Prevalence and heterogeneity of circulating tumour cells in metastatic cutaneous melanoma

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We previously demonstrated that circulating tumour cells (CTCs) are detectable by the MelCAM and high molecular weight melanoma-associated antigen (HMW-MAA)-dependent CellSearch platform. However, CTCs which do not express these capture and detection markers are not detectable by CellSearch. Consequently, we explored the use of isolation by size of epithelial tumour cells (ISET), a marker independent, filtration-based device to determine the prevalence and heterogeneity of CTCs in metastatic cutaneous melanoma patients. Ninety patients were prospectively recruited and blood samples taken before treatment. Patients' blood was filtered using the ISET platform. CTCs were enumerated using dual immunohistochemistry with positive selection by S100 expression and exclusion of leucocytes and endothelial cells expressing CD45 or CD144, respectively. A panel of markers (Melan-A, MITF, MelCAM, high molecular melanoma-associated antigen, CD271 and MAGEC) was also examined. Fifty-one patients (57%) had CTCs (range 1–44 CTCs/4 ml blood) and 12 patients also had circulating tumour microemboli. Seven patients had S100− CTCs, 11 patients' CTCs were S100+ and 33 patients had S100+ and S100− CTCs. Substantial intrapatient and interpatient heterogeneity was observed for all other melanoma-associated markers. CTCs in metastatic cutaneous melanoma are detectable using the flexible marker-independent ISET platform. CTCs display significant marker expression heterogeneity implying that marker-dependent platforms would not detect all CTCs and multimarker assays are now required to reveal the biological significance of this CTC heterogeneity. *Melanoma Res* 00:000–000 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The incidence of melanoma is increasing worldwide. The majority of patients are cured by surgical excision but 20% of patients die from metastatic disease. Therapeutic advances have improved outcomes in metastatic disease but median overall survival remains poor [1,2].

Tumour biopsies are required to test for the presence of the BRAF mutation to stratify patients for MAPK pathway targeted drugs and assess emerging patterns of drug resistance. This can be challenging in metastatic disease and if a biopsy is archival, for example from a nodal dissection, it may not reveal interim tumour evolution [3].

Circulating tumour cells (CTCs) can be assessed in a less invasive blood sample with potential utility as prognostic, pharmacodynamic and predictive biomarkers. CTC characterization may reveal therapeutic targets and enable assessment of drug–target heterogeneity and downstream indicators of drug–target interaction [4].

A variety of CTC platforms have been used for melanoma CTC detection [5]. RT-PCR is the most widely used method where sensitivity is high but cell number and morphology are not assessable and circulating RNA may emanate from nonviable CTCs and necrotic tumour. Immunomagnetic-based enrichment techniques such as the semiautomated CellSearch platform (Veridex LLC, Raritan, New Jersey, USA) are melanoma marker [MelCAM and high molecular weight melanoma-associated antigen (HMW-MAA)] dependent and restricted to detection of CTCs expressing these markers [6,7].

The isolation by size of epithelial tumour cells (ISET) platform is a marker-independent filtration platform enriching for CTCs based on cell size. This filtration approach has been used to isolate CTCs in breast, liver, lung, pancreatic carcinomas and melanoma [8–15]. CTCs were detected in AJCC stage III melanoma patients and in five out of eight patients with metastatic melanoma

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using the ISET platform. The aims of the present study were to evaluate the prevalence of ISET detected melanoma CTCs in 90 metastatic cutaneous melanoma patients, to correlate CTC number with known clinical factors and to explore CTC heterogeneity based on melanoma marker expression. A subset of recruited patients had dual CTC enumeration by ISET and the Veridex CellSearch platform. A comparison of CTC numbers as detected by each platform in these patients was also performed. The results from this exploratory study would thus inform on the potential biomarker utility of CTCs (as detected by ISET) and enable their incorporation into larger homogenous studies for further validation.

Materials and methods

Study design

Patients with metastatic cutaneous melanoma and with evidence of radiologically progressive disease were recruited prospectively at the Christie Hospital, Manchester, UK. The study was conducted according to Good Clinical Practice and the Declaration of Helsinki. Blood samples were obtained before treatment. Patients' demographics, AJCC stage, performance status, time from diagnosis of metastatic disease to study enrolment and treatment received were collected.

CTC detection by ISET

Peripheral blood samples (up to 10 ml blood) were collected in EDTA tubes (Becton Dickinson, Oxford, UK), stored at 4°C and processed within 4 h of collection. ISET (Rarecell Diagnostics, Paris, France) was performed according to manufacturer's instructions as described previously [11,16]. The ISET module has 10 wells making it possible to process up to 10 ml blood per patient producing up to 10 '6-mm^2 membrane spots' on which cells within 1 ml aliquot of whole blood are deposited. Four to 10 spots per patient were generated for analysis.

Enumeration of CTCs by ISET

CTCs were enumerated after ISET filtration and dual immunohistochemical (IHC) staining, first with a CD45 and CD144 antibody cocktail and second with a S100 antibody using the following staining protocol.

Individual membrane spots were allowed to equilibrate to room temperature before incubating in pH 6 citrate antigen retrieval buffer (#S1699; Dako, Cambridgeshire, UK) in a 100°C water bath for 40 min. Membranes were washed in tris-buffered saline (TBS) before placing in 0.2% Triton for 10 min. Membranes were washed in TBS and then subjected to dual staining using the Envision TM G/2 double stain system according to manufacturer's instructions (Rabbit/Mouse, DAB + /Permanent Red, #K5361; Dako). Membranes were placed in dual endogenous enzyme block for 10 min and washed in TBS twice before primary CD45 antibody (1 : 30 dilution clone T29/33; Dako) and primary CD144 antibody (1 : 50 clone 16B1; eBiosciences, Hatfield, UK) in S0809 antibody diluent (Dako) were added and incubated for 1 h at room temperature. Envision Liquid DAB + Substrate Chromagen System (Dako) was used to visualize CD45 and CD144 staining. A double stain block was applied for 3 min after which membranes were incubated with S100 antibody (polyclonal #MZ196; Dako) for an hour at room temperature. A rabbit/mouse link was added for 10 min after which liquid permanent red chromogen was used to visualize S100 staining. Membranes were washed and counterstained with 1 × Gill's haematoxylin. The membrane spots were mounted on glass slides using Fairmount aqueous mounting medium (Dako) and coverslips applied. The Bioview duet microscope system (Olympus BX52 microscope and Image analysis software; Rehovat, Bioview, Israel) was used to scan membranes at × 40 magnification. Manual image review and scoring of tumour cells was performed in a blinded fashion by an experienced pathologist (P.S.). S100 + and S100 − cells that were CD45 − /CD144 − , with a high nuclear to cytoplasmic ratio, irregular nuclear contours, hyperchromatic nuclei and diameter more than 12 μm were enumerated as melanoma CTCs.

Four membrane spots were used for CTC enumeration per patient as this has been shown to give a robust average CTC count [15].

Immunophenotypic characterization of CTCs

Further characterization of melanoma CTCs was performed using a previously described IHC protocol [11] for a panel of six melanoma-associated markers; Melan-A (1 : 25 clone A103; Dako), MITF (1 : 100 clone D5; Dako), HMW-MAA (1 : 100 clone 9.2.27), MelCAM (1 : 100 clone COM 7A4; Biocytex, Marseille, France), MAGE-C1 (1 : 50, polyclonal #ab60049; Abcam, Cambridge, UK) and CD271(1 : 100 clone NGFR5; Abcam). Melan-A expression was assessed in 84 patients' samples, MITF in 82, HMW-MAA in 57, MelCAM in 19, MAGE-C in 16 and CD271 in 19 patients' samples.

The number of markers analysed for each patient depended upon the number of membrane spots generated from their blood sample.

All IHC staining protocols used appropriate positive and negative controls performed in tandem (Supplementary Fig. 1).

CTC detection by CellSearch

Samples were collected and processed as described previously [7].

Statistical analysis

Associations between CTCs and clinical characteristics were examined using Fisher's exact test. Statistical analysis was performed using Stata (version 10; StataCorp LP, College Station, Texas, USA) where a P value of 0.05 or less was deemed to be significant.

Results

Recruitment and clinical characteristics of patients

Ninety patients with stage IV disease were recruited between December 2009 and December 2011. Patient characteristics are shown in Table 1. Fifty-seven patients had died at the time of analysis. Average follow-up time for the remaining 33 patients was 6 months (median 5 months, SD 5 months, range 0.3–23 months). Seventy-one patients were recruited before first-line treatment. Nineteen patients were recruited before second-line treatment (six of whom had metastectomies in addition to medical treatment before enrolment).

CTC prevalence

Of the 90 patients recruited, 39 (43%) patients had no CTCs and 51 (57%) patients had detectable CTCs (range 1–44 CTC/4 ml). There were four patient subgroups based on their CTC counts, (a) those with no CTCs, (b) those whose CTCs all expressed S100 (S100+), (c) those whose CTCs were all negative for S100 (S100−) and (d) those whose CTCs were heterogeneous for S100 expression (S100 mix). Seven patients had S100− CTCs, 11 patients had S100+ CTCs and 33 patients had S100+ and S100− CTCs (S100 mix) (Fig. 1).

Contaminating leucocytes and circulating endothelial cells (CECs) were distinguishable by their staining pattern (CD45+/CD114+) and characteristic morphology, CECs tended to have elongated shape with large round nuclei and were larger than leucocytes (Fig. 2).

Table 1 Patient characteristics

Clinical characteristics	*N* (%) (*N*=90)
Age at baseline (years)	
Median	60
Range	32–89
Sex	
Male	48 (53)
Female	42 (47)
Stage at enrolment	
M1a	4 (4)
M1b	16 (18)
M1c	70 (78)
Period of time from diagnosis with metastases to enrolment (months)	
Median	5
Range	1–72
WHO performance status	
0	37 (41)
1	41 (46)
2	9 (10)
3	1 (1)
Unknown	2 (2)
Lactate dehydrogenase (IU/l)	
Normal (≤ 550)	53 (59)
High (>550)	36 (40)
Unknown	1 (1)
BRAF mutation	
Wild type	34 (38)
Mutant	31 (34)
Unknown	25 (28)
Stage of treatment	
First line	71 (79)
Second line	19 (21)
Treatment after enrolment for all patients	
Dacarbazine/temozolamide	19 (21)
BRAF/MEK inhibitor	20 (22)
Ipimulimab	6 (7)
Other	7 (8)
No treatment	38 (42)
Treatment after enrolment for first-line patients	
Dacarbazine/Temozolamide	18
BRAF/MEK inhibitor	19
Other	4
No treatment	30
Treatment post enrolment for second-line patients	
Dacarbazine/temozolamide	1
BRAF/MEK inhibitor	1
Ipilimumab	6
Other	3
No treatment	8

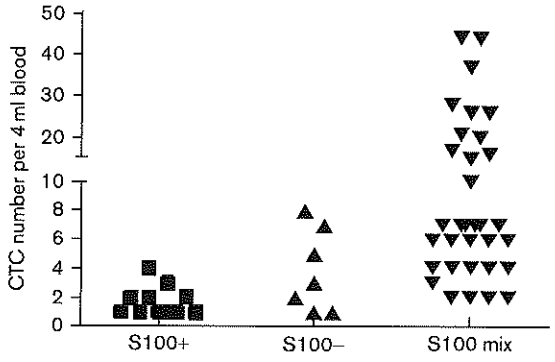
Associations between CTCs and clinical factors

Associations between patients according to CTC phenotype (S100+, S100− and S100 mix) and clinical characteristics were explored (Table 2). Males tended to have S100 mix CTCs ($P = 0.005$), patients with S100+ only CTCs had higher levels of lactate dehydrogenase (IU/l) ($P = 0.019$) and there was a trend towards a longer period with metastatic disease, that is the period between diagnosis with metastatic disease and study enrolment, in patients with S100 mix CTCs ($P = 0.064$).

Melanoma-associated marker expression on CTCs

At least two additional markers out of a panel of six (Melan-A, MITF, HMW-MAA, MelCAM, CD271 and MAGE-C1) were assessed in all patients' samples. Table 3 shows the number of patients with detectable CTCs/ml and their

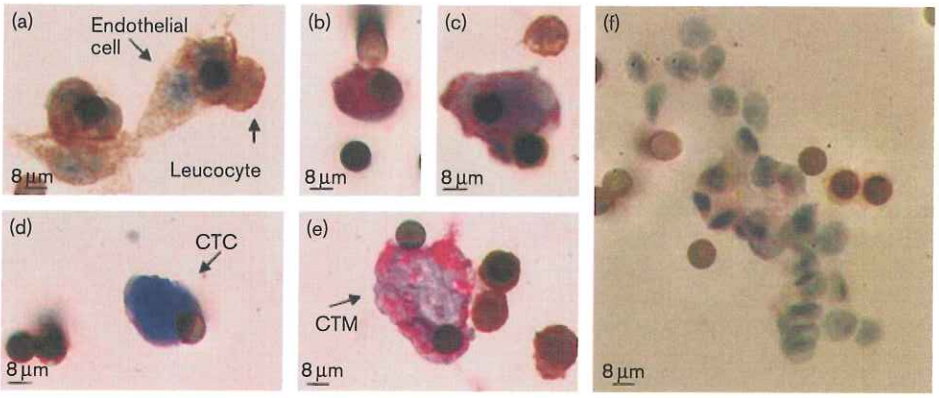
Fig. 1



Patients with CTCs (*N*=51)	S100+ (*N*=11)	S100− (*N*=7)	S100 mix (*N*=33)
Range	1–4	1–8	2–44
Mean/4 ml	2	4	12
Median/4 ml	1	3	7

CTC enumeration within 51 cutaneous melanoma patients' blood samples (4 ml) after ISET filtration and exclusion of CD45+ and CD144+cells with patient categorization according to CTC phenotype based on S100 staining. CTC, circulating tumour cell; ISET, isolation by size of epithelial tumour cells.

Fig. 2



Representative images of single CTCs and/or CTM with contaminating leucocyte and endothelial cells after ISET filtration and IHC for CD144 and CD45 (brown stain) and S100 (red stain). (a) Endothelial and leucocytes (brown staining with DAB), (b, c) S100+ CTC in patient 30 and patient 44, respectively (red chromogen staining), (d, e) S100− and S100+ CTC and CTM in patient 35, (f) a CTM in patient 39 with negative and weakly positive S100 staining within the same CTM. CTC, circulating tumour cell; CTM, circulating tumour microemboli; ISET, isolation by size of epithelial tumour cells.

Table 2 Populations of CTCs and association with clinical characteristics

Clinical characteristics	No CTCs	S100+ CTCs only	S100− CTCs only	Mixed CTCs
Sex				
Male	15	7	2	24
Female	24	4	5	9
Fisher's exact test *P* value*	–	0.178	1	0.005
Mean LDH (IU/l)				
≤ 550	26	3	3	19
>550	8	7	2	12
Fisher's exact test *P* value*	–	0.019	0.587	0.282
Performance status				
P0	17	5	2	13
P1, P2, P3	22	5	4	20
Fisher's exact test *P* value*	–	0.737	1	0.812
Period with metastasis				
>5 months	16	5	5	21
<5 months	23	6	2	12
Fisher's exact test *P* value*	–	1	0.220	0.064
Stage				
M1a, M1b	11	0	0	9
M1c	28	11	7	24
Fisher's exact test *P* value*	–	0.093	0.171	1
BRAF mutation				
Wild type	15	3	1	15
Mutant	17	3	2	9
Fisher's exact test *P* value*	–	1	1	0.288
Stage of treatment				
First line	20	3	1	17
Second line	2	2	0	7
No treatment	17	6	6	9
Fisher's exact test *P* value*	–	0.201	0.140	0.088

CTC, circulating tumour cell; LDH, lactate dehydrogenase.
**P* values represent associations between clinical characteristics and each of the three CTC populations, with the 'no CTCs' group used as a comparator.

CTC melanoma marker expression profile. The overall finding was that of significant marker heterogeneity (Fig. 3).

Circulating tumour microemboli

After evaluation of all patient ISET spots by IHC, 12 out of the 90 (13%) patients had circulating tumour microemboli (CTM) (i.e. cluster with >2 nuclei). Two patients had CTM within which all tumour cells were S100−, five patients had CTM in which all tumour cells were strongly S100+, one patient had a weakly S100+ CTM and a further patient had a S100 mix CTM. CTM were detected in a further three patients during melanoma marker characterization, one CTM contained MITF+ cells (Fig. 3g), one contained HMW-MAA+ cells and one patient had a CTM which was negative for Melan-A expression. Two of these patients had S100 mix CTCs and the patient with the Melan-A− CTM had no single CTCs. Two patients had multiple CTM: one patient had three CTM (S100+, MITF+ and MelCAM+; Fig. 3c) and the other patient had two CTM (S100− and CD271+). There were no associations between the presence of CTM and patient characteristics.

Table 3 Heterogeneity of melanoma-associated marker expression in CTCs

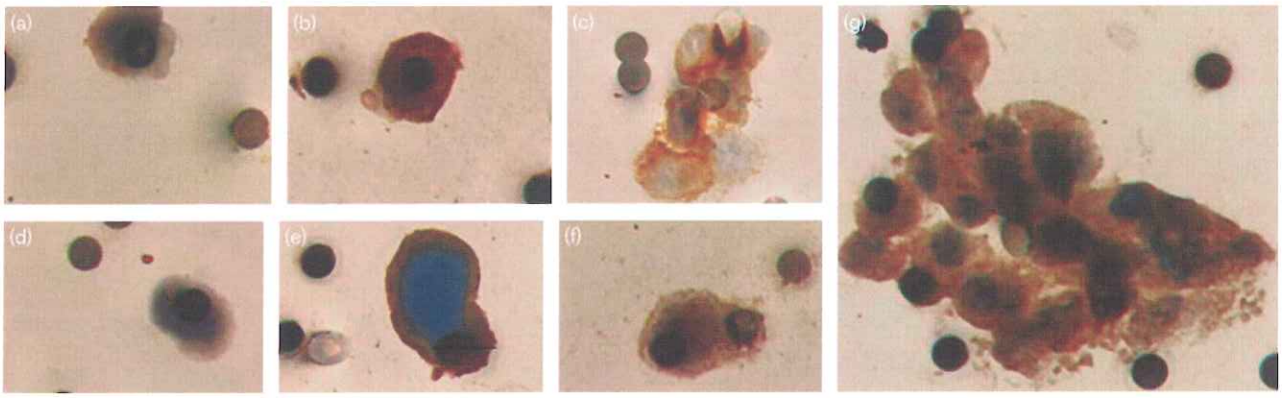
Melanoma-associated marker (number of patients where CTCs detected in 1 ml blood)	Patient number Marker positive	Patient number Marker negative	Patient number Heterogeneous Marker expression
Melan-A (25)	7	11	7
MITF (29)	7	17	5
HMW-MAA (23)	18	2	3
MelCAM (12)	1	11	0
CD271 (7)	1	6	1
MAGE-C1 (6)	1	5	0

CTC, circulating tumour cell.

Comparison of CTC numbers detected by ISET or CellSearch

Comparison of CTC enumeration between the CellSearch and ISET platforms was possible for 86 patients. Heterogeneity in CTC detection by each platform was found across patients; 21 patients had CTCs detected by both platforms, 14 patients had CellSearch only detectable CTCs and 26 patients had ISET detected

Fig. 3



Molecular characterization of CTCs using a panel of melanoma-associated markers. (a) Melan-A+ CTC in patient 106, (b) MITF-positive CTC in patient 31, (c) MelCAM+ CTM in patient 40, (d) weakly positive MAGE-C1 CTC in patient 119, (e) HMW-MAA+ CTC in patient 48, (f) CD271+ CTC in patient 46, (g) MITF+ CTM in patient 46. CTC, circulating tumour cell; HMW-MAA, high molecular weight melanoma-associated antigen.

CTCs alone. Twenty-five patients had no detectable CTCs by either platform. The mean and median for CellSearch CTC count was 2 and 0 per 7.5 ml blood (range 1–36) and for total ISET CTC count was 5 and 1 per 7.5 ml blood (range 1–44), respectively. ISET detected CTCs were S100+ or S100−; patients were S100+ alone ($n = 11$), S100− alone ($n = 7$) or S100 mix ($n = 29$) (Fig. 4). Linear regression of CellSearch CTC number versus ISET CTC number (of any phenotype or total ISET CTC number) did not show any significant correlation.

Discussion

We sought to examine the prevalence and heterogeneity of CTCs in metastatic cutaneous melanoma patients using the marker-independent ISET platform. The downstream applications to which ISET membranes can be subjected make the platform flexible with the potential to probe CTC biology. However, the lack of standardized criteria that define a melanoma CTC using ISET may hinder its implementation in clinical trial scenarios. Leucocyte and CEC contamination on ISET membranes has been reported previously [15] and previous ISET studies have used Giemsa staining [10] or CD45-negative selection with characteristic tumour cell morphology for CTC enumeration [15]. However, the larger size of CECs (compared with leucocytes) makes them more comparable with tumour cells and thus problematic for filtration-based approaches. This is important to address given the higher number of CECs in patients with cancer. Here for the first time we used a dual IHC protocol that allows exclusion of CD144+ and CD45+ endothelial cells and leucocytes, respectively.

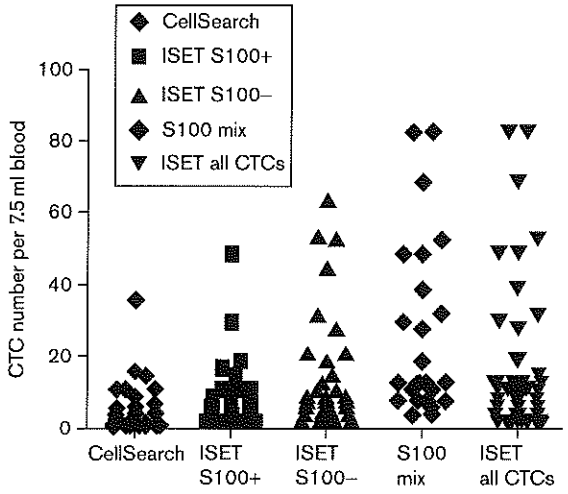
IHC staining for tumour-associated antigen expression can increase specificity of CTC enumeration and characterize isolated CTCs [12,17]. However, there is no accepted universally expressed melanoma marker. In IHC studies, the melanoma marker S100 is reported to have a sensitivity of 97–100% but a specificity of 75–87% [17]. Other melanoma markers such as MITF, MART-1/Melan-A, tyrosinase and HMB-45 are less sensitive than S100 (sensitivity can be as low as 75%) but specificity can range from 88 to 100%. Therefore even with a panel of melanoma markers up to 10% of melanomas may be marker negative. With these data in mind, we chose S100 as the melanoma marker and assessed CTC prevalence by enumerating S100+ and S100− CTCs with characteristic tumour morphology and negativity for CD45 or CD144 to optimize the robustness of the analysis.

There was heterogeneous CTC expression for every single melanoma marker assessed in this study. Given the paucity of CTCs, multiparameter staining protocols are now needed to explore simultaneously the expression of multiple markers on CTCs.

Our study enrolled a heterogenous group of patients and although we explored associations between clinical factors and CTCs, we acknowledge that these findings are preliminary and require further evaluation. Although sex is a weak-independent prognostic factor for survival (males do worse than females), it is most likely that the differences in CTC detection between sexes in our study reflects the size and heterogeneity of our sample set. Simultaneous detection of multiple CTC marker expression would address more fully their biological (including prognostic) significance in homogenous patient groups. We are conducting ongoing studies in this respect: within the context of clinical trials and collecting serial samples to examine the effect of treatment on CTC detection. These studies will consist of training and validation sets to evaluate more robustly our initial findings presented here.

The finding of CTM raises the possibility that cells enter the bloodstream via collective cell migration [18]. Most

Fig. 4



Platform (*N*=patients with CTCs (total patients=86)	CellSearch (*N*=22)	ISET S100+ (*N*=40)	ISET S100– (*N*=36)	ISET S100 mix (*N*=29)	ISET all CTCs (*N*=47)
Range (CTCs detected)	1–36	2–49	2–64	2–84	2–83
Mean	5	7	14	24	17
Median	2	4	8	13	9

CTC number as detected by ISET and CellSearch platform. The number of CTCs detected by each platform (for patients with paired samples) is shown along with range, mean and median. Patients with CTC detectable by ISET were divided into three groups according to S100 phenotype, S100+ alone, S100– alone and S100 mix. The total CTC numbers detected by ISET are also shown. CTC, circulating tumour cell; ISET, isolation by size of epithelial tumour cells.

single CTCs are thought to undergo apoptosis in the circulation brought about by sheer stress and anoikis [19], and it may be that cells within CTM have a survival advantage.

The comparison of CTC number in patients who had dual CellSearch and ISET CTC enumeration showed that these platforms detect different CTC populations. ISET detects more CTCs and in more patients than the CellSearch platform, a finding which is consistent with previous comparative published studies [11,15,20,21].

In summary, we have shown that the ISET platform provides a flexible method for melanoma CTC detection (enumeration, morphological assessment and immunophenotypic characterization), that we can exclude contaminant leucocytes and CECs, and that CTCs are present in more than 50% patients with metastatic cutaneous melanoma and are heterogeneous in melanoma marker expression profile. The ability to examine CTCs in a more unbiased manner (based on cell size and negative selection of leucocytes and CECs) distinguishes ISET from immunomagnetic methods [6]. While early studies of CTCs using the CellSearch platform suggest that they are prognostic for overall survival ($\geq$ 2 CTCs/7.5 ml blood) [6,7], the substantial heterogeneity of melanoma CTCs observed here using ISET cannot be readily explored using the CellSearch platform. This present study reveals the potential to monitor the biology of melanoma CTCs in real-time and this may be particularly useful as patients initially respond to treatment and then relapse with resistant disease.

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Conflicts of interest

There are no conflicts of interest.

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