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ORIGINAL ARTICLE

# Characterisation of circulating tumor-associated and immune cells in patients with advanced-stage non-small cell lung cancer

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## Abstract

**Objectives.** Globally, non-small cell lung cancer (NSCLC) is the most prevalent form of lung cancer and the leading cause of cancer-related deaths. Tumor-associated circulating cells in NSCLC can have a wide variety of morphological and phenotypic characteristics, including epithelial, immunological or hybrid subtypes. The distinctive characteristics and potential clinical significance of these cells in patients with NSCLC are explored in this study. **Methods.** We utilised a spiral microfluidic device to enrich large cells and cell aggregates from the peripheral blood samples of NSCLC patients. These cells were characterised through high-resolution immunofluorescent imaging and statistical analysis, correlating findings with clinical information from our patient cohort. **Results.** We have identified varied populations of heterotypic circulating tumor cell clusters with differing immune cell composition that included a distinct class of atypical tumor-associated macrophages that exhibits unique morphology and cell size. This subtype's prevalence is positively correlated with the tumor stage, progression and metastasis. **Conclusions.** Our study reveals a heterogeneous landscape of circulating tumor cells and their clusters, underscoring the complexity of NSCLC pathobiology. The identification of a unique subtype of atypical tumor-associatedmacrophages that simultaneously express both tumor and immune markers and whose presence correlates with late disease stages, poor clinical outcomes and metastatic risk infers the potential of these cells as biomarkers for NSCLC staging and prognosis. Future studies should focus on the role of these cells in the tumor microenvironment and their potential as therapeutic targets. Additionally, longitudinal studies tracking these cell types through disease progression could provide further insights into their roles in NSCLC evolution and response to treatment.

**Keywords:** circulating tumor cell clusters, circulating tumor cells, metastasis, microfluidics, NSCLC, tumor associated macrophages

## INTRODUCTION

Lung cancer, the second most frequent cancer worldwide, is the main cause of cancer-related deaths.[1,2] NSCLC accounts for 80–90% of lung cancer cases.[3] Normally upon diagnosis of NSCLC, over 60% of patients have advanced-stage disease, which includes distant or locoregional metastases. Patients with advanced-stage disease have a 5-year survival rate less than 15%.[1,4] NSCLC subtypes include squamous cell carcinoma and adenocarcinoma and can be further divided based on oncogenic driver mutations, dictating therapeutic strategies.[5] The variety of NSCLC subtypes emphasises the disease complexity and calls for individualised treatment strategies.[6] Furthermore, the identification of accurate biomarkers for NSCLC patients is a challenge because of tumor heterogeneity, and difficulties in accessing tissue biopsy.[7] CT, MRI, ultrasonography and PET are imaging-based tools that provide non-invasive, reproducible techniques for screening, surveillance and measuring therapy response. Their performance, however, fluctuates and is dependent on radiologists' assessments.[8] The current gold standard, tissue biopsy, is invasive and has limitations.[9] Up to 30% of NSCLC patients lack appropriate tissue for molecular profiling, a substantial restriction because of a lack of accessible tissue in quantity and quality.[10] The development of biomarkers that can overcome these obstacles is critical for successful cancer surveillance and management. A reliable biomarker should be simple to collect, non-invasive, broadly applicable and independent of operator expertise.[11]

Liquid biopsy, which contains a wide range of molecular and cellular components, including disseminated tumor-associated cells, has emerged as an ideal source for cancer biomarkers, allowing cell-based assays to reveal the developing tumor biology.[12,13] Liquid biopsy is (1) repeatable, (2) less invasive and (3) thought to represent systemically dispersed tumor heterogeneity.[14,15] Another advantage of liquid biopsy is that it can be used to monitor metastasis, a process that causes more than 90% of cancer deaths by shedding tumor cells from the primary tumor into the lymphovasculature and metastasizing to other organs.[16,17]

Circulating tumor cells (CTCs) can serve as biomarkers in liquid biopsy. In patients with no clinically detectable metastases, their presence indicates the possibility of seeding at the main site or distant organs.[18] These cells, known as seeds for metastasis, might be solitary CTCs, clusters of CTCs or mixed with white blood cells (WBCs).[19] CTC-WBC clusters, in particular, have a significant impact on disease progression and metastasis by regulating cell cycles and driving tumor growth. This interaction between CTCs and WBCs is critical in disease progression, both in circulation and in the tumor microenvironment (TME).[20,21] Furthermore, counting and characterising CTCs at several levels (DNA, RNA, proteins and morphology) provides information for a better understanding of disseminated tumor biology. Moreover, the variability of CTC composition and the critical role they play in tumor metastasis emphasise the need to investigate the diverse CTC subtypes and CTC-WBC clusters. In this study, we report on a wide range of tumor-associated cells, including CTCs and CTC clusters, tumor-associated macrophages (TAMs), cancer-associated macrophage-like cells (CAMLs) and tumor-immune cell fusions.

Macrophages that normally reside in healthy tissues migrate to tumor sites that are hypoxic or necrotic when malignancy is developed. In addition, neoplastic cells secrete specialised chemokines that attract circulating monocytes into the tumor, where they then develop into macrophages.[22,23] Local factors impact the development of various macrophage populations upon recruitment to the TME leading to TAMs formation. In some instances, TAMs can make up as much as half of the tumor mass.[24] Adams *et al.*[25] first described CAMLs, which are macrophages found in prostate, pancreatic and breast cancer patients. They can enter the bloodstream while engulfing tumor materials. These CD14-expressing stromal cells remove tumor cells and debris from the tumor microenvironment. CAMLs can vary in shape and size from 25 to 300 μm, making them more varied in appearance than CTCs.[25,26] Both overall and progression-free survival are shortened in patients with higher CAML levels before treatment. Typically, CAML enumeration rises briefly in

reaction to chemotherapy and has an inverse association with the number of detected CTCs. It also appears to correlate with treatment response. An increase in the number of CAMLs is observed after effective treatment, suggesting that these cells are monitoring and engulfing dead cancer cells. In oesophageal cancer and NSCLC, larger CAMLs before and after therapy are associated with lower overall survival, suggesting more aggressive disease subtypes.[26,27]

Large, polymorphic, mono- or polynuclear hybrid cells with epithelial and myeloid features can be generated when M2 macrophages fuse with tumor cells. Patients with a variety of cancers, including NSCLC, have been observed to contain these hybrids within their blood circulation, which express $CK^+$/$EpCAM^+$ and $CD14^+$/$CD45^+$.[25,28] Instead of having a filamentous cytokeratin pattern like epithelial cells, these fused cells have a dispersed pattern more like mesenchymal cells.[29] Circulating hybrid E/M cells, gigantic epithelioid cells, tumor-macrophage fusion cells (TMFs), and macrophage-tumor cell fusion cells (MTFs) are some of the names given to these cells.[27,29–32] Manjunath *et al*.[33] found that having two or more circulating cancer-associated cells was correlated with much worse survival rates and greater rates of recurrence.

In this study, we comprehensively characterise individual tumor-associated cells, to find correlations between the presence of distinct cell types and NSCLC. For this study, we enrolled individuals diagnosed with early or advanced NSCLC and employed a previously validated label-free technique to detect tumor-associated circulating cells in blood samples taken from these patients.

## RESULTS

### Recovery of CTCs from spiked healthy controls

Increasing the number of spiked cancer cells that can be efficiently isolated from healthy blood samples using the spiral chip was the first goal of this research. The spiral device have a bifurcated trapezoidal cross-sectional channel with a single inlet as well as target and waste outlets. Predominantly, cells and clusters larger than 14–15 μm move into the 'target outlet', whereas those lower than this size proceed into the 'waste outlet'. We optimised for large cell/cluster enrichment and recovery using healthy blood spiked with A549 cells, assessing flow rates ranging from 1.3 to 2.1 mL $min^{-1}$. The device's capacity to effectively enrich tumor cells was substantially reduced when operating at flow rates greater than 1.7 mL $min^{-1}$ and less than 1.5 mL $min^{-1}$. The ideal flow rate, which was determined to be 1.7 mL $min^{-1}$, led to the greatest average recovery of the cancer cell line (81 ± 4.5%), as shown in Figure 1.

### Isolation of cancer-associated cells from patient samples

Following RBC lysis, prepared samples were run through the device. Immunological staining with DAPI, CD45 and pan-CK was performed on the collected cells. Characterised by a high nucleus-to-cytoplasmic ratio and being morphologically larger than background leukocytes, tumor associated cells, mostly CTCs and CTC clusters, were found to be $DAPI^+$, $CD45^-$ and $CK^+$ cells. Conversely, WBCs such as neutrophils, lymphocytes or macrophages were identified to be $DAPI^+$, $CD45^+$ and $CD14^+$, and $CK^-$. Figure 2 displays a gallery of detected tumor-associated cells in a fluorescent field, and Figure 4 illustrates the number of each category per patient; notably, no CTCs were identified in the healthy controls ($N = 5$).

### Characterisation of subpopulations of circulating tumor cell clusters and tumor associated immune cells in NSCLC patients

Based on morphological analysis of cytological samples taken from the target outlet of the spiral chip, a varied population of heterotypic circulating tumor cell clusters and different subsets of immune cells were identified, with differing frequencies across patient cohort. We identified various CTC clusters with different cell composition and morphology (Figure 2b and c). Within these clusters, we observed small immune cells characterised by dense nuclei and elevated CD45 expression levels. These immune cells formed in either one-to-one clusters with individual CTCs or clusters with one-to-multiple CTCs (Figure 2d and e).

We also observed a subpopulation of cells, hereafter referred to as atypical tumor associated macrophages (aTAMs) expressing tumor and immune markers simultaneously. These $CK^+$, $CD45^+$ and $CD14^+$ cells have a bi-lobe nucleus with less density than common CTCs. Although their sizes

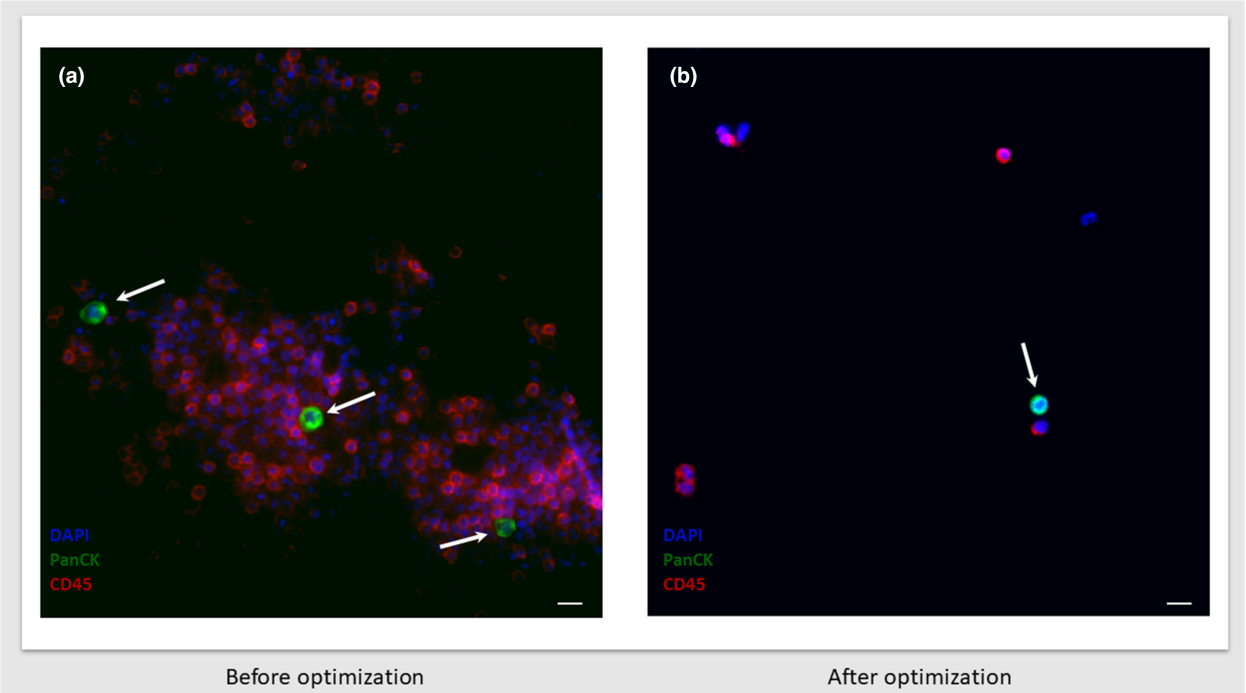


**Figure 1.** Optimisation of enrichment procedure. **(a)** High background cell in the target outlet before optimisation. **(b)** Reduced background cells in the target outlet at the optimal flow rate. White arrows = cancer cells. Scale = 15 μm.

vary between 10 and 15 μm in different patients, they mostly exhibit a regular rounded shape morphology. Interestingly, we found various levels of CD45 expression among these cells from very low to high. Moreover, they are positive for CD14, which has already been confirmed to be expressed by CAMLs.[25] Microscopic images depict these cells circulating individually or along with CTCs or other immune cells in clusters (Figure 3a–e). Morphological analysis of this cohort shows there are few CTCs with similar nuclear patterns circulating either alone or within the clusters (Figure 2a and e). Of the 40 patients in the cohort, 37 tested positive for heterotypic clusters, while only 10 tested positive for homotypic clusters. Additionally, atypical tumor-associated macrophages were observed in 25 patients. The distribution of all cell types in the samples can be seen in Figure 4 and also in Supplementary table 1.

### Atypical tumor associated macrophages correlate with stage, progression and metastasis

While the association of CTCs and CTC clusters with stage and metastasis has been documented by previous studies,[34,35] we found no such associations, possibly because of the relatively small sample size. A univariate correlation analysis was performed to explore the association between aTAMs and clinical parameters including stage, progression and metastasis in our study cohort. Progression of an existing lesion is defined by an increase in size that meets the RECIST criteria; specifically, a 20% or greater increase in the sum of diameters of the targeted lesions, or the emergence of new lesions. The results revealed a positive correlation between aTAMs and each of the assessed clinical conditions: stage ($P = 0.028$), progression ($P = 0.002$) and metastasis ($P = 0.038$).

## DISCUSSION

The detection of circulating aTAMs, both as a part of CTC clusters and as single cells, and their association with disease stage, progression and metastasis are novel discoveries, along with the detection of CTC clusters that include other non-neutrophil immune cells. As CTCs can vary from patient to patient and there is currently no standardised way to accurately detect them, the technique used in each study can affect

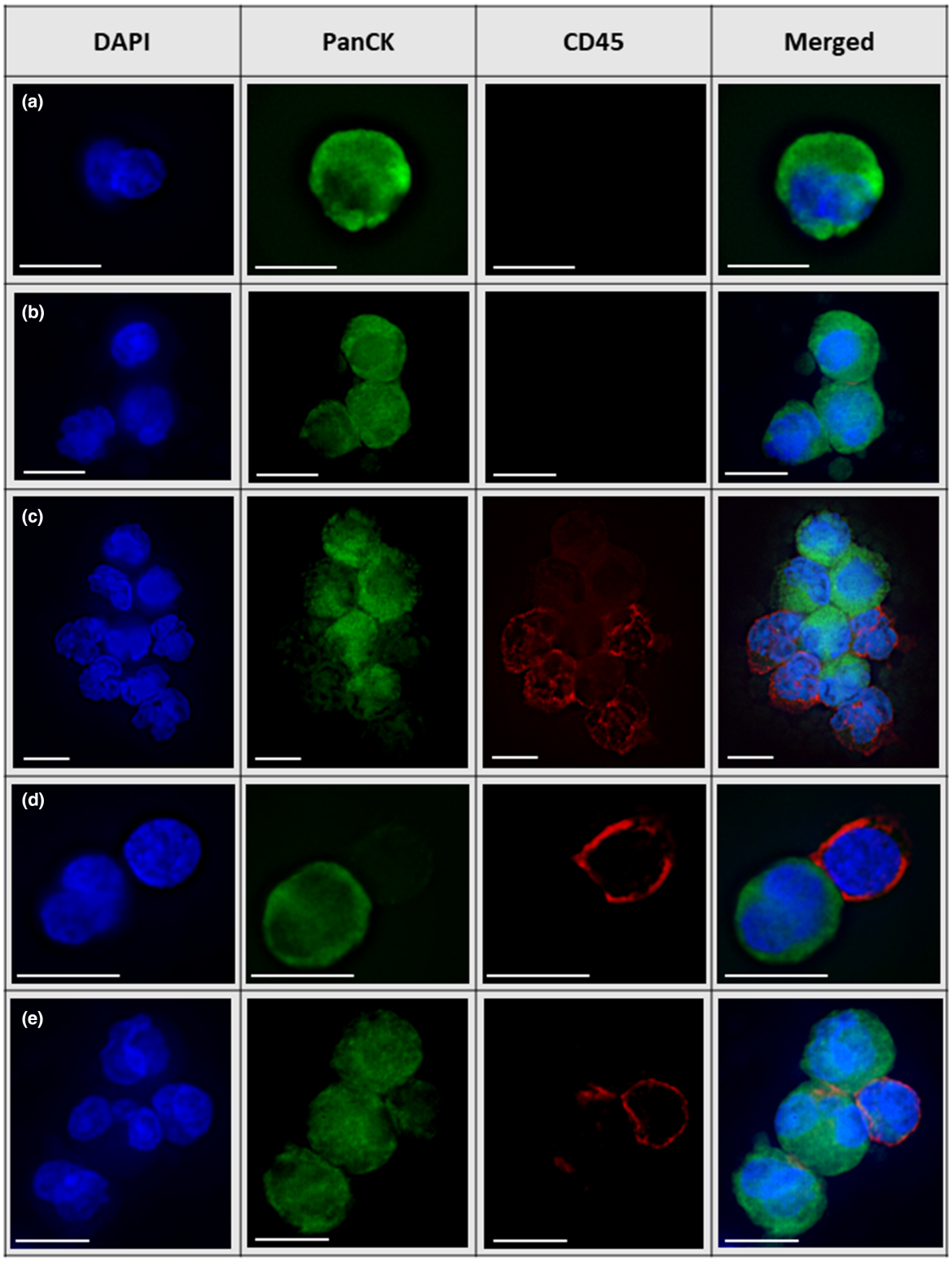


**Figure 2.** Examples of CTC types detected in NSCLC patient's blood samples. **(a)** Single CTC. **(b)** Small homotypic cluster. **(c)** Large heterotypic cluster. **(d)** Small heterotypic cluster with close proximity to immune cells. **(e)** Large heterotypic cluster with close proximity to immune cells. Scale = 10 μm.

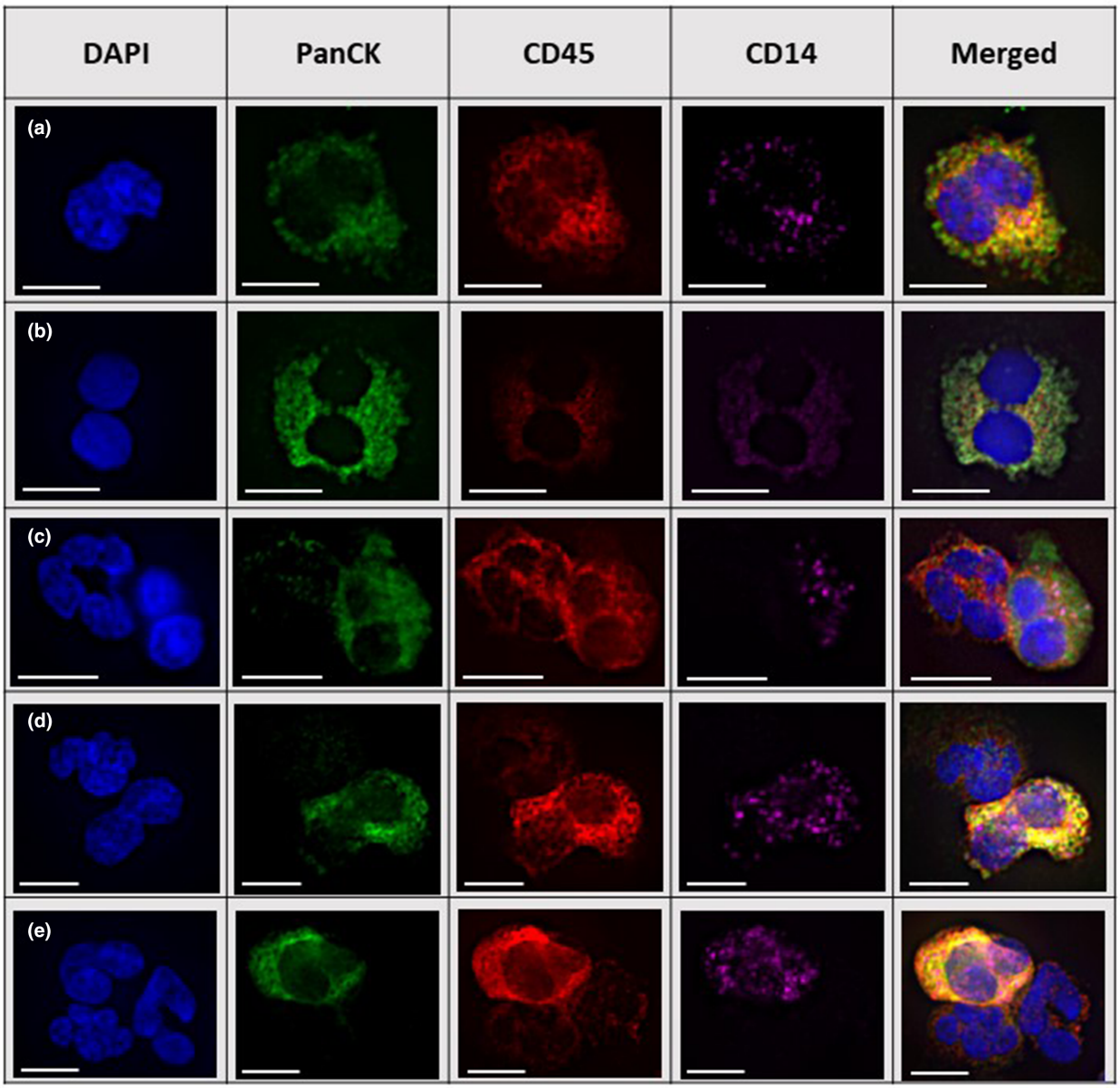


**Figure 3.** Examples of atypical tumor associated macrophages in NSCLC samples. **(a, b)** Single atypical tumor associated macrophages. **(c-e)** Doublets and triplets of atypical tumor associated macrophages and immune cells. Scale = 10 μm.

the operational definition for quantifying circulating cells. In a study that analysed CTCs in stage I–III NSCLC, researchers discovered that a size-based isolation method paired with cytomorphological analysis had a higher detection rate of CTCs than an EpCAM capture method.[36,37] Distinguishing between individual CTCs and CTC clusters is a challenge for current CTC enrichment approaches that rely on positive selection through surface marker expression or negative selection, which involves removing non-cancer cells. The possibility of losing circulating clusters containing CD45-positive cells at the aggregate's periphery is typical of negative enrichment methods like CD45 depletion targeting WBCs.[38] Most research has utilised circulating tumor cell enrichment methods that rely on the physical properties of tumor cells. In our study, we employed inertial microfluidics for CTCs, CTCs clusters and other potential tumor associated circulating cells enrichment, a method

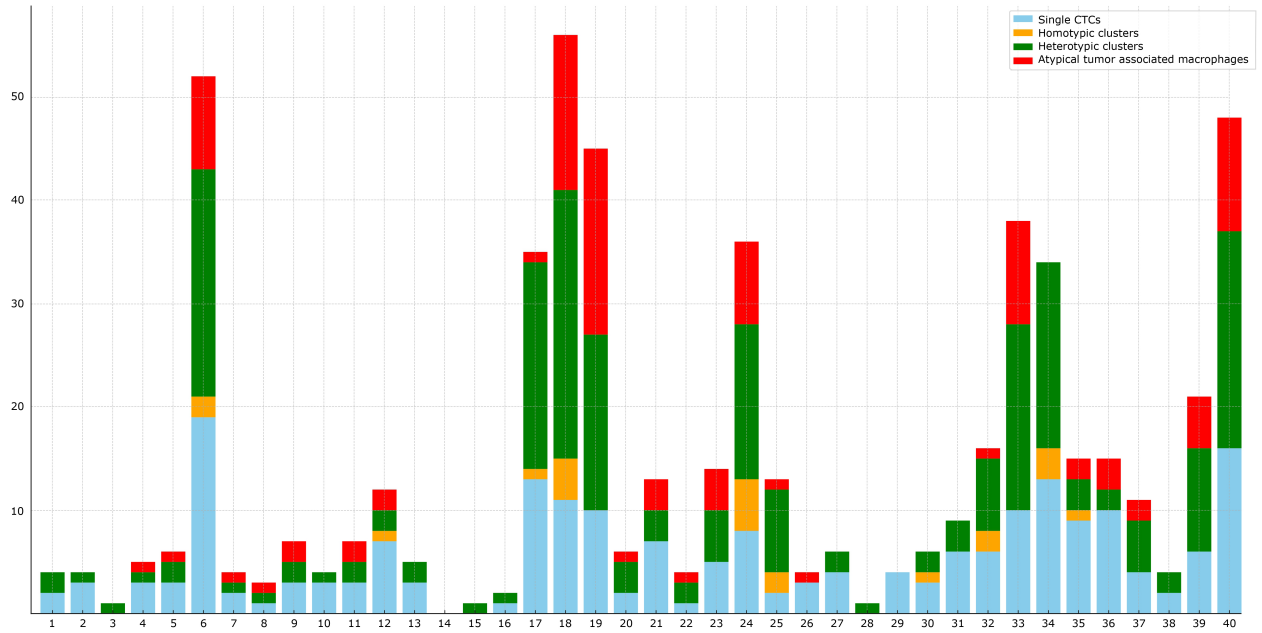


**Figure 4.** The prevalence of CTCs, homo- and heterotypic clusters, and atypical tumor associated macrophages in the NSCLC study.

previously validated for its high throughput, label-free and straightforward approach.[39] Notably, our results obtained from one cytospot per sample, which represents about 2 mL of the blood sample, were used for this study allowing for precise interpretation of the results, reproducibility and consistency in the experimental workflow.

Other than single CTCs, a number of cell subsets, which include homotypic and heterotypic clusters, are showing promise as diagnostic biomarkers because of the high specificity with which they differentiate lung cancer patients from healthy controls.[40,41] Clusters of cancer cells in conjunction with other cancer cells generate homotypic clusters while the composition of cancer cells and immune cells within a cluster turn it into a heterotypic cluster.[39] Furthermore, CAMLs and tumor-macrophage hybrids that express markers specific to both epithelial cells and leukocytes and macrophages have been discovered.[25,29] In our study, we found a similar type of cells expressing tumor, immune and macrophage markers with two main differences with CAMLs. Firstly, they are not as large as CAMLs and also formed clusters with immune cells. These cells, which have a morphology similar to some rare CTCs, could be a combination of TAMs fused with CTCs, forming a hybrid cell. Alternatively, they may have formed through a specific method of cell-to-cell communication that exploits membrane protrusions. These thin and temporary membrane structures play a role in facilitating communication between cells. These protrusions allow for the transmission of targeted messages across both short (tens of microns) and long (hundreds of microns) distances by means of direct cell-to-cell contact. Tunnelling Nanotubes (TNTs) and cytonemes, which are protrusions formed from filopodia, are thin and temporary membrane structures that have been found in a variety of tissues and disease models, both *in vitro* and *in vivo*.[42,43] This might be the other strategy that tumor cells are applying to be able to express immune markers like CD45 and CD14 saving them from immune surveillance responses.

Regardless of the various names used for stratification of tumor associated macrophages like CAMLs or TMFs, the consistent observation across all studies is their co-expression of both epithelial and macrophage markers, large cell size and heterogeneous cellular structure, often featuring multiple nuclei. Secondly, detected atypical macrophages in our study exhibit all the characteristics of common CAMLs except size, reaching a maximum of 15 μm. Multiple studies have shown that TMFs and CAMLs can be detected in the blood of cancer patients at frequencies exceeding 40% and 90%, respectively.[25,44–47] Our results show that aTAMs

were present in over 60% of the samples and are associated with poor outcomes in NSCLC patients. A study by Adams *et al.*[25] suggested that CAMLs can be an independent diagnosis indicator of cancer progression. Similarly, several papers reported a positive correlation between TMFs and the progression.[48–50] We could also find a similar trend in our samples so that the presence of aTAMs correlates with disease progression.

According to a study by Manjunath *et al.*, higher numbers of TMFs were observed with increasing NSCLC tumor stages, while the presence of CAMLs was recorded in all samples regardless of stage in another study by Sulaiman *et al.*[47,50] Of the patients from which we detected aTAMs, more than 80% were at advanced stages (III and IV). We found a positive association between the number of aTAMs detected and stage, progression and metastasis, inferring a possible direct link between the presence of aTAMs and metastatic events. It is suggested that large, multinucleated cancer cells like CAMLs exhibit higher tumorigenicity and may play a crucial role in metastasis and resistance to chemotherapy.[51–53] A pilot study by Gardner *et al.*[54] on CAMLs demonstrated the potential roles of some G protein-coupled receptors (GPCRs) in metastasis and disease progression. Likewise, genetic evidence supports that the tumor-immune fusion cells, TMFs for instance, are linked to metastasis through the acquisition of traits like genetic/epigenetic diversity, immune evasion and chemoresistance.[50]

In addition to macrophage-derived populations, CTC clusters interact with polymorphonuclear/myeloid-derived suppressor cells, which increase metastatic potential, and this suggests that CTC clusters may be a predictor or contributor to therapy resistance.[55] Studies on CTC clusters have been mostly focused on the heterotypic clusters containing neutrophils within their structures.[21,56] Patients with CTC-WBC clusters in their blood had a much lower overall survival rate after 6 months of treatment than those without clusters, regardless of whether they were present at baseline or at 6-month time points.[57] More evidence (in accordance with our results) suggests that immune cells, such as macrophages, help with immune evasion and CTC seeding when they interact closely or cluster together.[58,59] Cancer cells in these heterotypic cluster configurations use other surrounding cells to shield themselves and increase the metastatic capacity throughout the body.[60–63] While confirming the identity of these small immune cells with higher expression of CD45 and dense nuclei as T cells requires further investigation using more specific markers and larger sample sizes, detecting them in both small and large clusters with CTCs could provide a compelling insight that has not been explored before. Another compelling question is whether the contribution of various immune cells in generating heterotypic clusters affects the clinical outcomes. Are they immune suppressive or activators?

In conclusion, our study sheds light on the diverse landscape of circulating tumor cells and associated macrophage populations in NSCLC patients. We have detected various cell subpopulations, including aTAMs and CTC clusters that exhibit varied cell compositions and may serve as diagnostic biomarkers. Notably, the presence of aTAMs correlates with late disease stages and poor clinical outcomes, highlighting their potential roles in tumor progression and metastasis. Further research is warranted to elucidate the mechanisms underlying the formation and function of these cell populations and their implications for NSCLC prognosis and treatment strategies.

## METHODS

### Patient cohort

Ethics approval was obtained from the Metro South Health District Human Research Ethics Committee in accordance with the National Health and Medical Research Council guidelines (HREC/11/QPAH/331) to collect samples from the Princess Alexandra Hospital. This study has institutional approval from the Queensland University of Technology Human Research Ethics Committee (1100001420). All patients provided signed informed consent. We enrolled consecutive patients diagnosed with NSCLC, aged 18 or above, treatment-naïve, without a prior cancer diagnosis within the last 5 years. Eligible participants for chemotherapy (Carboplatin, Pemetrexed, Cisplatin, Paclitaxel, Gemcitabine and Docetaxel), immunotherapy (Pembrolizumab, Atezolizumab and Durvalumab) or combination therapy were admitted to the Princes Alexandra Hospital thoracic and lung cancer clinic from March 2023 to March 2024. Blood samples were collected from lung cancer patients on the day of or the day before the first round of systemic therapy. Blood samples from a cohort of healthy volunteers 30–50 years of age were evaluated as controls to refine target cell identification. Patients whose status information was lacking were removed from the analysis (Table 1).

**Table 1.** Patient clinicopathological and tumor data.

| Variables | N | Circulating atypical tumor associated macrophages per 2 mL of whole blood | | | |
|---|---|---|---|---|---|
| | | Present | Mean (±SEM) | Median (Range) | *P*-value |
| Total | 40 (100%) | – | – | – | – |
| Samples with atypical tumor associated macrophages | – | 25 (62.5%) | 4.24 (±0.96) | 2 (1–18) | |
| Gender | | | | | |
| Male | 26 (60%) | 18 (69.2%) | 3.67 (±1.08) | 2 (1–18) | |
| Female | 14 (40%) | 7 (50%) | 5.71 (±2.04) | 3 (1–15) | 0.33[a] |
| Age | | | | | |
| < 60 | 21 (52.5%) | | | | |
| > 60 | 19 (47.5%) | | | | |
| Histologic subtype | | | | | |
| Adenocarcinoma | 30 (75%) | 19 (63.3%) | 3.84 (±1.1) | 2 (1–18) | |
| Squamous cell carcinoma | 9 (22.5%) | 6 (66.6%) | 5.5 (±2) | 5 (1–11) | 0.766[a] |
| Pleomorphic | 1 (2.5%) | 0 | – | – | |
| Tumor staging | | | | | |
| I | 7 (17.5%) | 2 (28.5%) | 12.5 (±2.5) | 12.5 (10–15) | |
| II | 3 (7.5%) | 2 (66.6%) | 1.5 (±0.5) | 1.5 (1–2) | |
| III | 12 (30%) | 5 (41.6%) | 2 (±0.5) | 2 (1–4) | |
| IV | 18 (45%) | 16 (88.8%) | 4.25 (±1.2) | 2 (1–18) | 0.176[a] |
| PDL1 TPS | | | | | |
| < 50 | 22 (55%) | | | | |
| ≥ 50 | 11 (27.5%) | | | | |
| Unknown | 7 (17.5%) | | | | |

[a]Kruskal–Wallis test.

## Microfluidic device and its fabrication

The microfluidic device architecture, first described by Warkiani *et al*., was used to isolate CTCs, CTC clusters and large circulating cells from blood samples.[64–66] This device was made up of two polydimethylsiloxane (PDMS) layers (Sylgard 184 from Dow Corning, Midland, MI, USA) that were created by combining liquid PDMS and a curing agent in a 10:1 (w/w) ratio followed by a baking process inside the device aluminium mould. After a 2.5-min treatment with oxygen plasma (Harrick Plasma, Ithaca, NY, USA), the top layer adhered to a flat PDMS layer using the previously described moulding procedure. Furthermore, 1.5-mm holes were made at the beginning and end of the microfluidic channel, and 1.5-mm Tygon tubes (Saint-Gobain, Akron, USA) were placed into each inlet/outlet.

## Device optimisation

We evaluated the spiral chip's performance using a commercial cancer cell line to optimise its efficiency in enriching and retrieving CTCs. To fine-tune the device for CTC separation, A549 cells with 0, 10, 100, 500 and 1000 counts were injected into 5 mL of healthy blood. Enrichment was performed at five tested flow rates (1.3, 1.5, 1.7, 1.9 and 2.1 mL $min^{-1}$) to find the most effective flow rate for optimising cancer cell recovery into the target outlet. An optimal flow rate of 1.7 mL $min^{-1}$ was achieved (Figure 1). The microfluidic chip's output was separated into two parts: the 'target' part comprised the majority of the tumor cells, and the 'waste' part had the majority of the white blood cells. To prepare for antibody labeling, the cell mixture in the target fraction was fixed with a 4% paraformaldehyde (PFA) solution in DPBS for 10 min. Centrifuged at 300 *g* after fixation, 3 mL of DPBS was added to the supernatant. To confirm the validity of the results and confirm the optimum flow rate, the optimisation experiment was carried out three times.

## Processing of blood samples

Blood samples were collected from 40 patients with localised NSCLC and 5 healthy male and female volunteers under the age of 40 with no lung illnesses and were transported to the testing lab in a thermal container for microfluidic processing.

A volume of 7.5 mL of blood was mixed with a 3× volume of RBC lysis buffer and incubated for 10 min at room temperature in the dark. Afterward, the sample was centrifuged, and the resulting pellet was resuspended in 10 mL Sheath buffer. This suspension was then loaded into a 10-mL syringe and passed through a spiral chip at a flow rate of 1.7 mL $min^{-1}$ using a syringe pump. The output from the target outlet was collected, centrifuged at 300 g for 3 min at room temperature, and the cell pellet was resuspended in 1 mL of 4% PFA followed by a 10-min incubation. Subsequently, the cell suspension was washed in the same procedure previously described and resuspended in 2% BSA for a 60-min blocking step at room temperature. The pellet was resuspended in 3 mL DPBS after being incubated with 4% PFA for 10 min. Cell solution then washed with DPBS at 300 g for 3 min and

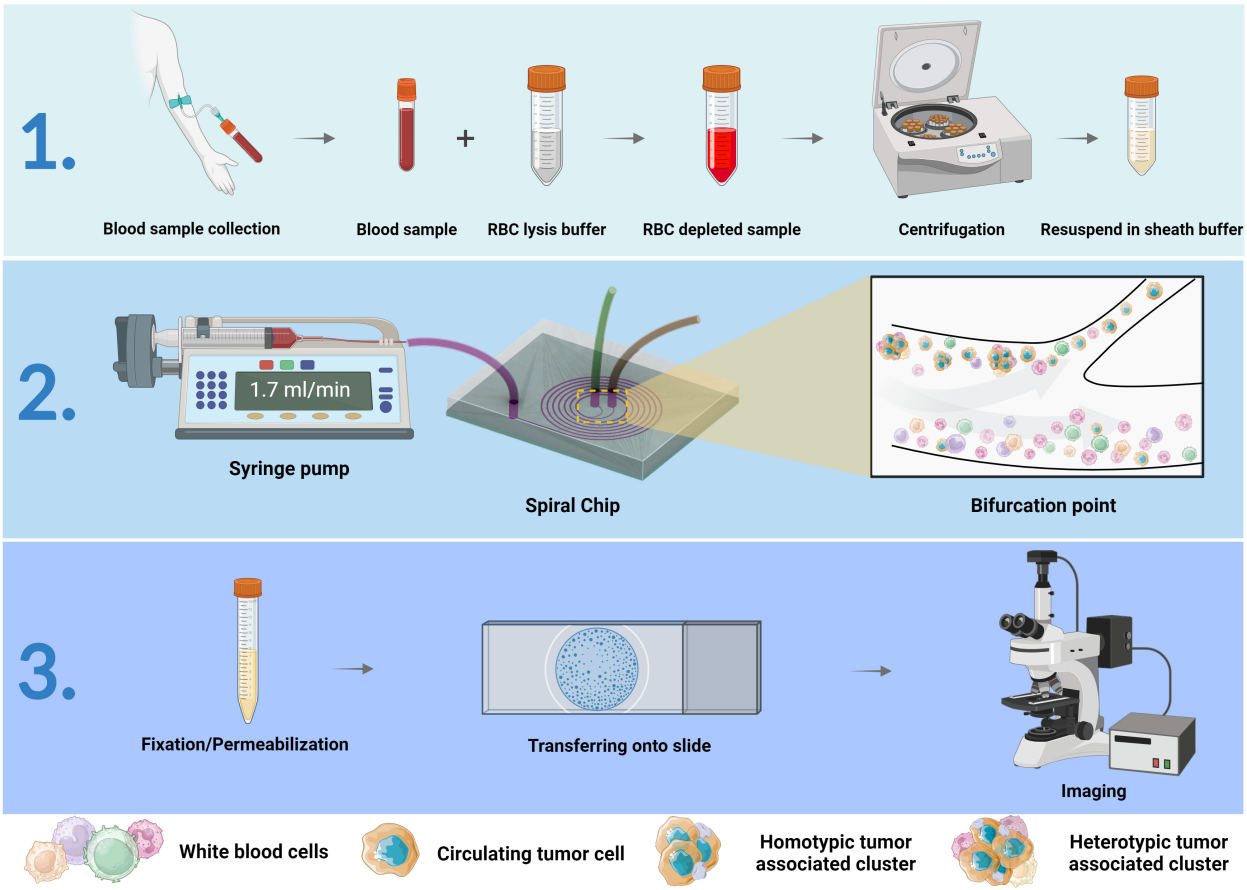


**Figure 5.** Schematic illustration of the technique for isolation of circulating tumor-associated cells from blood sample, with further detection of the isolated cells. The figure was generated by Biorender.

blocked with 2% BSA for 60 min. Another washing step was applied after the blocking procedure and then the cell suspension was then transferred to glass slides for phenotyping using a Shandon CytoCentrifuge 4 (ThermoFisher Scientific, WA, USA). Fractions of 250 μL were centrifuged at 300 g for 5 min to have 4 cytospots per sample. The slides were dried and placed in long-term storage cases at either −20 °C for short-term storage or −80 °C for long-term storage (Figure 5).

Slides were permeabilised using 0.1% Triton-X 100 for 10 min at room temperature and then washed with DPBS (3X). An antibody panel was used to stain and identify circulating tumor cells, immune cells, macrophages and other tumor-associated cells isolated from patient samples. The following antibody dilutions were optimised for staining: anti-CD14 at 1:100, anti-PanCK at 1:500 and anti-CD45 at 1:20, all diluted with BSA. Once the antibody cocktail applied to the slides, they were left to incubate for 2 h at room temperature or overnight at 4 °C with gentle agitation. Slides then were washed in TBS-T three times, each for 5 min. For nuclear staining, slides were then incubated at room temperature with DAPI (1:1000; stock solution 1 mg $mL^{-1}$) for 15 min. Before imaging with a DeltaVision ultra-focus microscope (Applied Precision, Issaquah, WA, USA), the slides were rinsed once more and mounted with ProLongTM Gold Antifade (Invitrogen, WA, USA).

## Image processing and analysis

Fiji software (National Institutes of Health, Bethesda, MD, USA) was used to process and analyse images. The software was used to perform picture improvement, pixel intensity quantification and cellular feature measurement with all images analysed consistently.

## Statistical analysis

For the association analysis, we utilised the Chi-square statistic. A *P*-value of 0.05 or below is set as the cut-off to assess significance. Python 3 was used to perform statistical analyses.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

**Vahid Yaghoubi Naei:** Formal analysis; investigation; methodology; resources; software; visualization; writing – original draft; writing – review and editing. **Ekaterina Ivanova:** Formal analysis; investigation; methodology. **William Mullally:** Investigation; methodology; project administration; writing – review and editing. **Connor G O'Leary:** Investigation; methodology; project administration; writing – review and editing. **Rahul Ladwa:** Investigation; methodology; project administration; resources; writing – review and editing. **Ken O'Byrne:** Conceptualization; funding acquisition; investigation; project administration; supervision; writing – review and editing. **Majid E Warkiani:** Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; supervision; writing – review and editing. **Arutha Kulasinghe:** Conceptualization; funding acquisition; investigation; methodology; project administration; supervision; writing – review and editing.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics 2021. *CA Cancer J Clin* 2021; **71**: 7–33.
2. Sung H, Ferlay J, Siegel RL *et al*. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021; **71**: 209–249.
3. Eslami-S Z, Cortés-Hernández LE, Sinoquet L *et al*. Circulating tumour cells and PD-L1-positive small extracellular vesicles: the liquid biopsy combination for prognostic information in patients with metastatic non-small cell lung cancer. *Br J Cancer* 2024; **130**: 63–72.
4. Huang C-Y, Chen B-H, Chou W-C, Yang C-T, Chang JW-C. Factors associated with the prognosis and long-term survival of patients with metastatic lung adenocarcinoma: a retrospective analysis. *J Thorac Dis* 2018; **10**: 2070–2078.
5. Torres JA, Brito ABC, Silva VSE *et al*. CD47 expression in circulating tumor cells and circulating tumor microemboli from non-small cell lung cancer patients is a poor prognosis factor. *Int J Mol Sci* 2023; **24**: 11958.
6. Andrikou K, Rossi T, Verlicchi A *et al*. Circulating tumour cells: detection and application in advanced non-small cell lung cancer. *Int J Mol Sci* 2023; **24**: 16085.
7. Uramoto H, Tanaka F. Recurrence after surgery in patients with NSCLC. *Transl Lung Cancer Res* 2014; **3**: 242.
8. O'connor JP, Aboagye EO, Adams JE *et al*. Imaging biomarker roadmap for cancer studies. *Nat Rev Clin Oncol* 2017; **14**: 169–816.
9. Ariga R, Bloom K, Reddy VB *et al*. Fine-needle aspiration of clinically suspicious palpable breast masses with histopathologic correlation. *Am J Surg* 2002; **184**: 410–413.
10. Pisapia P, Malapelle U, Troncone G. Liquid biopsy and lung cancer. *Acta Cytol* 2019; **63**: 489–496.
11. Strimbu K, Tavel JA. What are biomarkers? *Curr Opin HIV AIDS* 2010; **5**: 463–466.
12. Cooperman AM, Iskandar ME, Wayne MG, Steele JG. Prevention and early detection of pancreatic cancer. *Surg Clin North Am* 2018; **98**: 1–12.
13. Locker GY, Hamilton S, Harris J *et al*. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol* 2006; **24**: 5313–5327.
14. Heitzer E, Haque IS, Roberts CE, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet* 2019; **20**: 71–88.
15. Mathai RA, Vidya RVS, Reddy BS *et al*. Potential utility of liquid biopsy as a diagnostic and prognostic tool for the assessment of solid tumors: implications in the precision oncology. *J Clin Med* 2019; **8**: 373.
16. Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. *Science* 2011; **331**: 1559–1564.
17. Dasgupta A, Lim AR, Ghajar CM. Circulating and disseminated tumor cells: harbingers or initiators of metastasis? *Mol Oncol* 2017; **11**: 40–61.
18. Massagué J, Obenauf AC. Metastatic colonization by circulating tumour cells. *Nature* 2016; **529**: 298–306.
19. Zhu HH, Liu YT, Feng Y *et al*. Circulating tumor cells (CTCs)/circulating tumor endothelial cells (CTECs) and their subtypes in small cell lung cancer: predictors for response and prognosis. *Thorac Cancer* 2021; **12**: 2749–2757.
20. Guan Y, Xu F, Tian J *et al*. The prognostic value of circulating tumour cells (CTCs) and CTC white blood cell clusters in patients with renal cell carcinoma. *BMC Cancer* 2021; **21**: 826.
21. Szczerba BM, Castro-Giner F, Vetter M *et al*. Neutrophils escort circulating tumour cells to enable cell cycle progression. *Nature* 2019; **566**: 553–557.
22. Henze A-T, Mazzone M. The impact of hypoxia on tumor-associated macrophages. *J Clin Invest* 2016; **126**: 3672–3679.
23. Hirayama D, Iida T, Nakase H. The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis. *Int J Mol Sci* 2017; **19**: 92.
24. Kim J, Bae J-S. Tumor-associated macrophages and neutrophils in tumor microenvironment. *Mediat Inflamm* 2016; **2016**: e6058147.
25. Adams DL, Martin SS, Alpaugh RK *et al*. Circulating giant macrophages as a potential biomarker of solid tumors. *Proc Natl Acad Sci USA* 2014; **111**: 3514–3519.

26. Gironda DJ, Adams DL, He J *et al*. Cancer associated macrophage-like cells and prognosis of esophageal cancer after chemoradiation therapy. *J Transl Med* 2020; **18**: 413.
27. Augustyn A, Adams DL, He J *et al*. Giant circulating cancer-associated macrophage-like cells are associated with disease recurrence and survival in non–small-cell lung cancer treated with chemoradiation and atezolizumab. *Clin Lung Cancer* 2021; **22**: e451–e465.
28. Shabo I, Midtbö K, Andersson H *et al*. Macrophage traits in cancer cells are induced by macrophage-cancer cell fusion and cannot be explained by cellular interaction. *BMC Cancer* 2015; **15**: 92.
29. Adams DL, Adams DK, Alpaugh RK *et al*. Circulating cancer-associated macrophage-like cells differentiate malignant breast cancer and benign breast conditions. *Cancer Epidemiol Biomarkers Prev* 2016; **25**: 1037–1042.
30. Gast CE, Silk AD, Zarour L *et al*. Cell fusion potentiates tumor heterogeneity and reveals circulating hybrid cells that correlate with stage and survival. *Sci Adv* 2018; **4**: 7828.
31. Mu Z, Benali-Furet N, Uzan G *et al*. Detection and characterization of circulating tumor associated cells in metastatic breast cancer. *Int J Mol Sci* 2016; **17**: 1665.
32. Ramakrishnan M, Mathur SR, Mukhopadhyay A. Fusion-derived epithelial cancer cells express hematopoietic markers and contribute to stem cell and migratory phenotype in ovarian carcinoma. *Cancer Res* 2013; **73**: 5360–5370.
33. Manjunath Y, Suvilesh KN, Avella DM *et al*. Abstract 5375: large, polymorphonuclear circulating cancer-associated cell with dual epithelial and macrophage/myeloid phenotype is prognostic in non-small cell lung cancer and has an impact on anti-tumor immune responses. *Cancer Res* 2020; **80**: 5375.
34. Aceto N, Bardia A, Miyamoto DT *et al*. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell* 2014; **158**: 1110–1122.
35. Aceto N, Toner M, Maheswaran S, Haber DA. En route to metastasis: circulating tumor cell clusters and epithelial-to-mesenchymal transition. *Trends Cancer* 2015; **1**: 44–52.
36. Alix-Panabières C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. *Clin Chem* 2013; **59**: 110–118.
37. Hofman V, Ilie MI, Long E *et al*. Detection of circulating tumor cells as a prognostic factor in patients undergoing radical surgery for non-small-cell lung carcinoma: comparison of the efficacy of the CellSearch assay™ and the isolation by size of epithelial tumor cell method. *Int J Cancer* 2011; **129**: 1651–1660.
38. Kulasinghe A, Zhou J, Kenny L, Papautsky I, Punyadeera C. Capture of circulating tumour cell clusters using straight microfluidic chips. *Cancer* 2019; **11**: 89.
39. Yaghoubi Naei V, Bordhan P, Mirakhorli F *et al*. Advances in novel strategies for isolation, characterization, and analysis of CTCs and ctDNA. *Ther Adv Med Oncol* 2023; **15**: e17588359231192401.
40. Manjunath Y, Upparahalli SV, Suvilesh KN *et al*. Circulating tumor cell clusters are a potential biomarker for detection of non-small cell lung cancer. *Lung Cancer* 2019; **134**: 147–150.
41. Mascalchi M, Maddau C, Sali L *et al*. Circulating tumor cells and microemboli can differentiate malignant and benign pulmonary lesions. *J Cancer* 2017; **8**: 2223–2230.
42. Desir S, O'Hare P, Vogel RI *et al*. Chemotherapy-induced tunneling nanotubes mediate intercellular drug efflux in pancreatic cancer. *Sci Rep* 2018; **8**: 9484.
43. Lou E, Fujisawa S, Morozov A *et al*. Tunneling nanotubes provide a unique conduit for intercellular transfer of cellular contents in human malignant pleural mesothelioma. *PLoS One* 2012; **7**: e33093.
44. Clawson GA, Kimchi E, Patrick SD *et al*. Circulating tumor cells in melanoma patients. *PLoS One* 2012; **7**: e41052.
45. Joosse SA, Pantel K. Biologic challenges in the detection of circulating tumor cells. *Cancer Res* 2013; **73**: 8–11.
46. Kaifi JT, Kunkel M, Das A *et al*. Circulating tumor cell isolation during resection of colorectal cancer lung and liver metastases: a prospective trial with different detection techniques. *Cancer Biol Ther* 2015; **16**: 699–708.
47. Sulaiman R, De P, Aske JC *et al*. Identification and morphological characterization of features of circulating cancer-associated macrophage-like cells (CAMLs) in endometrial cancers. *Cancers (Basel)* 2022; **14**: 4577.
48. Clawson GA, Matters GL, Xin P *et al*. "Stealth dissemination" of macrophage-tumor cell fusions cultured from blood of patients with pancreatic ductal adenocarcinoma. *PLoS One* 2017; **12**: e0184451.
49. LaBerge GS, Duvall E, Grasmick Z, Haedicke K, Pawelek J. A melanoma lymph node metastasis with a donor-patient hybrid genome following bone marrow transplantation: a second case of leucocyte-tumor cell hybridization in cancer metastasis. *PLoS One* 2017; **12**: e0168581.
50. Manjunath Y, Porciani D, Mitchem JB *et al*. Tumor-cell–macrophage fusion cells as liquid biomarkers and tumor enhancers in cancer. *Int J Mol Sci* 2020; **21**: 1872.
51. Mirzayans R, Andrais B, Murray D. Roles of polyploid/multinucleated giant cancer cells in metastasis and disease relapse following anticancer treatment. *Cancer* 2018; **10**: 118.
52. Tang CM, Zhu P, Li S, Makarova OV, Amstutz PT, Adams DL. Blood-based biopsies—Clinical utility beyond circulating tumor cells. *Cytometry A* 2018; **93**: 1246–1250.
53. Zhang S, Mercado-Uribe I, Xing Z, Sun B, Kuang J, Liu J. Generation of cancer stem-like cells through the formation of polyploid giant cancer cells. *Oncogene* 2014; **33**: 116–128.
54. Gardner KP, Cristofanilli M, Chumsri S *et al*. Beta 2-adrenergic receptor in circulating cancer-associated cells predicts for increases in stromal macrophages in circulation and patient survival in metastatic breast cancer. *Int J Mol Sci* 2022; **23**: 7299.
55. Sprouse ML, Welte T, Boral D *et al*. PMN-MDSCs enhance CTC metastatic properties through reciprocal interactions via ROS/notch/nodal signaling. *Int J Mol Sci* 2019; **20**: 1916.
56. Raskov H, Orhan A, Gaggar S, Gögenur I. Neutrophils and polymorphonuclear myeloid-derived suppressor cells: an emerging battleground in cancer therapy. *Oncogenesis* 2022; **11**: 22.
57. Jansson S, Bendahl PO, Larsson AM, Aaltonen KE, Rydén L. Prognostic impact of circulating tumor cell apoptosis and clusters in serial blood samples from patients with metastatic breast cancer in a prospective observational cohort. *BMC Cancer* 2016; **16**: 433.

58. Harney AS, Arwert EN, Entenberg D *et al*. Real-time imaging reveals local, transient vascular permeability, and tumor cell intravasation stimulated by TIE2hi macrophage–derived VEGFA. *Cancer Discov* 2015; **5**: 932–943.
59. Roh-Johnson M, Bravo-Cordero JJ, Patsialou A *et al*. Macrophage contact induces RhoA GTPase signaling to trigger tumor cell intravasation. *Oncogene* 2014; **33**: 4203–4212.
60. Liu Q, Liao Q, Zhao Y. Myeloid-derived suppressor cells (MDSC) facilitate distant metastasis of malignancies by shielding circulating tumor cells (CTC) from immune surveillance. *Med Hypotheses* 2016; **87**: 34–39.
61. Ward MP, E. Kane L, A. Norris L *et al*. Platelets, immune cells and the coagulation cascade; friend or foe of the circulating tumour cell? *Mol Cancer* 2021; **20**: 59.
62. Liu X, Zuo F, Song J *et al*. Immune checkpoints HLA-E: CD94-NKG2A and HLA-C:KIR2DL1 complementarily shield circulating tumor cells from NK-mediated immune surveillance. *Cell Discov* 2024; **10**: 16.
63. Spicer JD, McDonald B, Cools-Lartigue JJ *et al*. Neutrophils promote liver metastasis via Mac-1-mediated interactions with circulating tumor cells. *Cancer Res* 2012; **72**: 3919–3927.
64. Kulasinghe A, Tran THP, Blick T *et al*. Enrichment of circulating head and neck tumour cells using spiral microfluidic technology. *Sci Rep* 2017; **7**: 42517.
65. Warkiani ME, Guan G, Luan KB *et al*. Slanted spiral microfluidics for the ultra-fast, label-free isolation of circulating tumor cells. *Lab Chip* 2014; **14**: 128–137.
66. Warkiani ME, Khoo BL, Wu L *et al*. Ultra-fast, label-free isolation of circulating tumor cells from blood using spiral microfluidics. *Nat Protoc* 2016; **11**: 134–148.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.