The value of characterising Circulating Tumour Cells using Epithelial and Mesenchymal markers in Breast Cancer

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Introduction

Circulating Tumour Cells (CTCs) contain essential information that may help monitor and inform disease management. Most CTC isolation platforms use epitope-dependent methods, such as EpCAM expression, for enrichment. However, it is well understood that CTCs can undergo Epithelial-to-Mesenchymal (EMT) transition during extravasation (**Figure 1**), switching to a mesenchymal (Mes+) or a transitioning (EMT+) phenotype as they enter the bloodstream to establish distant metastases (*Gu X., et al, 2024*). Moreover, in a previous study (*Ciccioli M., et al, 2024*), we identified a population of cytokeratin-positive (Epi+) CTCs that were EpCAM-negative in metastatic breast cancer (MBC) patients, suggesting heterogeneity within the Epi+ CTC population as well. EpCAM-based CTC isolation methods may fail to detect clinically relevant populations of both Mes+ CTCs or EpCAM negative Epi+ CTCs, highlighting the need for an epitope-independent CTC enrichment method. In this study, we present a Research Use Only assay, combining CTC harvesting using ANGLE's Parsortix® device and downstream immunofluorescence (IF) targeting epithelial and mesenchymal markers in Ductal and Lobular MBC patients.

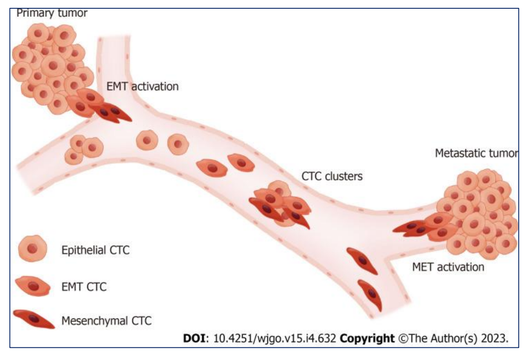
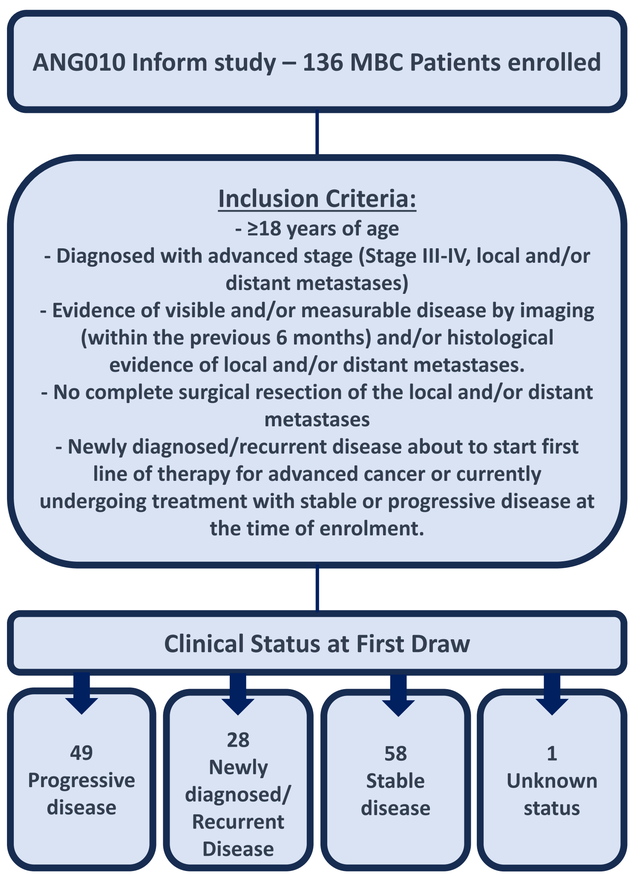


Figure 1. Epithelial to Mesenchymal transition in cancer. Figure adapted from Ko, S.W et al (2023). Clinical implications and perspectives of portal venous circulating tumor cells in pancreatic cancer. World Journal of Gastrointestinal Oncology.

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

Study Design

Peripheral blood (up to 15 mL) was drawn into Streck Cell-Free DNA blood tubes from 136 MBC patients, for up to 6 draws per patient (minimum 30 days apart) totalling 1048 total samples **(Figure 2).**



Demographics	Count	Percent (%)
Patients		
Total patients	136	-
Total samples	1048	-
Total deceased patients	21	15.00%
Age		
Mean ± Standard Deviation (SD)	62.0 ± 11.2	-
Range	32-83	-
Age at first blood draw		
30-39	3	2.00%
40-49	17	13.00%
50-59	36	26.00%
60-69	43	32.00%
≥70	37	27.00%
Gender		
Female	132	97.10%
Male	3	2.20%
Not specified	1	0.70%
Date of Diagnosis		
Diagnosis range	1992 to 2024	-
Cancer type		
Breast	136	100.00%
Histologic Subtype		
Ductal	86	63.20%
Lobular	19	14.00%
Not specified/Unknown	31	22.80%
HER2		
Negative	91	66.90%
Positive	19	14.00%
Not specified/Unknown	26	19.10%
ER		
Positive	115	84.60%
Negative	13	9.60%
Not specified/Unknown	8	5.90%
PR		
Positive	75	55.20%
Negative	28	20.60%
Not specified/Unknown	33	24.20%

Figure 2. Schematic representation of the study criteria and demographic summary. Diagram on the left details the total number of metastatic breast cancer patients enrolled in this study, along with the inclusion criteria and a breakdown on clinical status. Table on the right is a summary of the total patients, number of patients who died during course of study, total samples included in the study, breakdown on the age (mean, median, percentage per age group), gender, date of diagnosis, cancer type and histological subtype.

Workflow

All samples were processed on Parsortix instruments up to 144 hours post-draw, harvested onto ANGLE's Cellkeep™ slides and stained with ANGLE's IF assay comprising of epithelial and mesenchymal markers (for positive selection) and blood lineage markers (for negative selection).

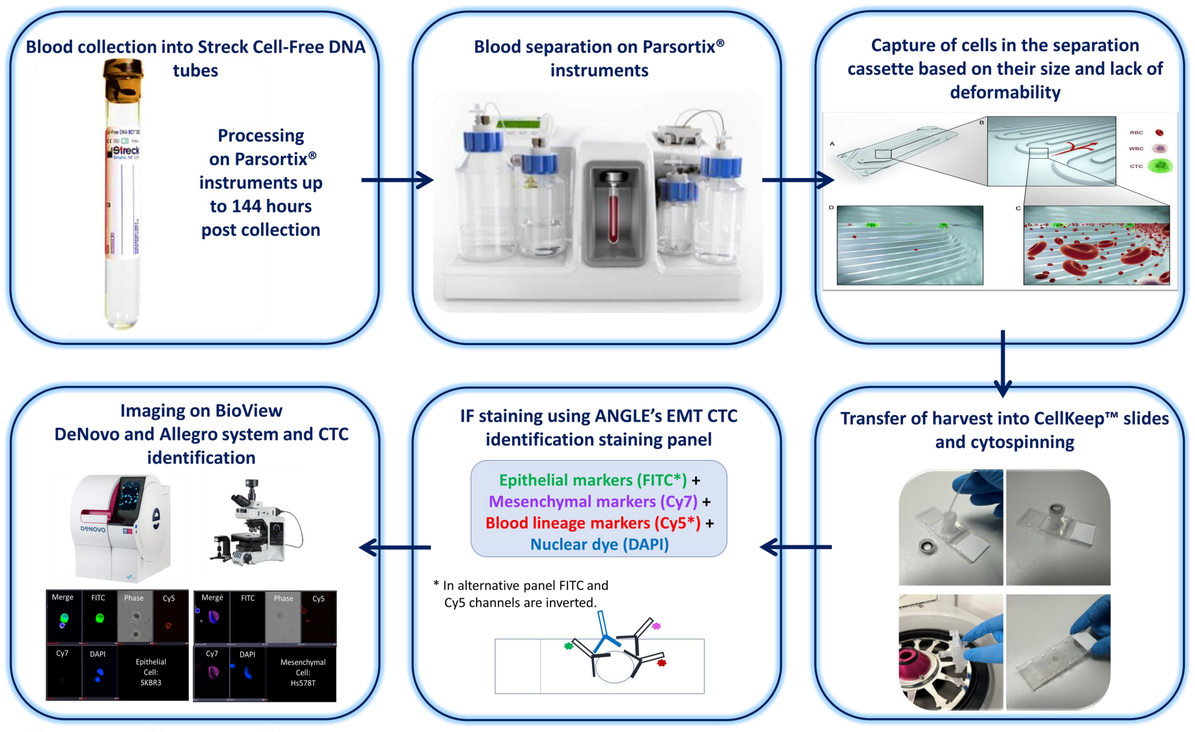


Figure 3. Schematic representation of the assay workflow. Two tubes of 7.5 mL of peripheral blood were collected into Streck Cell-Free DNA tubes from 136 metastatic breast cancer patients for up to six draws per donor. Blood was stored up to 144 hours from collection before processing. Blood samples 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 where CTCs were defined as epithelial (FITC+, Cy7-, Cy5-, DAPI+), mesenchymal (FITC-, Cy7+, Cy5-, DAPI+), or EMT transitioning (FITC+, Cy7+, Cy5-, DAPI+). Different versions of the same panel, using antibodies against the same targets with different fluorophores, were applied to some samples in this study. Stained slides were imaged using a BioView Allegro Plus system or the BioView DeNovo system, platforms equipped with a deep learning algorithm for automated imaging, CTC candidate identification and reporting.

Results

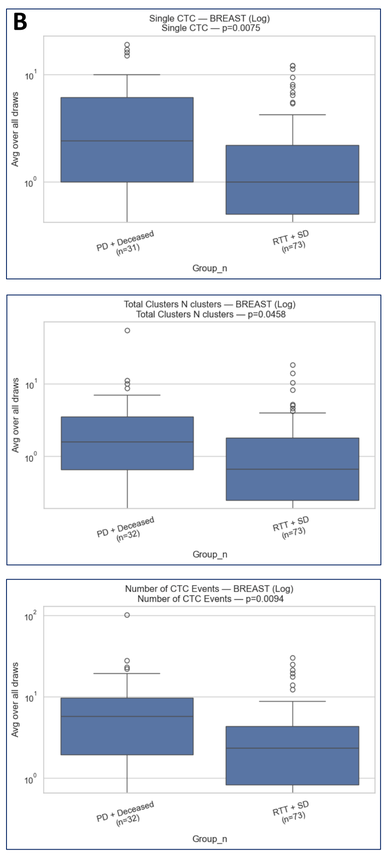
CTC Summary data

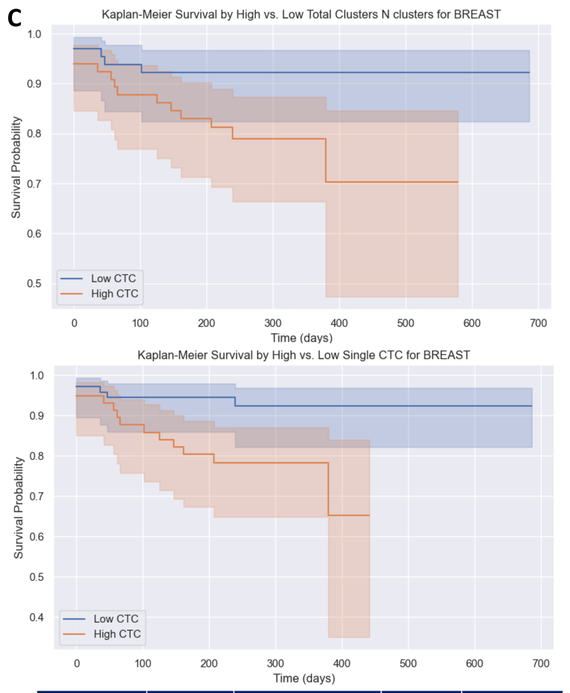
- **Figure 4.A** shows that CTCs (≥1) were identified in at least one draw of 92% of patients and ≥5 CTCs were found in 76% of patients.
- CTC clusters (≥1) were identified in 81% of CTC positive patients, with the size of clusters ranging from 2 to 313 CTCs.
- 35% donors had only Mes+ CTCs, whilst just 8% of donors showed only Epi+ CTCs. Most donors (57%) showed a mix of both Epi+ and Mes+/or EMT CTC phenotypes.

A	All patients
N – Processed	136
N – Analysable	133
N (%) with ≥1 CTC	122 (92%)
N (%) with ≥5 CTCs	101 (76%)
Median CTCs	6
Mean CTCs	24
CTC Range	1-496
N (%) with ≥1 CTC Cluster	108 (81%)
N CTCs per Cluster (range)	2-313
N Clusters (range)	1-130
N (%) Epithelial + only	11 (8%)
N (%) Mesenchymal + only	46 (35%)
N (%) Epithelial + and Mesenchymal +/or EMT+	76 (57%)

Survival Analysis

- CTCs were assessed by clinical status. **Figure 4.B** shows a statistically significant increase in single CTCs (all phenotypes), as well as all CTC clusters and all CTC events in the deceased + Progressive disease (PD) group as compared to the group comprising patients responding to treatment (RTT) and stable (SD).
- Higher CTCs count (both single and clusters) was associated with decreased survival probability **(Figure 4.C).** This was statistically significant only when considering all CTCs, not when accounting for either only Epi+ or only Mes+ CTCs.





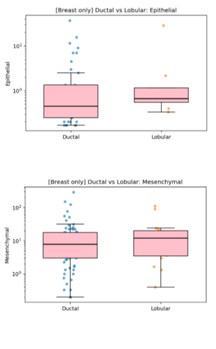
CTC Type	Baseline Comparison (Median)	Does a higher CTC count decrease survival probability?	Log – rank test p – value	Statistically Significant
Epithelial (Single + In Cluster)	0	Yes	0.25	No
MZ (Single + In Cluster)	3.36	Yes	0.26	No
Total Clusters	**0.64**	**Yes**	**0.04**	**Yes**
Total Single	**1**	**Yes**	**0.01**	**Yes**

Subtype Analysis – Lobular vs Ductal MBC

- **Figure 4.D** shows that median total, as well as Epi+ and Mes+, CTCs were comparable between Lobular and Ductal MBC.
- A higher proportion of patients with CTC clusters were identified in Lobular MBC (90% vs. 78%), along with higher mean and median cluster counts. Higher CTC and cluster counts in the Lobular MBC cohort did not correlate with mortality (0%), whereas 16% Ductal MBC patients died.
- In this group, **Figure 4.E** shows that deceased patients had a statistically significant higher median total and Epi+ CTCs, as well as a higher occurrence of CTC clusters (p = 0.0029).

D

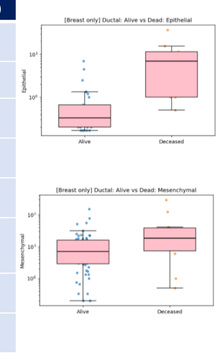
Cancer Subtype	Ductal (86 donors)	Lobular (19 donors)
% Deceased	16%	0%
All CTC Positivity (≥1) %	93%	90%
Mean/Median All CTCs	20/9	23/13
Epithelial Positivity (≥1) %	46%	42%
Mean/Median Epithelial CTCs	3/<1	4/<1
Mesenchymal Positivity (≥1) %	84%	84%
Mean/Median Mesenchymal CTCs	20/8	23/12
CTC Cluster positivity (≥1) %	78%	90%
Mean/Median CTC Cluster #	6/3	9/5



Comparison	P value	Test
All CTCs Lobular vs Ductal	0.294286	Mann-Whitney U
Epithelial Lobular vs Ductal	0.288212	Mann-Whitney U
Mesenchymal Lobular vs Ductal	0.427974	Mann-Whitney U
CTC Clusters Lobular vs Ductal	0.430683	Mann-Whitney U

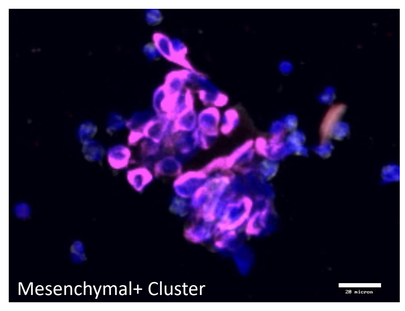
E

Cancer Subtype	Alive (72 donors)	Deceased (14 donors)
All CTC Positivity (≥1) %	88%	93%
Mean/Median All CTCs	14/7	48/16
Epithelial Positivity (≥1) %	40%	64%
Mean/Median Epithelial CTCs	<1/<1	10/7
Mesenchymal Positivity (≥1) %	82%	71%
Mean/Median Mesenchymal CTCs	14/7	54/19
CTC Cluster positivity (≥1) %	76%	86%
Mean/Median CTC Cluster #	2/1	8/3



Comparison	P value	Test
All CTCs Alive vs Deceased	**0.014337**	Mann-Whitney U
Epithelial Alive vs Deceased	**0.000271**	Mann-Whitney U
Mesenchymal Alive vs Deceased	0.086666	Mann-Whitney U
CTC Clusters Alive vs Deceased	**0.002964**	Mann-Whitney U

F



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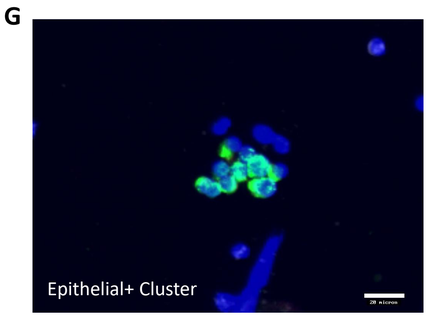

Figure 4. CTC positivity, survival and cancer subtype analysis. (A) Table showing descriptive statistics for all patients processed including % positivity, mean, median, range of CTCs and CTC clusters, breakdown of positivity by phenotype per patient. **(B)** Box and whisker plots (logarithmic scale) of (from top) total single CTCs, total clusters and all CTC events (single and clustered CTCs counted as one) compared between progressive (PD) and deceased patients with patients who were either classed as responding to treatment (RTT) or stable (SD). A statistically significant difference (p = <0.05) was identified (Mann Whitney U test for independent non - parametric data) between the two patient populations. **(C)** Kaplan-Meier survival plots showing survival probability as it relates to (from top) high versus low CTC clusters and total single CTCs, regardless of phenotype. High and low groups were defined based on the median value (baseline comparison). Summary table below denotes the p value. **(D)** Summary table displays comparison, in Lobular and Ductal metastatic breast cancer cohorts, of percentage positive donors and mean/median number of total (all CTC phenotypes), epithelial, mesenchymal positive CTCs and number of CTC clusters. Box and whisker plots (right) show differences in median Epithelial (top) survival and Mesenchymal (bottom) CTC populations. Bottom table displays statistical testing and p value of the different comparisons. **(E)** Summary table displays comparison between alive and deceased patients, in Ductal metastatic breast cancer cohort, of percentage positive donors and mean/median number of total (all CTC phenotypes), epithelial, mesenchymal positive CTCs and number of CTC clusters. Box and whisker plots (right) show differences in median Epithelial (top) survival and Mesenchymal (bottom) CTC populations. Bottom table displays statistical testing and p value of the different comparisons. **(F)** and **(G)** are representative merged images of mesenchymal positive and epithelial positive CTC clusters, respectively. Images were taken using a 10X magnification using the Bioview Allegro automated imaging system. Scale bar = 20 µm, **FITC** = epithelial markers, **Cy7** = mesenchymal markers , **Cy5** = (white in merged image) blood lineage markers, **DAPI** = nucleus.

Conclusions

- This study demonstrates that a high proportion of MBC patients have either only Mes+ CTCs, Mes+ CTC clusters, or both Mes+ and Epi+ CTCs. Greater number of total CTCs which includes Mes+ and clusters, was correlated with worse overall survival.
- This study highlights the importance of using an epitope-independent CTC detection method, in order to identify all CTC phenotypes and CTC clusters. This may allow for more informative longitudinal monitoring, potentially enabling a more complete understanding of disease progression.