDR marker detection (ɣ-H2AX and pKAP1 assays).

Figure 2. Schematic representation of the assay workflow. In analytical samples, cultured cancer cell lines expressing Epithelial and/or Mesenchymal markers were induced for DNA damage, fixed with 4% Formaldehyde and spiked into healthy volunteer blood samples collected in Streck Cell-Free DNA tubes. For patient-derived samples two tubes of 7.5 mL of peripheral blood were collected into Streck Cell-Free DNA tubes from 17 breast, 21 prostate and 16 ovarian cancer patients for up to six draws per donor. Blood was stored for up to 144 hours from collection before processing. Blood samples were processed on a Parsortix® instrument, an epitope-independent microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability. Harvested CTCs were cytospun onto ANGLE's CellKeep™ slides. Slides were stained using ANGLE's IF-based CTC identification assay combined with DDR markers (ɣ-H2AX and pKAP1). CTCs were defined as epithelial (FITC+, Cy7-, Cy5-, DAPI+), mesenchymal (FITC-, Cy7+, Cy5-, DAPI+), or EMT transitioning (FITC+, Cy7+, Cy5-, DAPI+). CTCs were then investigated for the presence of DNA damage signal. ɣ-H2AX positivity was identified mostly by the presence of distinct nuclear foci and, occasionally, diffuse nuclear signal, while pKAP1 positivity was identified by the presence of a distinct diffuse, pan-nuclear signal. Stained slides were imaged using a BioView Allegro Plus system or the BioView DeNovo system, a platform equipped with a deep learning algorithm for automated imaging, CTC candidate identification and reporting.

Analytical Results

- **Linearity** was established by plotting the number of stained and harvested Etoposide-treated cancer cells against the number of spiked cells. A linear relationship between the number of harvested and stained cells was confirmed, with R^2= 0.92, slope = 0.42 over the range of 0-250 cells (**Figure 3B**) .
- For both ɣ-H2AX and pKAP1 markers, a statistically significant increase (*p <0.0001*) in DNA damage was observed in the positive/treated cell model vs the negative/untreated cell model **(Figure 3C,D**).
- **Analytical Sensitivity** and **Specificity**, referring to the percentage of harvested cells known to express/not express a marker that had a mean fluorescence intensity (MFI) above/below the established thresholds, respectively, were all ≥ 90% in both DDR assays **(Figure 3E).**
- Example images of spiked epithelial cancer cell, with induced DNA damage, and neighbouring White Blood Cell from analytical samples shown below **(Figure 3A**).





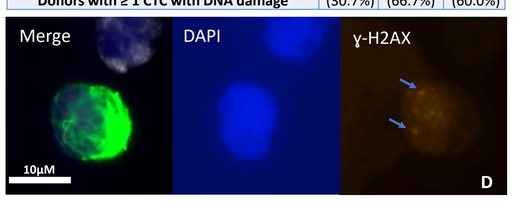
E		Epithelial Markers	Mesenchymal Markers	Blood lineage Markers
IF CTC/DDR staining assay	Analytical Sensitivity	>99%	>90%	>99%
	Analytical Specificity	>94%	>93%	>92%

Figure 3. Analytical performance of the IF CTC identification assay combined with detection of DNA damage (ɣ-H2AX or pKAP1) markers. (A) Representative image of spiked induced MCF7 cell, positive for ɣ-H2AX (FITC+, Cy7-, Cy5-, DAPI+, Cy3+), and white blood cell. Epithelial markers (FITC) in green, Mesenchymal markers (Cy7) in magenta, Blood lineage markers (Cy5) in red or white (in merge), Nuclear dye (DAPI) in blue and DDR markers (Cy3) in orange. **(B)** Dot plot shows the number of cancer cells spiked (x-axis) vs the number of cancer cells harvested (y-axis) across spiking levels for Etoposide treated fixed H226 cells. The trendline equation and R^2 value are included for the first order polynomials. Histograms show the mean ± SEM of the mean percentage of **(C)** ɣ-H2AX positive cancer cells and **(D)** pKAP1 positive cancer cells in respective positive and negative cell models (Unpaired t test, *p**** <0.0001)*. **(E)** Summary table shows the analytical sensitivity (proportion of cells known to express the marker(s) of interest which were positive) and specificity (proportion of cells known to not express the markers of interest which were marker negative in the assay) of both DDR assays.

Results from Patient Samples

- Approximately 76% of Breast, 86% of Prostate and 94% of Ovarian cancer donors showed ≥ 1 CTC, with a CTC range of 1-254 in positive samples (**Figure 4A**).
- DNA damage (either ɣ-H2AX or pKAP1) was identified in all cancer types, in 31%, 67% and 61%, respectively.
- Case studies show the potential clinical benefit of assessing the presence of CTCs and DDR+ CTCs over time.
  - CTCs were present when a breast cancer patient was classed as progressive disease (PD) (**Figure 4B**). A decrease in CTCs was identified once the patient stabilised and was responding to treatment (RTT). DDR+ CTCs were identified ahead of change to RTT.
  - CTC numbers in ovarian cancer patient (**Figure 4C)** increased at the 3rd draw, despite a reported clinical status of RTT, prior to change in clinical status to progressive disease. DDR+ CTCs were observed after receiving platinum treatment. Increase recorded in 6th draw, a few weeks after Paclitaxel treatment.

A	Breast	Prostate	Ovarian
N (Donors)	17	21	16
N Donors with ≥1 CTC	13 (76.4%)	18 (85.7%)	15 (93.8%)
N CTC+ donors with ≥1 CTC Cluster	9 (69.2%)	17 (94.4%)	9 (60.0%)
CTC range	1-52	1-227	1-254
CTC mean	9	23	18
CTC median	6	6	3
Donors with ≥1 Epithelial only CTCs	1 (7.7%)	0 (0.0%)	0 (0.0%)
Donors with ≥1 Epithelial and Mesenchymal CTCs	1 (7.7%)	5 (27.8%)	4 (26.7%)
Donors with ≥1 EMT CTCs	0 (0.0%)	4 (22.2%)	2 (13.3%)
Donors with ≥1 Mesenchymal only CTCs	11 (84.6%)	9 (50%)	9 (60.0%)
Donors with ≥ 1 CTC with DNA damage	4 (30.7%)	12 (66.7%)	9 (60.0%)



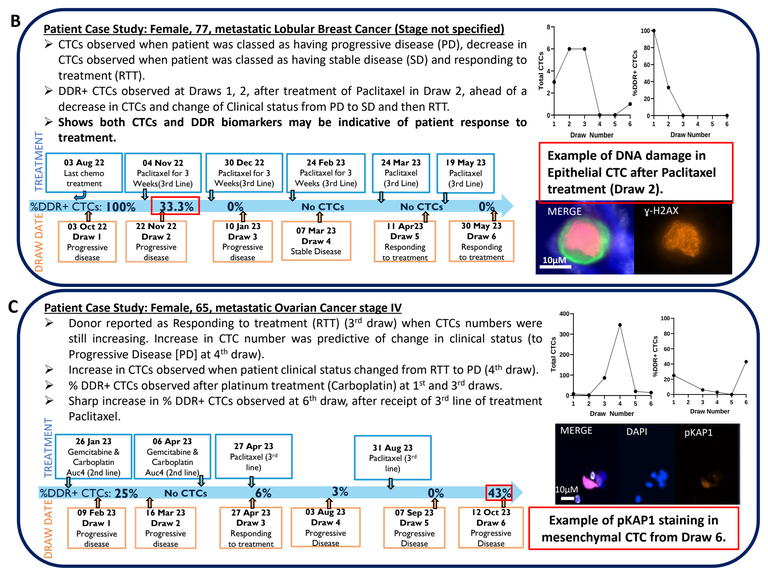


Figure 4. CTC identification and phenotyping in metastatic breast, prostate and ovarian cancer patient samples. **(A)** Table showing number (N) of donors, percentage of CTC+ donors (≥1 CTC), percentage of CTC cluster+ donors (≥1 CTC cluster), mean, median, range of total CTCs and different CTC phenotypes by donor, percentage of CTC+ donors with ≥1 DDR+ CTC. Case study of one **(B)** breast cancer and **(C)** ovarian cancer patient with changes in CTC numbers and DDR+ expression in CTCs identified across draws. **(D)** Representative image an epithelial positive CTC from a prostate cancer patient with ɣ-H2AX foci (indicated by blue arrows) signal (image taken using 60× objective lens). Epithelial markers (FITC) in green, Mesenchymal markers (Cy7) in magenta, Blood lineage markers (Cy5) in white, Nuclear dye (DAPI) in blue and DDR markers (Cy3) in orange.

Ongoing Research

- **Dual DDR biomarker detection**
  - IF assay currently under development that combines both markers for Epithelial and Mesenchymal markers in one channel (FITC), freeing up two channels (Cy7 and Cy3) which allows for the study of two markers of interest **(Figure 5.AB).** Proof-of-concept study demonstrates the feasibility of combining both ɣ-H2AX and RAD51 in the same assay **(Figure 5.C).** Detecting presence of RAD51 in cancer cells may be beneficial for identifying patients likely to respond to PARP-inhibitor treatment
- **Other DNA damage markers**
  - Micronuclei (MN), another biomarker of genomic instability, have been identified with ANGLE's IF CTC identification assays DAPI **(Figure 5.D).**

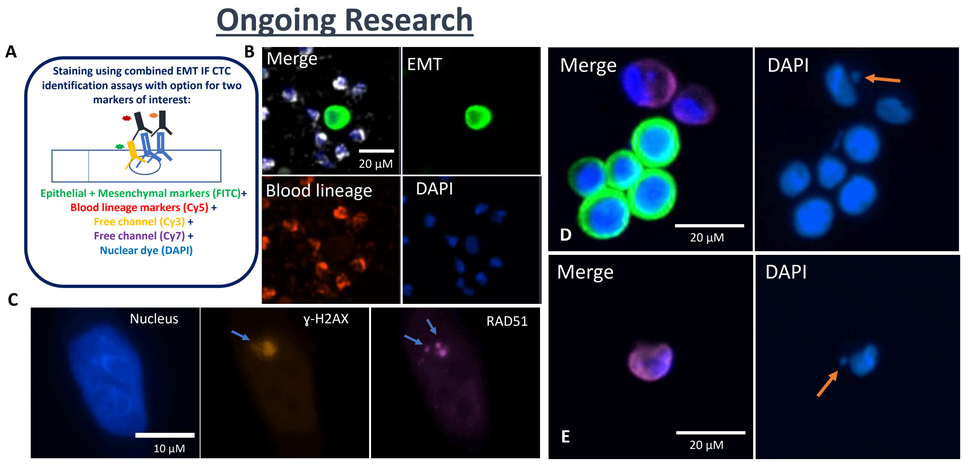


Figure 5. Current assay development research at ANGLE. (A) Diagram showing concept of combined EMT IF CTC staining assay where markers for Epithelial and Mesenchymal are both in FITC (green) channel, allowing for insertion of two biomarkers of interest in Cy7 and Cy3 channel. **(B)** Spiked cancer cell line stained with combined EMT IF CTC assay, Epithelial and mesenchymal (EMT) markers (FITC) in green, Blood lineage markers (Cy5) in red (white in merge), Nuclear dye (DAPI). **(C)** Image of BT549 cell demonstrates feasibility of combining two DDR biomarkers of interest, ɣ-H2AX and RAD51 in free Cy3 (orange) and Cy7 (magenta) channels, respectively. Images show example of Micronuclei (indicated by orange arrows) identified using ANGLE's IF assay workflow in single Hs 578t (Cy7+) cell in **(D),** alongside SKBR3 cells (FITC+) and **(E)** in single Hs578t cell (Cy7+) with IF CTC staining assay with Epithelial markers (FITC) in green, Mesenchymal markers (Cy7) in magenta, Nuclear dye (DAPI) in blue.

Conclusions

- Analytical verification demonstrated that ANGLE's ɣ-H2AX and pKAP1 IF assays produce linear data, with high analytical sensitivity and analytical specificity for epithelial, mesenchymal, blood lineage and DDR markers.
- ANGLE's ɣ-H2AX and pKAP1 assays identified CTCs in breast, prostate and ovarian cancer patient cohorts, with ≥1 CTC observed in 76%, 86% and 94% of patients, respectively. DDR markers were detected in CTCs from all cancer cohorts.
- ANGLE's DDR workflow demonstrated the possibility to monitor number of CTCs, and DNA damage in CTCs over time. Applied in a clinical setting, this workflow could allow for minimally invasive monitoring of treatment response.
- Proof-of-concept studies have demonstrated feasibility of combining markers for EMT into one channel, allowing for two DDR biomarkers to be added in free channels. ANGLE's DDR workflow may also be able to detect structures such as MN.