



# Evaluation of PD-L1 expression on circulating tumour cells in small-cell lung cancer

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**Background:** Antibodies against the programmed death-1 (PD-1) receptor and its ligand (PD-L1) have been recently approved for small-cell lung cancer (SCLC) treatment. Circulating tumour cells (CTCs) have emerged as an appealing liquid biopsy candidate that could enhance treatment decision-making in systemic therapy for SCLC patients. Several current technologies enrich CTCs using specific surface epitopes, size, rigidity, or dielectric properties. However, they are hampered by the heterogeneity of the enriched cells from blood samples.

**Methods:** We evaluated two CTC enrichment systems: EpCAM conjugated to magnetic beads and a microfluidic device (Parsortix, Angle plc). PD-L1 expression was evaluated on the isolated CTCs. Twenty-three blood samples were collected from 21 patients with SCLC. PD-L1 expression was determined on CTCs through immunofluorescent staining.

**Results:** CTCs were found in 14/23 (60.9%) of the samples, with 11/23 (47.8%) through EpCAM-coated magnetic beads (range, 4–1,611 CTCs/8 mL; median =5) and 11/20 (55.0%) using the Parsortix system (range, 1–165 CTCs/8 mL; median =4). Notably, a total of 17 EpCAM-negative CTCs were isolated using the Parsortix system. PD-L1 expression was detected on 268 of the 3,501 (7.7%) CTCs isolated with EpCAM-coated beads and in 33/366 (9.0%) of the CTCs isolated with the Parsortix system. No vimentin expression was observed in any of the detected CTCs.

**Conclusions:** Overall, we identified a population of EpCAM-negative SCLC CTCs and showed that PD-L1 expression can be assessed on CTCs from SCLC patients. Comparison to tumour and treatment outcomes is needed to validate the potential of CTCs as an alternative sample for the assessment of PD-L1 expression in SCLC.

**Keywords:** Circulating tumours cells; PD-L1 expression; small-cell lung cancer (SCLC)

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## Introduction

Small-cell lung cancer (SCLC) is a highly aggressive subtype of lung cancer that accounts for approximately 15% of all lung cancers (1). It is characterised by rapid cellular proliferation and early extensive metastases (2). About 60% of patients have an extensive-stage disease at the time of diagnosis (3). Despite extensive studies, limited therapeutic advances have done little to improve SCLC patients' outcomes.

Chemotherapy and/or radiotherapy remain the principal treatment modalities for SCLC patients, who often show a high response to treatment early on (4-6). However, recurrence occurs in most cases, resulting in a poor prognosis. The 5-year overall survival (OS) rate for early-stage disease is around 15–27%, and for metastatic disease, it is reduced to ~2.8% (4-7). The use of monoclonal antibodies to block the interaction between programmed death-1 (PD-1) and its ligand (PD-L1) has appeared recently as a treatment option in non-small cell lung cancer (NSCLC), and more recently, SCLC (8,9). Expression of PD-L1 by tumour cells allows them to escape immune effector mechanisms (10). Recently, the anti-PD-L1 agents atezolizumab and durvalumab in combination with chemotherapy gained US Federal Drug Administration (FDA) approval as a first-line treatment for extensive-stage SCLC. Despite immune checkpoint inhibitors (ICIs) becoming a primary component of SCLC treatment, their efficacy is modest, with only 2 months of OS benefits and limited to a small subset of patients (8,9,11). Hence, there is a need to identify biomarkers that will help determine a subgroup of SCLC patients most likely to benefit from these treatments.

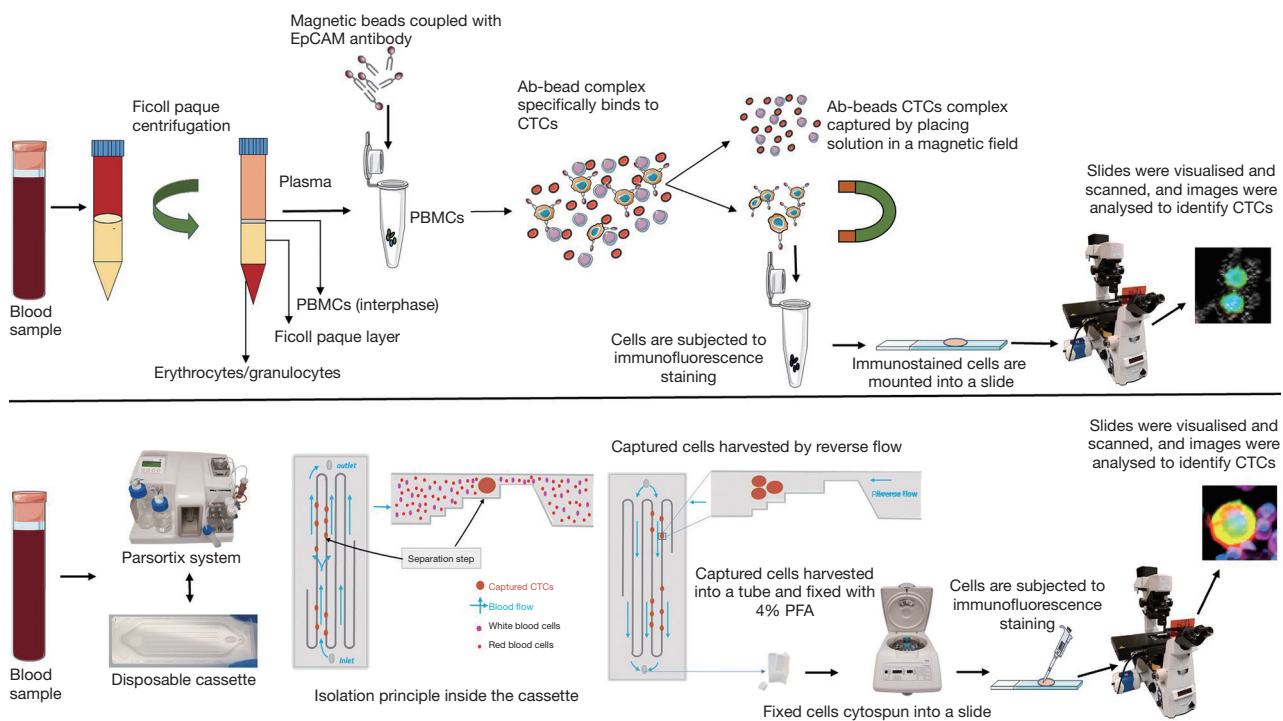
Generally, expression of PD-L1 is assessed on fine-needle aspiration biopsy or core needle biopsy tissue specimen. However, acquisition of tumour tissue is both laborious and invasive for patients. In metastatic SCLC, surgical resection and repeat tumour biopsies are not standard of care and consequently, there can be insufficient tissue for clinical analysis (12,13). Circulating tumour cells (CTCs) offer an appealing liquid biopsy modality for SCLC due to their abundance in the blood of these patients. CTCs can serve as a minimally invasive and serially acquirable substitute for tumour biopsies for tumour characterisation and evaluation of PD-L1 expression in SCLC (14,15).

CTCs are malignant cells shed into the blood by both primary and metastatic solid tumours and their presence

in circulation represents a critical step in the metastatic process (16,17). CTCs can reflect the heterogeneity of SCLC tumours because they arise from different tumour sites (18,19). SCLC is distinguished by exceedingly high but variable numbers of CTCs ranging from single to thousands of CTCs per 7.5 mL of peripheral blood compared with other solid malignancies (20). The number of CTCs present are prognostic and reflect the changing disease burden throughout treatments (21,22). Yet, detection of CTCs after isolation is a challenge due to tumoural heterogeneity. Different well-established approaches to isolate and identify SCLC CTCs with different definitions of tumour cells have been published with detection rates ranging from 60% to 96% (23-25).

CellSearch, an EpCAM-based system, remains the only FDA-approved system and the most used SCLC CTC isolation platform in the clinical setting (26). With the CellSearch platform, CTCs are detectable in most SCLC patients due to the abundance of high EpCAM expressing CTCs (20-22). However, some CTCs might not express EpCAM or might have downregulated EpCAM and therefore remain undetectable with this method. To overcome the above limitation, alternative strategies based on the biophysical properties of the cells other than EpCAM protein expression are necessary (27). Such non-marker-based strategies may allow for broader coverage of CTCs subpopulations. Currently, there are several developed size-based platforms (28). Amongst them, the Parsortix system, which isolates cells based on a combination of size and deformability, has been shown to isolate CTCs where CellSearch was unable to (29).

The assessment of PD-L1 on CTCs (PD-L1<sup>+</sup> CTCs) has been extensively studied in NSCLC (30) but to our knowledge, no exhaustive report exists for SCLC. We, therefore, developed an EpCAM targeting magnetic bead-based CTC isolation method as a surrogate for CellSearch, the gold standard for CTC enumeration. Using our immunomagnetic isolation technique, we compared detection rates of CTCs isolated using EpCAM-based immunomagnetic capture to those isolated using the Parsortix system. Secondly, we established a workflow to determine the prevalence of PD-L1<sup>+</sup> CTCs in SCLC utilising EpCAM-coated magnetic beads and the Parsortix system. We present the following article in accordance with the MDAR reporting checklist (available at <https://tclr.amegroups.com/article/view/10.21037/tclr-21-819/rc>).



**Figure 1** Workflow for assessment of PD-L1 expression on SCLC patient CTCs. CTC isolation workflow: blood is collected from SCLC patients and processed through Parsortix system and EpCAM-coated magnetic beads. Enriched cells are collected, permeabilised and fixed, and then immunostained with immunofluorescence markers for imaging. Medical elements in this image are from smart.servier.com. CTCs, circulating tumour cells; PBMCs, peripheral blood mononuclear cells; Ab-bead, antibody conjugated beads; PFA, paraformaldehyde; SCLC, small-cell lung cancer.

## Methods

### Patient recruitment and sample collection

For this pilot study, a total of 21 SCLC patients were recruited in the study between August 2018–March 2021 at Sir Charles Gairdner Hospital (SCGH) and Fiona Stanley Hospital (FSH) in Perth, Western Australia. Written informed consent was obtained from all patients under approved Human Research Ethics Committee protocols from Edith Cowan University (No. 18957) and Sir Charles Gairdner Hospital (No. 2013-246, RGS0000003289). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). At least 8 mL of blood was collected from each patient into K2EDTA (BD, Franklin Lakes, NJ, USA) tubes for CTC analysis. Samples were processed within 6 hours of blood collection. Demographic and clinical information such as age, gender, disease stage, performance status, smoking status, number of metastases, and type of treatment of patients were collected. Smoking status was collected as smokers and non-smokers. The

smokers included those who smoked at least 10 packs a year (i.e., one pack a day for 10 years) either former or current.

### Enrichment and identification of CTCs

Plasma was isolated from samples by centrifugation for 20 minutes at 300  $\times$ g before CTC enrichment with anti-EpCAM coated magnetic beads (Appendix 1). The CTC capture process was carried out using anti-EpCAM beads in a modified protocol developed in our laboratory (31). Captured cells were immunostained with antibody cocktail containing three mixed pan-cytokeratin antibodies to ensure broad cytokeratin coverage, CD45, CD16, and CD66b antibodies to exclude hematopoietic cells and anti-PD-L1 antibody (28.8) to detect PD-L1 expression as detailed in Appendix 1.

In parallel, another blood sample was processed using the Parsortix system at 99-mbar through a 6.5- $\mu$ m cassette (Figure 1). Enriched cells were harvested according to the manufacturer's instructions and fixed for 10 minutes at room temperature with 4% paraformaldehyde (PFA). A total of

**Table 1** Clinical and demographic characteristics of patients

Variables	Frequency (n)	Percentage (%)
Age (years), median (IQR)	67.5 (63.5–83.0)	–
Age group (years)		
<67	9	42.9
≥67	12	57.1
Gender		
Male	9	42.9
Female	12	57.1
Disease stage		
Limited	2	9.5
Extensive	19	90.5
Performance status (ECOG)		
0	9	42.9
1	8	38.1
≥2	4	19.0
Smoking status		
Yes	20	95.2
No	1	4.8
Number of metastasis		
1	5	23.8
≥2	16	76.2
Type of treatment		
Chemotherapy	7	33.3
Chemotherapy + ICI	13	61.9
Radiation	1	4.8

IQR, interquartile range; ECOG, Eastern Cooperative Oncology Group; ICI, immune checkpoint inhibitor.

8–9 mL of blood was processed through each method. To increase the numbers of markers to be interrogated, such as EpCAM expression separate from cytokeratins, vimentin, and PD-L1 expression (29), we adapted the quenching and re-staining protocol described by Adams *et al.* (32). This protocol utilises borohydride to quench fluorescent signals after an initial round of immunostaining followed by a second round of staining for additional markers, allowing for multi-phenotype analysis of CTCs. The PD-L1 detection, quenching, and restaining methods were standardised using MCF7, MCF7 induced with IFN- $\gamma$ , MDA-MB-231 cell lines

spiked into white blood cells (WBCs) from healthy control donors as detailed in [Appendix 1](#).

### Imaging and image analysis

Slides were visualised and scanned using a Nikon Eclipse Ti-E inverted fluorescent microscope (Nikon, Chiyoda, Japan). Images were analysed using the NIS-Elements Analysis software, version 5.21 (Nikon).

### Statistical analysis

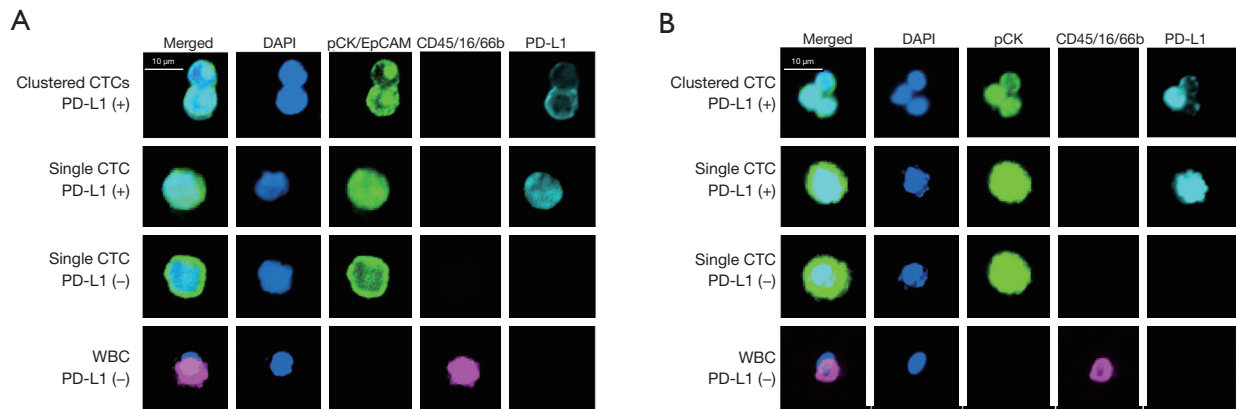
All data was entered into Microsoft Excel and analysed with GraphPad Prism (version 8.0.2). Demographic data is presented as numbers, ranges, or counts, percentages, means, and medians where applicable using GraphPad version 8. Cohen's kappa test was used to analyse the difference in CTC detection rates between EpCAM-coated magnetic beads and the Parsortix system as well as an agreement between the two isolation methods.

Survival analysis was performed using the Kaplan-Meier method and differences in patient survival rates were determined using log-rank tests. Univariate and multivariate Cox regression hazard models for OS were performed for CTC count, number of metastases, Eastern Cooperative Oncology Group (ECOG) performance status, sex, and age using SPSS version 26. All survival plots were performed in R (version 4.05) using the “survplot” package (33,34) with  $P < 0.05$  considered statistically significant.

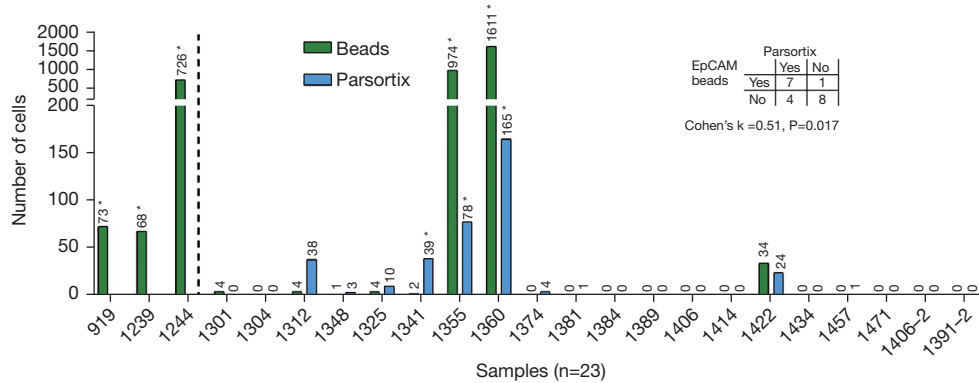
## Results

### Clinical characteristics of patients

A total of 23 blood samples were collected from 21 SCLC patients for the analysis of the presence of CTCs before the commencement of treatment. Blood samples for CTC enumeration were collected before treatment in 19 patients, while bloods were collected before treatment and at the time of relapse in 2 patients. The clinical characteristics of the study population are summarised in [Table 1](#). Of the 21 SCLC patients in this study, 2 patients (9.5%) had limited stage disease and 19 (90.5%) had extensive disease. The median age of SCLC patients at the time of diagnosis was 67.5 (range, 63.5–83.0) years and there were 12 females and 9 males. Patients were treated with chemotherapy alone ( $n=7$ ) or in combination with either atezolizumab or durvalumab ( $n=13$ ). One patient was treated with cyberknife radiation.



**Figure 2** Representative images of SCLC CTCs identified by immunofluorescence staining. (A) CTCs enriched with Parsortix system. Cells were immunostained with pan-cytokeratins and EpCAM (green), CD45/16/66b (pink), and TSA PD-L1 (cyan). (B) CTCs enriched by EpCAM coated magnetic beads. Cells were immunostained with pan-cytokeratins (pCK, green), CD45/16/66b (pink), and TSA PD-L1 (cyan). WBC were included for comparison. Scale bar (top left) represents 10  $\mu$ m. CTCs, circulating tumour cells; WBC, white blood cells; SCLC, small-cell lung cancer.



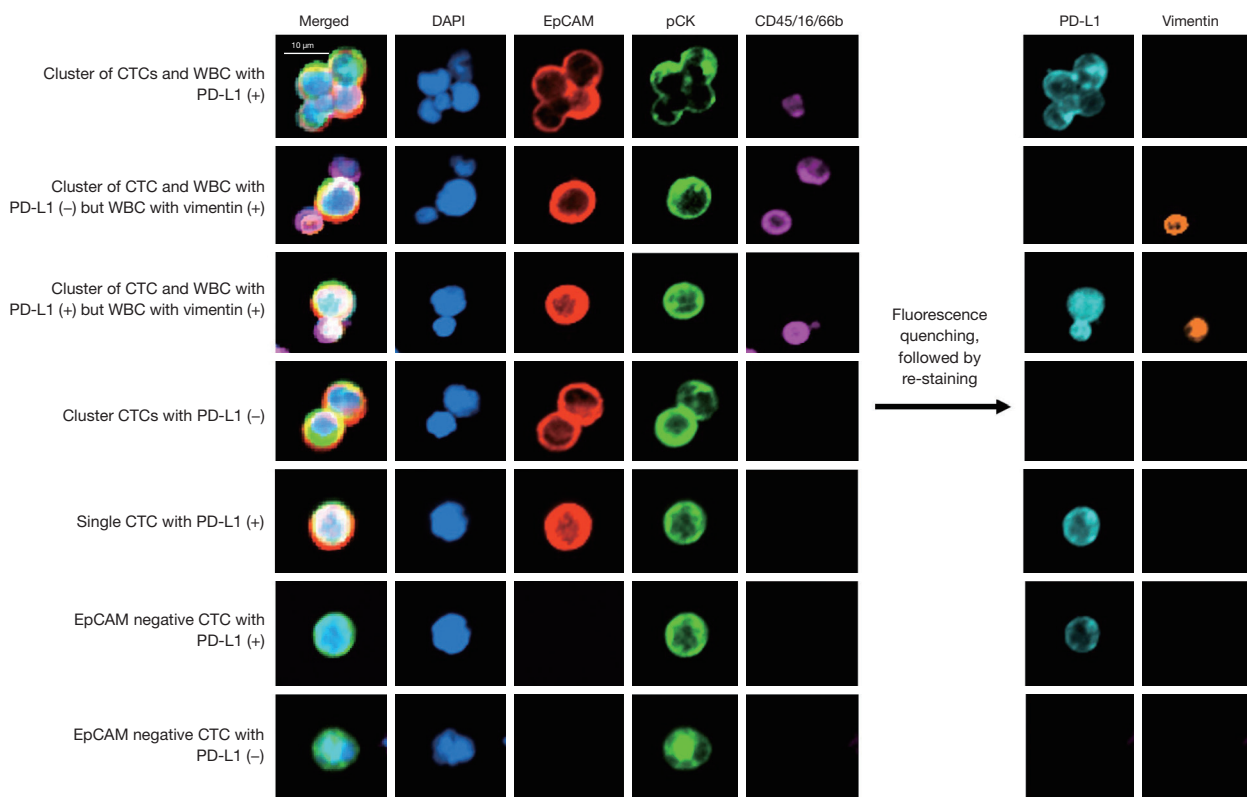
**Figure 3** CTC counts in SCLC patients. Samples were processed with EpCAM-coated magnetic beads (n=23, green bars), and a proportion of them (to the right of the dashed line) was also enriched for CTCs using the Parsortix system (n=20, blue bars). The number of cells on each sample is indicated on top of the bars. \*, indicate samples with CTC clusters. A contingency table comparing the number of positive samples by each method and associated statistics have been inserted. CTC, circulating tumour cell; SCLC, small-cell lung cancer.

**CTC enumeration and characterisation**

CTCs were isolated from 23 blood samples using anti-EpCAM immunomagnetic beads. Only 20 of these patients had a second blood sample available for CTC isolation using the Parsortix system. Enriched CTCs were identified through immunofluorescence staining, as exemplified in *Figure 2*. CTCs were detected in 11 of 23 (47.8%) samples processed with EpCAM-coated magnetic beads [median =5 (range, 1–1,611)] and in 11 of 20 (55.0%) samples processed with the Parsortix system [median =4 (range,

1–165) (*Figure 3*). Combining both methods, CTCs were found in 14/23 (60.9%) of the SCLC samples. Comparison of CTC detection in the 20 matched samples using Cohen's kappa coefficient indicated a moderate agreement ( $\kappa$  = 0.51; P = 0.017) between the detection rate of the two methods. In samples with a large number of CTCs (cases 1355 and 1360 in *Figure 3*), EpCAM beads recovered 10 times more CTC than using Parsortix. However, six samples (1312, 1318, 1325, 1341, 1374, and 1434) exhibited a higher number of CTC recovered using the Parsortix system than using





**Figure 4** Representative cells enriched with the Parsortix system. Cells were stained with pan-cytokeratins (pCK, green), EpCAM (red), CD45/16/66b (pink) to identify classical SCLC CTCs, followed by fluorescence quenching and re-immunostained for PD-L1 expression (cyan) and vimentin (orange). Scale bar (top left) represents 10  $\mu$ m. CTCs, circulating tumour cells; WBC, white blood cells; SCLC, small-cell lung cancer.

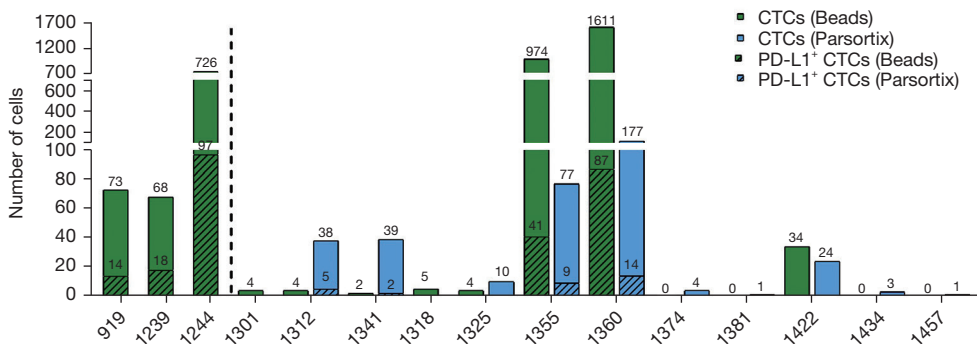
EpCAM-coated beads.

CTC clusters were found in 6/23 of samples. Of the 6 samples with clusters, 3 samples were processed with EpCAM beads only. The remaining 3 cluster-containing samples were processed with both isolation methods, where clusters were found in all three Parsortix samples and 2/3 EpCAM beads samples (*Figure 3*). Additionally, we found WBCs paired with single CTCs or with CTC clusters in all 3 samples with clusters processed on the Parsortix system, but not in any of the EpCAM-captured samples (*Figure 4*, *Figure S4*). A subgroup of 14 samples enriched using Parsortix was also assessed for the expression of EpCAM and CK on CTCs separately, as well as for vimentin expression (*Figure 4*). No vimentin expressing CTCs were detected in any of the patients. A total of 17 EpCAM-negative CK-positive CTCs were detected in 3/14 (21.4%) patients, and these cells were always found as single CTCs (*Figure 4*, *Figure S5*). Notably, in one sample (1374

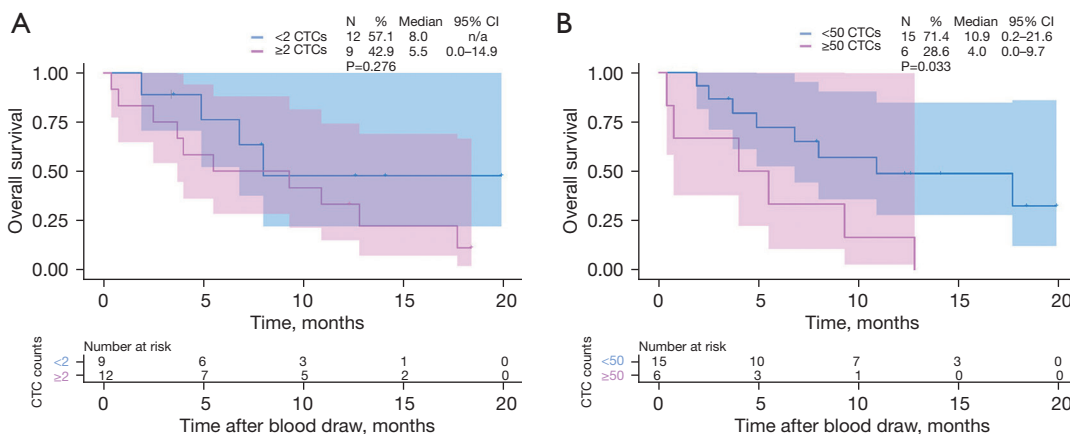
in *Figure 3*) only EpCAM-negative CTCs were detected, which was consistent with the sample found to be negative using the EpCAM-beads capturing approach.

#### ***PD-L1 expression on CTCs***

PD-L1 expression was assessed on the 14 CTC-positive samples found among the 23 blood samples analysed. Overall,  $\geq 2$  PD-L1<sup>+</sup> CTCs were detected in 7/23 (30.4%) samples regardless of the isolation method. PD-L1<sup>+</sup> CTCs were found in 5 samples processed with EpCAM-coated magnetic beads and in 3 samples processed with Parsortix (*Figure 5*). PD-L1 expression was analysed on a total of 3,501 CTCs isolated with EpCAM-coated beads with 268 (7.7%) found positive for PD-L1. In comparison, 33 of 366 (9.0%) CTCs isolated on the Parsortix system expressed PD-L1. Three of the 17 EpCAM-negative CTCs identified were positive for PD-L1 expression.



**Figure 5** Comparison of PD-L1 expressing CTCs isolated by immunomagnetic beads and the Parsortix system. Bars represent counts in CTC positive samples processed with EpCAM-coated magnetic beads (n=15, green bars), and a proportion of them (to the right of the dashed line) was also enriched for CTCs using the Parsortix system (n=12, blue bars). PD-L1 positive CTC counts are indicated by hatched bars. CTCs, circulating tumour cells.



**Figure 6** Kaplan-Meier curves for OS for analysed CTCs thresholds. (A) <2 vs. ≥2 CTCs. (B) <50 vs. ≥50 CTCs. Log-rank P values, group numbers, median, and 95% CIs are indicated for each plot. CTCs, circulating tumour cells; CI, confidence interval; OS, overall survival.

**Survival analysis**

The median OS of patients after blood draw was 9.3 months (95% CI: 4.3–14.2 months). We assessed the correlations of CTC counts with OS using the previously validated thresholds of 2 and 50 CTC per 7.5 mL of blood (24,35,36). Neither of the two CTC threshold groupings showed statistically significant differences between the clinical characteristic of the patients (Table S2). There was no statistically significant difference in median OS between patients with ≥2 CTCs compared to those with <2 CTCs (5.5 vs. 8 months, P=0.276). However, patients with ≥50 CTCs had significantly shorter median OS compared with those with <50 CTCs (4.0 vs. 10.9 months, P=0.033) (Figure 6). Univariate Cox regression analysis revealed that

≥50 CTCs was significantly associated with shorter OS (HR =3.11; 95% CI: 1.01–9.32; P=0.043). Multivariate analysis showed that ≥50 CTCs was an independent prognostic factor for shorter OS (HR =6.15; 95% CI: 1.35–27.99; P=0.019) (Table 2). Among the 14 SCLC patients with ≥2 CTCs, there was no statistical difference in the survival of patients with PD-L1-CTCs compared with patients with PD-L1+ CTCs (10.9 vs. 4.0 months, P=0.103) (Figure S6).

**Discussion**

CTCs have emerged as appealing liquid biopsy candidates that could enhance treatment decision-making (14,15). In this study, we employed a size-based CTC enrichment method, the Parsortix system, that has been demonstrated

**Table 2** Univariate and multivariate Cox hazard regression analysis

Variables	Groups	Univariate		Multivariate	
		HR (95% CI)	P value	HR (95% CI)	P value
Age (years)	≥67 (n=12)	–	–	–	–
	<67 (n=9)	1.29 (0.43–3.87)	0.641	1.14 (0.27–4.77)	0.858
Sex	Male (n=9)	–	–	–	–
	Female (n=12)	2.14 (0.17–6.50)	0.176	2.45 (0.64–9.35)	0.189
CTC count	<50 (n=15)	–	–	–	–
	≥50 (n=6)	3.11 (1.01–9.32)	0.043*	6.15 (1.35–27.99)	0.019*
Performance status (ECOG)	0 (n=9)	–	–	–	–
	1 (n=8)	1.26 (0.38–4.18)	0.194	0.45 (0.09–2.25)	0.337
	≥2 (n=4)	2.62 (0.61–11.23)	0.480	4.23 (0.64–27.98)	0.134
No of metastases	1 (n=5)	–	–	–	–
	≥2 (n=16)	2.79 (0.59–13.02)	0.230	3.56 (0.62–20.48)	0.156

\*, P<0.05, considered statistically significant. HR, hazard ratio; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group.

to harvest CTCs in a greater proportion in different tumour types including SCLC (29,37–39). We also validated a simple, rapid, and affordable method to detect CTCs based on magnetic cell separation. We compared CTCs detection rates between the two isolation methods, using matched samples, and evaluated the potential of CTCs for PD-L1 expression other than SCLC biopsy tissue.

Both epitope-dependent and epitope-independent enrichment methods have been shown to isolate high numbers of CTCs in SCLC patients compared with other types of cancers (40). The overall frequency of patients with detectable CTCs in our study was 61%, in line with previous reports in SCLC, showing detectable CTCs in between 60–95% of patients. However, CTC detection rate was higher in samples processed with the Parsortix system (55%) compared to EpCAM-coated magnetic beads (48%) in a matched comparison. Chudziak *et al.* (29) also reported similar results reporting that cytokeratin positive CTCs were detectable in all the 12 samples processed on the Parsortix platform while CellSearch only detected cytokeratin positive CTCs in 10 (83%) of the SCLC patients tested. These results may be explained by the fact that EpCAM-based isolation methods may fail to capture EpCAM low/negative expressing CTCs. In line with this, we demonstrated here that EpCAM negative CTCs were isolated using Parsortix in 14.3% of the processed samples. On the other hand, the number of Parsortix-isolated

CTCs was lower those isolated using EpCAM-beads, in particular for the two patients with the largest number of CTCs. SCLC CTCs are relatively small, compared to other carcinomas (ref) and may not be efficiently retained by the Parsortix system which isolates CTCs based on size and deformability (41).

It has been proposed that primary and metastatic tumours release cells into the bloodstream through a process of the epithelial to mesenchymal transition (EMT) (42). Given the loss of EpCAM observed in CTCs isolated using Parsortix, we assessed the potential expression of vimentin on these cells. Results revealed the absence of vimentin in all the CTCs interrogated. This result suggests that EMT is not homogenously expressed in tumour cells within the circulation of SCLC patients and supports the importance of other types of motility shift such as amoeboid cell invasion which has been demonstrated to be typical of SCLC (43,44). Although, there are limited studies on amoeboid tumour cell invasion in SCLC and lack of EMT markers such as vimentin on SCLC CTCs suggest it might be an important subject area for further studies.

Multiple studies in the last decade have demonstrated that the presence of measurable CTCs in SCLC patients is associated with shorter survival (24,36,45). The presence of ≥2 and ≥50 CTCs per 7.5 mL of blood from SCLC patients before chemotherapy was highly significant for poor OS, regardless of other clinical prognostic variables (24,36).



Consistent with these studies (24,36,45), we found that patients with  $\geq 50$  CTCs had significantly shorter median OS compared with the  $< 50$  CTCs group.

The biology of clustered CTCs is an evolving area of research. CTC clusters in the peripheral blood have been reported in patients with SCLC (24). In this study, CTC clusters were detected samples processed on the Parsortix system and with EpCAM coated magnetic beads. The number of cells within the CTC clusters detected on the Parsortix were large, comprised of up to 8 CTCs and involving WBCs, compared to 2–3 clustered CTCs detected using the immunomagnetic beads. A number of studies have shown the role of clusters in the migration and survival of CTCs in breast and gastric cancer (38,46,47). However, no studies have directly addressed how SCLC CTC clusters may enable metastases and/or chemoresistance.

Until recently, far too little attention has been paid to the expression of PD-L1 on CTCs in SCLC, compared to its extensive study in NSCLC (30,48–51). This is partly due to the early approval of ICIs for the treatment of NSCLC and higher expression of PD-L1 protein in NSCLC. On the other hand, there is a wide difference in the prevalence of PD-L1 expression in tumour cells of SCLC patients reported in the literature, ranging from 0–86% (52,53). Even though ICIs combined with chemotherapy were recently approved for SCLC treatment, tumour PD-L1 expression has been demonstrated to be a non-discriminatory biomarker (8,9,11). However, it is possible that retaining PD-L1 might represent one of the mechanisms that CTCs use to survive immune system attack while in circulation and, therefore a better readout of a pre-existing anti-tumour response. Previous studies in melanoma and NSCLC have shown that PD-L1 expression on CTCs a promising prognostic biomarker in patients treated with ICIs, despite the lack of correlation with the expression on matching tumours (54,55).

Our study is the first to evaluate PD-L1 expression on CTCs in SCLC by both epitope-dependent and -independent enrichment techniques. Two other studies have assessed the expression of PD-L1 on CTCs in SCLC patients finding PD-L1 expression in 0–50% of samples (49,51). In our study,  $\geq 2$  PD-L1<sup>+</sup> CTCs were detected in 7/23 (30.4%) samples regardless of the isolation method. This discrepancy could be attributed to the antibody clones utilised, especially the high sensitivity of the PD-L1 antibody clone (28.8) or TSA amplification of the antibody signal for PD-L1 detection in our study.

The present study has some limitations such as the

fact that the study population was small with an inferred post-hoc power of 0.36, which hindered the feasibility of inferential statistics. In particular, the inclusion of a small number of patients treated with chemotherapy alone did not enable an analysis of the predictive value of PD-L1 expressing CTC for response to treatment as shown for other cancers (37,56,57). It was not possible to assess the association of PD-L1<sup>+</sup> CTCs with survival among patients treated with immunotherapy, given their small number of cases (9 of 21) in this subgroup. Finally, PD-L1 expression assessment is not a routine practice for SCLC. Thus, we could not compare the expression of PD-L1 on CTCs to that of the matching tumours as samples were not available for evaluation.

## Conclusions

The current findings extend our knowledge of the ability of epitope-independent technologies to detect subsets of CTCs. The study demonstrates that PD-L1 expression can be quantified on CTCs detected in SCLC patients. This could potentially serve as a marker to evaluate the likelihood of anti-PD-1 therapy response.

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## Footnote

*Reporting Checklist:* The authors have completed the MDAR reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-819/rc>

*Data Sharing Statement:* Available at <https://tlcr.amegroups.com/article/view/10.21037/tlcr-21-819/dss>

*Peer Review File:* Available at <https://tlcr.amegroups.com/article/view/10.21037/tlcr-21-819/prf>

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Written informed consent was obtained from all patients under approved Human Research Ethics Committee protocols from Edith Cowan University (No. 18957) and Sir Charles Gairdner Hospital (No. 2013-246, RGS000003289). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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