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A comparison of CellCollector with CellSearch in patients with neuroendocrine tumours

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Table 1, Figures 1.

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Dear Editor

Circulating tumour cells (CTCs) have been hypothesised to be mediators of metastases [1] but, with numbers as low as one per 10^7 white cells [2], their utility as biomarkers has been limited by low rates of detection and isolation. CTCs have been identified in patients with metastatic neuroendocrine tumours (NETs) using the FDA-cleared CellSearch (Janssen Diagnostics) technology, a semi-automated platform that uses immunomagnetic enrichment of CTCs based on expression of epithelial cell adhesion molecule (EpCAM) [3]. Using this platform, CTCs were found in 36% of patients with pancreatic NETs and 53% of those with midgut NETs. The presence of CTCs is associated with a worse overall survival, and early changes in CTC number following treatment in NET patients are also prognostic [4, 5]. CTCs may also be considered as 'liquid biopsies', offering the opportunity to interrogate the molecular characteristics of the tumour. For such an approach to be broadly applicable, alternative technologies are required to increase number of CTCs isolated and the proportion of patients in which they can be detected.

The CellCollector (GILUPI GmbH) is a novel medical device consisting of a 160mm sterile steel wire of which the terminal 20mm is coated with anti-EpCAM antibodies covalently coupled to a gold and hydrogel layer. The CellCollector is inserted into a peripheral vein enabling the circulating blood volume to be sampled. The wire is stained with fluorescently labelled antibodies and examined microscopically to identify CTCs. The clinical application of this device has been previously reported in patients with breast and lung cancer [6]

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In this study we sought to compare the performance of the CellCollector and CellSearch in patients with metastatic NETs. Thirty-four patients provided written informed consent and were recruited into the study (Figure 1A). The protocol was approved by the central ethical review board (IRAS Project ID 105772). The CellCollector was inserted into the cubital vein via a 20G cannula and left *in situ* for 30 minutes after which it was removed, washed in phosphate buffered saline (PBS) and fixed in acetone. The cells were permeabilized (Triton X-100 in PBS, 0.1% concentration) at room temperature, washed in PBS and incubated with blocking buffer (bovine serum albumin (BSA)/PBS,3% concentration). Immunostaining was performed with a solution containing FITC conjugated antibodies against EpCAM [1:50; HEA125, Acris antibodies, Germany], cytokeratin 19 conjugated with Alexa488 (1:50, A53-B/A2, Life technologies Corporation, US), pan-cytokeratin-Alexa488 (1:50, C11,eBioscience, California) and cytokeratin 7-FITC (1:50, LP5K Milipore,MA). An Alexa-Fluor 647 conjugated anti-CD45 rabbit polyclonal antibody was added as negative marker to exclude white blood cells (1:25, MEM-28Exbio, Czech Republic). Finally, the wire was incubated in the nuclear stain, Hoesch 33342 (Sigma), (concentration 1ug/ml). The wire was examined in a bespoke holder allowing inspection in four planes using an Axio Imager microscope with digital camera and AxioVision software.

CTCs were defined according to the following criteria: 1. Intact cellular morphology , 2. Cell diameter more than 4 μm, 3. Positive for cytokeratin and nuclear stain, but negative for CD45, 4. Nuclear stain distinct from the cytokeratin or EpCAM staining. Examples of positively identified CTCs are shown in Figure 1B. The number of CTCs was enumerated by two independent operators who were blind to the patient's

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clinical information. Where there was disagreement between the two operators, a third operator arbitrated. A 7.5 ml peripheral blood sample was collected concurrently into a CellSave tube and analysed within 72 hours by CellSearch as previously described [3].

The interobserver variation for CellSearch has been previously reported [7], and here we demonstrated good correlation between observers enumerating CTCs using the CellCollector achieving Spearman's correlation of 0.92 (95% CI 0.85, 0.96) ($p < 0.0001$) (Figure 1C). The median number of CTCs enumerated with CellCollector was 6 (range 2-49), compared to a median of 0 (range 0-57) with CellSearch ($P<0.0001$[Mann Whitney U test]). In 33/34 patients, there was ≥1 CTC found compared to only 16/34 patients with CellSearch. (Table 1). Therefore, CTCs were detected in greater numbers and a greater proportion of patients with the CellCollector (Figure 1D). The CellCollector identified CTCs in all midgut NETs, and 12/13 PNETS.

We explored the prognostic relevance of CTC count according to CellCollector. With a median follow-up period of 13 months, overall survival data was insufficiently mature so we examined progression free survival (PFS) as a surrogate. Overall, 14 patients had progression by RECIST criteria and applying a cut-off of 7 CTCs, there was a significant difference in PFS (Cox Hazard Ratio 3.4, $P<0.05$). Using the same threshold in the Kaplan Meier survival analyses (Figure 1E), median PFS was 11 months for patients with ≥7CTCs but not reached for those with <7 (Log Rank $P<0.05$).

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Here, we have demonstrated for the first time, that the CellCollector is able to detect CTCs in in more NET patients and in greater numbers than CellSearch. However, the CellSearch has been extensively validated and remains a robust method for prognostication whilst the CellCollector offers the potential to make molecular analysis of CTCs more widely applicable. Indeed, a recent study in lung cancer demonstrated both *KRAS* and *EGFR* mutations known to be present in the primary tumour, in CTCs derived from the CellCollector using chip-based digital PCR [8]. Other strategies to increase the volume of blood sampled for CTCs include the use of leukapheresis [9]. However, the leukapheresis product has a very high rate of contaminating leukocytes and requires downstream enrichment methods to isolate CTCs. Compared with CellCollector, leukapheresis is also more time-consuming, expensive and onerous for patients [10].

The CellCollector, like CellSearch, is limited by the dependence on EpCAM as a selection marker for CTCs, and a biologically important component of EpCAM negative CTCs will not be sampled by either technology. Marker agnostic devices based on size exclusion or biophysical properties rather than antigen expression, offer an alternative method of CTC isolation but remain limited by the small volume of blood that can be sampled.

In summary, the CellCollector appears to be a promising innovation that may help enhance our understanding of CTC biology and the mechanism of metastasis.

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Declaration of Interest; No conflict of interest

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A

Primary	Midgut n=18	PNET n=14	Other n=3
Age: median range	59 (40-74)	58.6 (36-66)	50 (40-56)
Sex: Female Male	5 13	6 8	3
Median duration	64.5	32	62
< 25% Liver disease > 25% Liver disease	8 10	7 7	1 2
Primary resection	11	2	2
Grade 1 Grade 2 Grade 3	15 3 0	2 10 2	0 2 1
Metastatic Sites Lymph Node Bone Lung Peritoneal Brain Other	 16 4 1 10 0 2	 12 3 1 1 0 1	 2 2 1 1 0 0
Previous therapy SST Analogues Chemotherapy TAE RFA PRRT Sunitinib Everolimus Interferon	 13 1 1 1 3 0 0 1	 5 5 0 0 2 0 0 0	 2 1 0 1 0 0 0 0

Figure 1A: Clinicopathological details of study cohort (SST= somatostatin, TAE=transarterial embolization, RFA = radiofrequency ablation, PRRT= Peptide radiotargeted receptor therapy)

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B

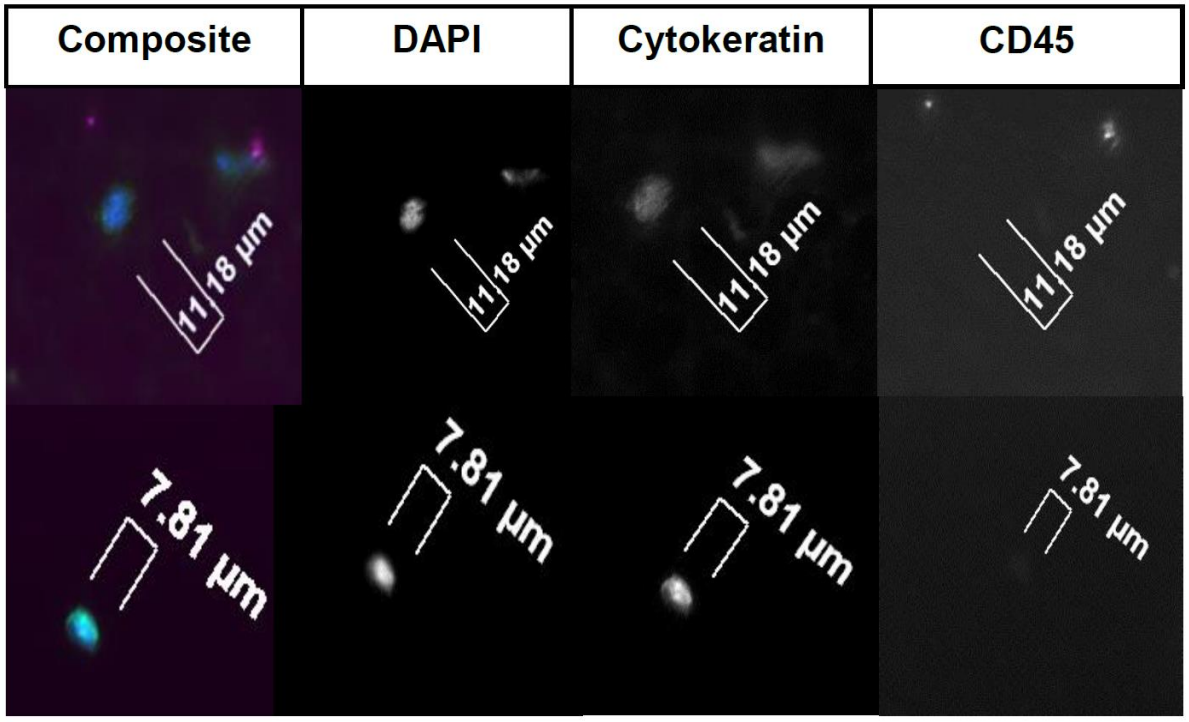

Figure 1B: Examples of CTCs identified using immunofluorescent microscope, with signal for each channel demonstrated alongside composite image.

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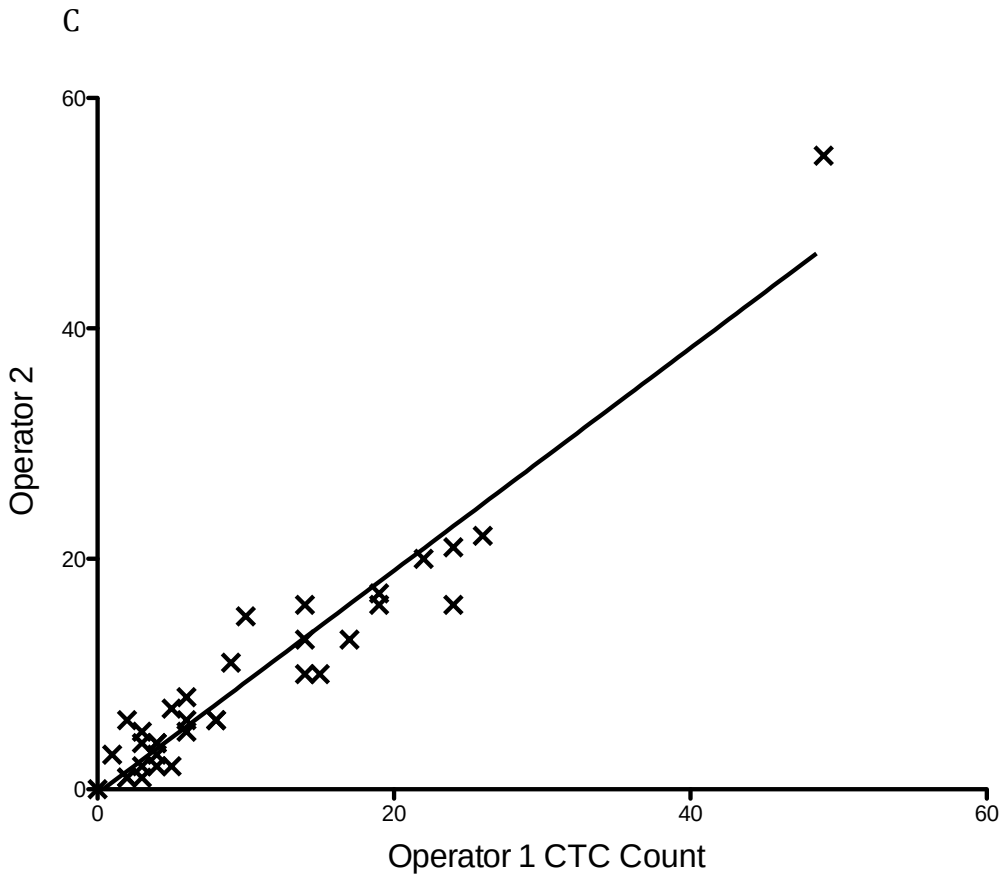

Figure 1C: Correlation between CTC identified by each operator for each wire enumerated

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D

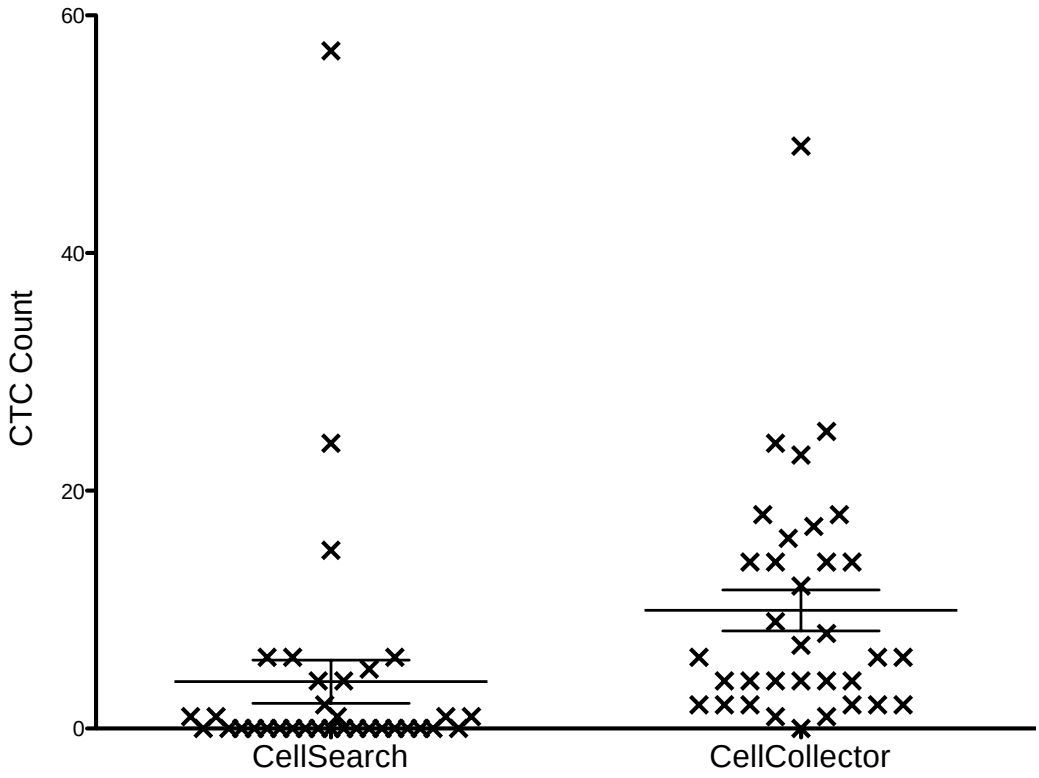

Figure 1D: Scattergram CTCs identified by CellCollector compared to CellSearch

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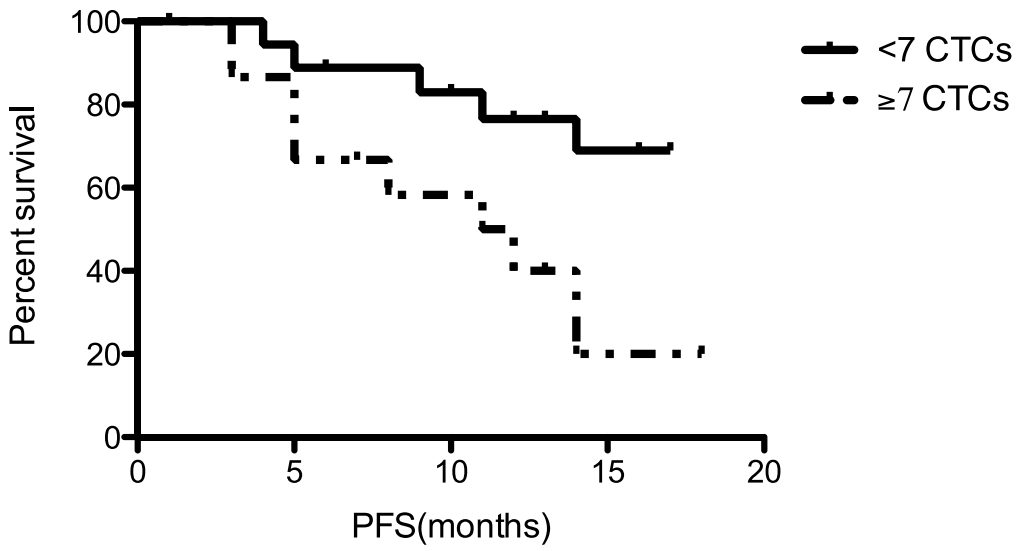

Figure 1E: Kaplan Meier survival for PFS when using 7 CTCs as threshold

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Pt No.	Primary	Grade	CTC by CellSearch	CTC by CellCollector	>25% Liver involvement	≥3 metastati sites
01	Midgut	1	4	14	Yes	Yes
02	Midgut	1	1	2	Yes	Yes
03	PNET	2	0	2	No	Yes
04	PNET	1	1	1	Yes	Yes
05	Hindgut	2	1	1	Yes	Yes
06	Midgut	1	6	2	No	Yes
07	PNET	2	0	4	Yes	Yes
08	Midgut	1	1	2	No	No
19	PNET	2	0	4	Yes	No
10	PNET	2	0	4	Yes	No
11	PNET	3	0	2	No	No
12	Midgut	1	1	4	No	No
13	PNET	3	6	9	Yes	Yes
14	Bronchial	2	0	2	No	Yes
15	Midgut	1	57	49	Yes	No
16	PNET	1	0	8	No	No
17	PNET	2	0	0	No	No
18	Midgut	2	0	4	Yes	Yes
19	Midgut	1	0	17	Yes	No
20	Midgut	1	0	6	No	No
21	PNET	2	0	24	No	Yes
22	Midgut	1	0	14	Yes	No
23	Midgut	1	0	14	Yes	Yes
24	Midgut	1	0	6	No	Yes
25	Unknown	3	24	25	Yes	Yes
26	Midgut	1	0	16	Yes	No
27	PNET	2	0	18	Yes	Yes
28	Midgut	2	0	4	No	no
29	PNET	2	5	14	Yes	Yes
30	Midgut	1	0	6	No	Yes
31	Midgut	1	4	18	No	No
32	Midgut	2	6	7	Yes	Yes
33	Midgut	1	15	23	Yes	Yes
34	PNET	2	2	12	no	Yes

Table 1: Demonstrates CTC count from both CellCollector and CellSearch for all 34 patients that underwent successful enumeration with each isolation method.

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