ANGLE

Investigating PD-L1 status in circulating tumor cells isolated from the blood samples of lung cancer patients

Morgan Spode [1], Chloe Goodwin [1], Aarabhi Varatharajah [1], Aaron Cottingham[1], Elisha Duhig [1], Laura Kaja [1], David Greaves [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

A038

INTRODUCTION

Programmed Death Ligand-1 (PD-L1) is a transmembrane protein which allows cancer cells to evade the host immune response when upregulated **(Figure 1).** Assessment of PD-L1 status in the tumor, as determined by traditional tissue biopsy, can indicate if immunotherapy has the potential to be an effective treatment. However, PD-L1 expression in tumor biopsies may not reflect metastatic sites in their entirety and can become outdated during tumor evolution, as the process cannot be repeated due its invasive nature. Liquid biopsy offers a minimally invasive option for dynamic testing of PD-L1 in Circulating Tumor Cells (CTCs) as patients undergo treatment.

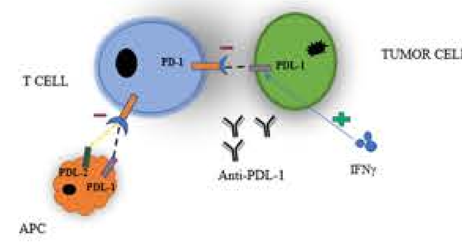


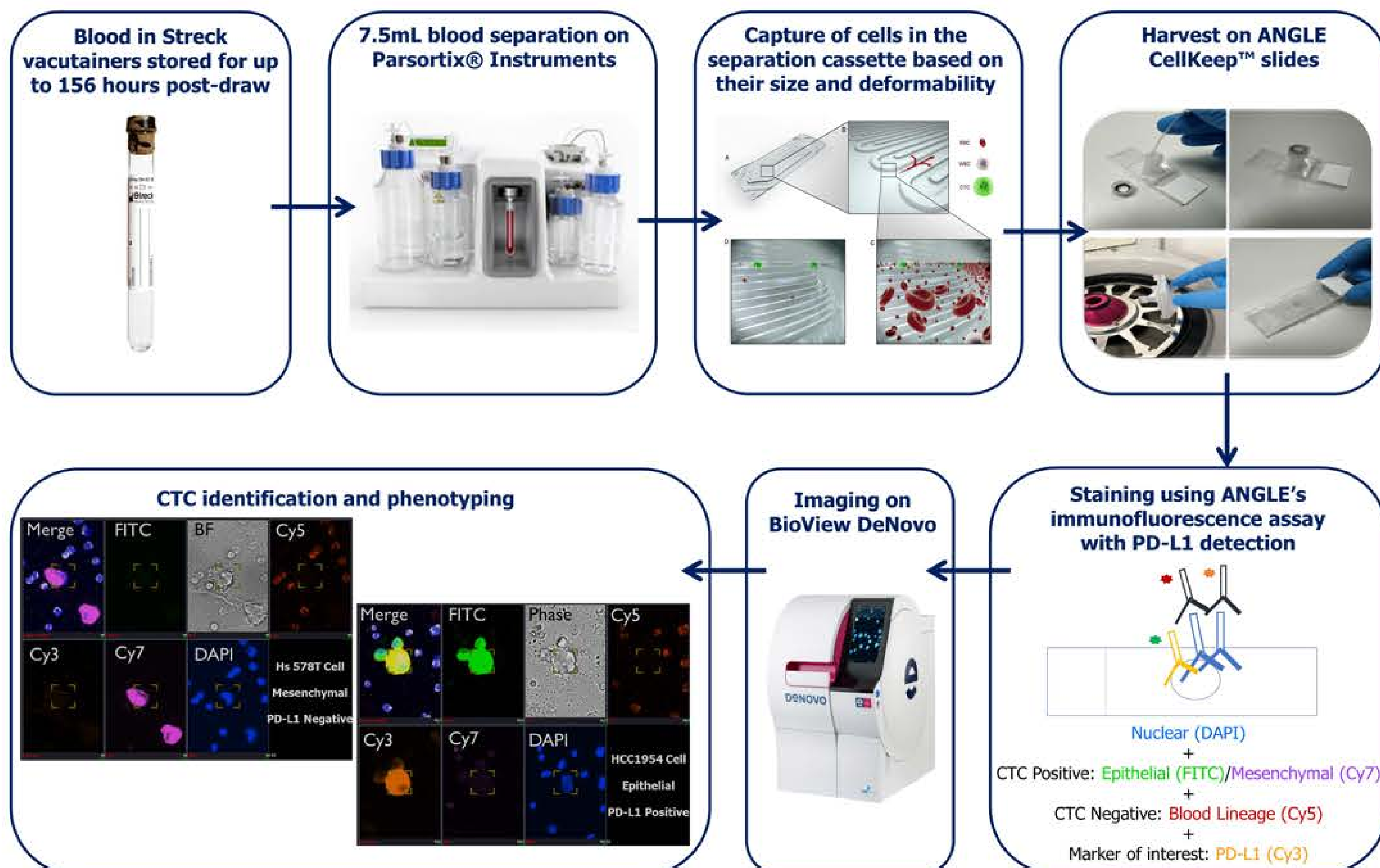
Figure 1. PD-1 and PD-L1 interaction on tumor cells and immune cells inhibits immune response. *Figure taken from Akinleye et al., J. Hematol. Oncol., 2019*

This study assessed the performance of a research use only (RUO) immunofluorescence (IF) assay targeting PD-L1 evolution in epithelial and/or mesenchymal CTCs isolated from patients' blood using Parsortix® microfluidic technology.

WORKFLOW

ANALYTICAL STUDIES: Blood samples from 34 healthy volunteers were collected into Streck Cell-Free DNA Blood Collection tubes (BCTs) and spiked with cancer cell lines. The HCC1954 cell line was used as an epithelial-positive, PD-L1-positive control. The Hs 578T cell line was used as a mesenchymal positive, PD-L1 negative control. Samples were processed between 72–136 hours post draw, as per ANGLE's workflow (**Figure 2**), for assessment of analytical sensitivity, specificity and linearity.

PATIENTS' FEASIBILITY STUDY: Blood samples from 47 lung cancer patients, on a variety of treatment programs, were collected into Streck Cell-Free DNA BCTs and processed per ANGLE's workflow (**Figure 2**). Blood was collected for up to six longitudinal draws per donor, with two tubes collected per draw, each processed between 24–156 hours post draw and stained using ANGLE's IF-based CTC identification assay combined with PD-L1 detection.



For Research Use Only. Not For Use In Diagnostic Procedures.

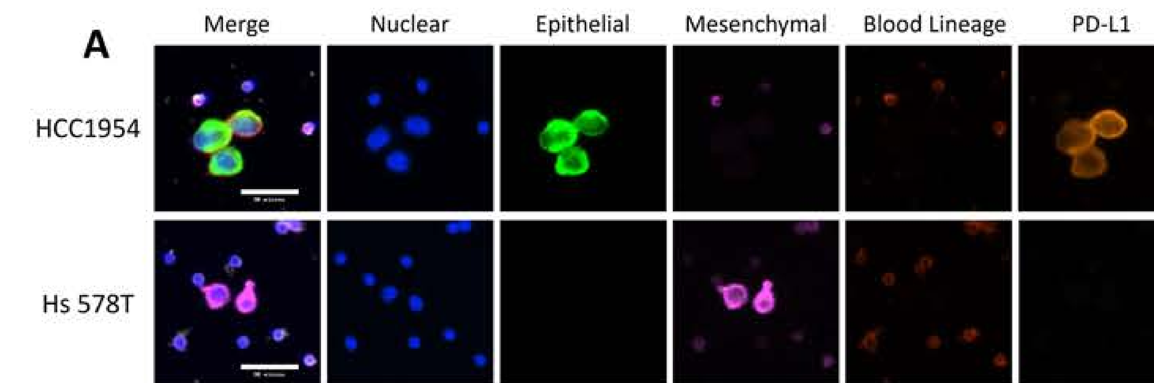
Figure 2. Schematic representation of the assay workflow. *Blood samples were collected from healthy volunteers (analytical study) or lung cancer patients (patients' feasibility study) into Streck Cell-Free DNA BCT for processing between 24–156 hours post draw. Blood samples (7.5 mL) were processed on a Parsortix® instrument, a microfluidic device capable of capturing and harvesting CTCs from bodily fluids based on cell size and lack of deformability. Cell harvests were cytospun onto ANGLE's CellKeep™ slides. Slides were stained using ANGLE's IF-based CTC identification assay combined with PD-L1 detection. Stained slides were imaged using a BioView DeNovo imaging system, a platform equipped with deep learning algorithms for automated imaging, CTC candidate identification, and reporting. For analysis CTCs were defined as epithelial (FITC+, Cy7-, Cy5-, DAPI+), mesenchymal (FITC-, Cy7+, Cy5, DAPI+) or EMT transitioning (FITC+, Cy7+, Cy5-, DAPI+). After identification, CTCs were investigated for the presence of PD-L1 positivity, seen as membranous Cy3+ signal.*

RESULTS: ANALYTICAL STUDIES

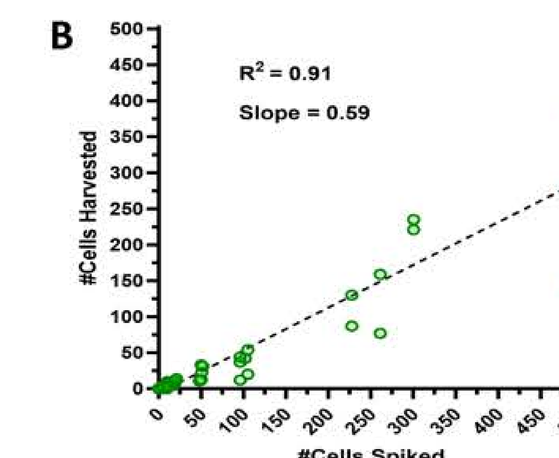
Cultured cancer cell lines were used to establish the assay performance **(Figure 3A)**.

- Linearity: The relationship between the number of harvested, epithelial stained HCC1954 cells (y-axis) and the number of spiked HCC1954 cells (x-axis) per sample, assessed using simple linear regression with a first-order polynomial. Linearity was established over a range of 0–500 cells with R^2 of 0.91 and slope of 0.59 (**Figure 3B**).
- Sensitivity: The percentage of harvested cells expected to express a marker which did express that marker above an established mean fluorescence intensity (MFI) threshold **(Figure 3C & 3D).**
- Specificity: The percentage of harvested cells expected to not express a marker which expressed that marker below an established MFI threshold **(Figure 3C & 3D).**

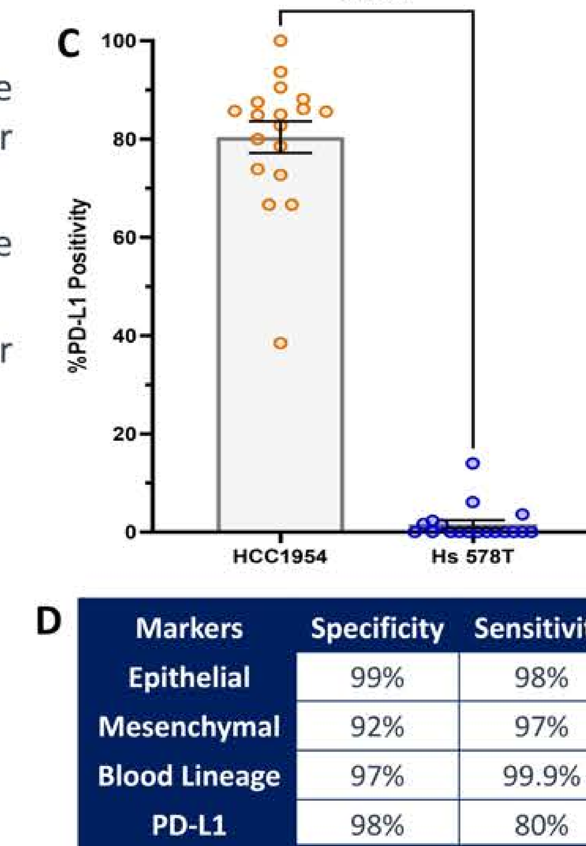
A



B



C



D

Markers	Specificity	Sensitivity
Epithelial	99%	98%
Mesenchymal	92%	97%
Blood Lineage	97%	99.9%
PD-L1	98%	80%

Figure 3. Analytical performance of ANGLE IF assay for CTC identification with PD-L1 detection. (A) *Representative images of HCC1954 cells (**top**) and Hs 578T cells (**bottom**) with healthy volunteer PBMCs. Epithelial markers (FITC) in green, Mesenchymal markers (Cy7) in magenta, blood lineage markers (Cy5) in white (merge) or red, Nuclear dye (DAPI) in blue, and PD-L1 (Cy3) in orange. **(B)** Dot plot shows the number of HCC1954 cells spiked (x-axis) vs the number of epithelial HCC1954 cells harvested (y-axis) for spiking levels of 0–500. Slope and R^2 values for the first order polynomial are displayed. **(C)** Histogram shows the mean ± SEM of the mean percentage of PD-L1 positive cancer cells in respective PD-L1 positive and negative cell models (Unpaired t test, p**** <0.0001) spiked into the blood of nine healthy volunteers as processed by two operators (n=18). **(D)** Summary table shows the overall analytical sensitivity and specificity of the assay.*

RESULTS: PATIENTS' FEASIBILITY STUDY

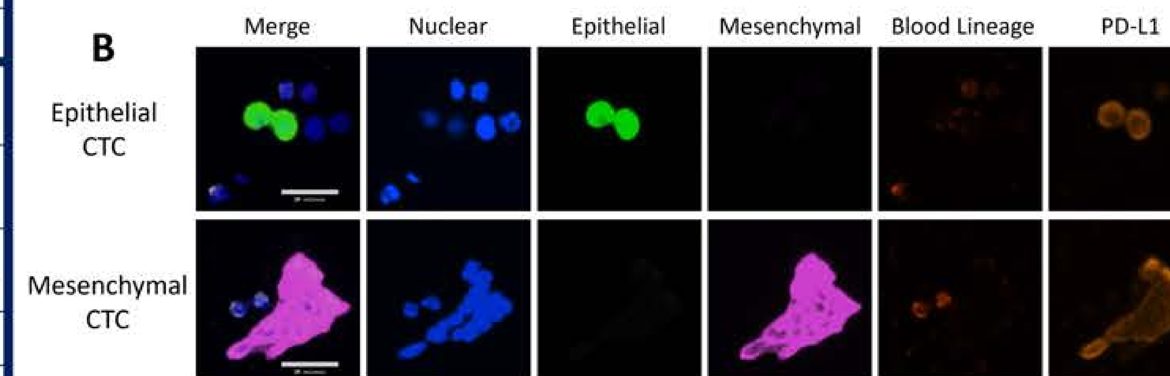
In an ongoing longitudinal study, 47 lung cancer patients provided up to 6 draws, consisting of 1–2 samples per draw. Tissue biopsy was performed prior to enrollment in the study. The PD-L1 tissue status was known in 32 donors (**Figure 4A**).

- CTC Positivity: ≥ 1 CTC was seen in ≥ 1 sample from 91% (29/32) of donors, with a range of 1–498 CTCs visualized in positive samples.
  - ≥ 1 CTC was seen in all draws taken for 52% (15/29) of CTC+ donors, while the remaining 48% (14/29) of CTC+ donors displayed some CTC negative draws, demonstrating the ability to assess dynamic change within a donor over time.
- PD-L1 positivity: ≥ 1 PD-L1+ CTC was seen in 72% (13/18) of donors with a PD-L1 positive tissue biopsy and in 27% (3/11) of donors with a PD-L1 negative tissue biopsy, indicating good concordance but with improved assessment of tumor heterogeneity to offer a more comprehensive, real-time assessment of PD-L1 status.
- Phenotypes: 62% (18/29) of donors presented Mesenchymal CTCs only, 31% (9/29) presented both Mesenchymal and Epithelial CTCs, and 7% (2/29) presented both Mesenchymal and Epithelial-to-Mesenchymal Transition (EMT) CTCs. No donors presented with Epithelial CTCs only.
  - Epithelial and mesenchymal CTCs with PD-L1 positivity were identified in lung cancer patient samples (**Figure 4B**).
  - A case study of a single patient (**Figure 4C**) shows that CTCs were identified in all longitudinal samples, despite the donor being classed as having Stable Disease from the second draw onwards. Expression of PD-L1 in CTCs was variable between draws, demonstrating discordance with the results of the initial tumor biopsy.

A

	PD-L1 Tissue Status	Positive		Negative	
	Immunotherapy	Yes	No	Yes	No
All Donors	Total	15	4	7	6
	Mean #Draws	4	3	4	3
CTC+ Donors	Total	14 (93%)	4 (100%)	6 (86%)	5 (83%)
	PD-L1+ (≥1 CTC)	11 (79%)	2 (50%)	1 (17%)	2 (40%)
	Cluster+ (≥1 Cluster)	14 (100%)	3 (75%)	6 (100%)	5 (100%)
	Range #CTCs	1 – 498	1 – 227	1 – 108	1 – 60
	Mean #CTCs	61	27	12	9
	Median #CTCs	26	5	3	3
Phenotype	Mesenchymal	11 (79%)	1 (25%)	3 (50%)	3 (60%)
	Mesenchymal & Epithelial	2 (14%)	2 (50%)	3 (50%)	2 (40%)
	Mesenchymal & EMT	1 (7%)	1 (25%)	0 (0%)	0 (0%)

B



C

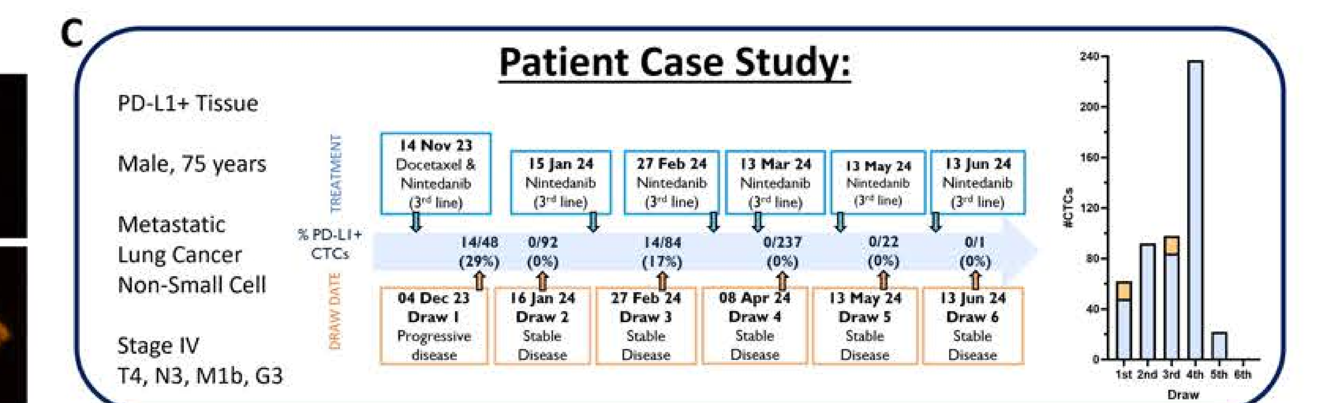


Figure 4. CTC identification and phenotyping in lung cancer patient samples. (A) *Table quantifying the donor data as categorized by the PD-L1 status of the tissue/metastasis and if the donor immunotherapy treatment courses included any immunotherapy. Data includes the total number of donors, and the mean number of draws processed per donor. For all CTC+ donors (≥1 CTC), the data reported includes total number of donors with subsets of PD-L1+ (≥1 PD-L1+ CTC), cluster presence (≥1 cluster of ≥2 CTCs), CTC quantification, and phenotype. **(B)** Representative images from patient samples of a PD-L1 positive epithelial CTC cluster and a PD-L1 positive mesenchymal CTC cluster found near peripheral blood mononuclear cells (images taken using 10x objective lens). Nuclear dye (DAPI) in blue, epithelial markers (FITC) in green, mesenchymal markers (Cy7) in magenta, blood lineage markers (Cy5) in white (merge) or red, and PD-L1 marker (Cy3) in orange. **(C)** Case study of one patient with changes in CTC numbers and PD-L1 expression across six draws. Each draw includes two 7.5 mL samples, totaling 15mL of blood processed.*

CONCLUSIONS

- Analytical verification demonstrated that ANGLE's IF assay for CTC detection with PD-L1 assessment produced linear data, with high analytical sensitivity and analytical specificity for epithelial, mesenchymal, blood lineage and PD-L1 markers.
- ANGLE's PD-L1 assay identified CTCs in 91% of lung cancer patients assessed in a longitudinal study, taken from cohorts with known PD-L1 status in the tissue, on a variety of treatment plans.
- PD-L1 positive CTCs (≥1) were identified in 27% of patients with a negative tissue status, demonstrating the feasibility of a more repeatable and accurate assessment of patients' PD-L1 status than can be achieved through a traditional tissue biopsy.
- This study demonstrated the ability to enumerate and phenotype CTCs from the blood of metastatic lung cancer patients and, subject to further study, the future potential to develop dynamic PD-L1 testing for advancement of personalized cancer treatments.