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# Biomarker Utility of Circulating Tumor Cells in Metastatic Cutaneous Melanoma

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The incidence of melanoma is increasing worldwide. Advances in targeted agents and immunotherapy have improved outcomes in metastatic disease, but biomarkers are required to optimize treatment. We determined the prevalence of circulating tumor cells (CTCs) and explored their utility as prognostic and pharmacodynamic biomarkers. A total of 101 patients with metastatic cutaneous melanoma were recruited prospectively. CTC number was determined using the CellSearch platform and melanoma kits in samples taken at baseline and serially during treatment. CTC numbers ranged between 0 and 36 per 7.5 ml blood; 26% of patients had $\geqslant 2$ CTCs. Baseline CTC number was prognostic for median overall survival (OS) in univariate analysis (2.6 vs. 7.2 months ($P<0.011$) for patients with $\geqslant 2$ CTCs vs. $<2$ CTCs, respectively). In multivariate analysis, CTC number was an independent prognostic biomarker of OS (hazard ratio (HR) 2.403, 95% confidence interval (CI) 1.303–4.430, $P=0.005$). Patients receiving treatment in whom CTC number remained $\geqslant 2$ CTCs during treatment had shorter median OS than those who maintained $<2$ CTCs (7 vs. 10 months, HR 0.34, 95% CI 0.14–0.81, log-rank test $P=0.015$). In conclusion, CTC number in metastatic cutaneous melanoma patients is prognostic for OS with a cutoff of 2 CTCs per 7.5 ml blood. CTC number measured before and throughout treatment provided additional prognostic information. Larger studies are warranted to confirm CTC biomarker utility in melanoma patients.

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## INTRODUCTION

Melanoma is the most serious form of skin cancer and the incidence is increasing worldwide. Approximately 80% of patients are cured with surgery. The prognosis of patients with metastatic disease has improved with targeted agents and immunotherapy (Hodi *et al.*, 2010; Chapman *et al.*, 2011), but there are few long-term survivors. Advances in molecular subtyping of melanoma have identified patient subgroups with specific genetic aberrations (Davies *et al.*, 2002; Curtin *et al.*, 2005, 2006). This has in turn guided the development of targeted therapies such as the BRAF selective inhibitor vemurafenib, which has been shown to improve survival when compared with standard chemotherapy with dacarbazine in patients with BRAF mutant tumors (Chapman *et al.*, 2011). However, subsequent drug resistance and disease progression are observed in the majority of patients (Ribas and Flaherty, 2011). Ipimulimab, a cytotoxic T-lymphocyte antigen 4 inhibitor, has also improved median overall survival (OS) in metastatic disease, with a subgroup of patients being long-term survivors. Patient selection is hindered by the absence of a predictive biomarker to stratify patients (Hodi *et al.*, 2010).

Assessment of genetic aberrations and evolving tumor biology, particularly with respect to emergent mechanisms of drug resistance, is thus increasingly relevant. Longitudinal monitoring of patients to report or, better still, give an early warning of treatment failures would be beneficial. However, as obtaining tumor biopsy before and after treatment is challenging and not without risk, more minimally invasive approaches are sought.

Circulating tumor cells (CTCs) hold potential as prognostic, predictive, and pharmacodynamic biomarkers. Although melanoma was the first solid tumor in which CTCs were detected (using PCR) (Smith *et al.*, 1991), lack of validated and standardized methodology hampered qualification of CTCs as a biomarker in clinical trials (Nezos *et al.*, 2009). CTC number determined using the CellSearch platform (Veridex, Raritan, NJ) is prognostic for OS in metastatic lung, breast, prostate, and colorectal carcinomas (Cristofanilli *et al.*, 2004; Cohen *et al.*, 2008; de Bono *et al.*, 2008; Hou *et al.*, 2009, 2012; Krebs *et al.*, 2011), where the ''cutoff'' (CTC number per

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*Abbreviations: BETI, β-expectation tolerance interval; CI, confidence interval; CTC, circulating tumor cell; HR, hazard ratio; HMW-MAA, high molecular weight–melanoma-associated antibody; LDH, lactate dehydrogenase; MEK, MAPK/ERK kinase; OS, overall survival; PS, performance status*

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7.5 ml blood) depends on the tumor type and prevalence of CTCs. A melanoma-specific CellSearch CTC kit developed recently uses Melcam and high molecular weight–melanoma-associated antibody (HMW-MAA) for melanoma CTC capture and detection, respectively (Rao *et al.*, 2011).

Here, we evaluate the prevalence, clinical significance, and biomarker potential of melanoma CTCs using this technology in patients with metastatic disease. Our primary objective was to evaluate the incidence and prognostic significance on OS of CTC number at baseline in metastatic cutaneous melanoma patients. Exploratory end points were pharmacodynamic changes in CTC number and prognostic significance of these changes. Our overall goal was to evaluate the biomarker utility of CTCs as a prelude to their incorporation in clinical trials, where they might also provide opportunity for molecular analyses to facilitate patient stratification and monitoring of development of drug resistance.

## RESULTS

### Patient demographics

Between December 2009 and December 2011, 101 patients were recruited into this study and their clinical characteristics are shown in Table 1. The majority of patients had stage M1C disease and performance status (PS) 0–1. BRAF mutational status was assessed in approximately two-thirds of patients and approximately half of them had a mutation detected. Sixty-one patients had died by the time of analysis and one was lost to follow-up. Average follow-up for the remaining 39 patients was $5.9 \pm 5.2$ months (SD, range 0.3–20 months). Of the patients studied, 68 were treatment naive and went on to receive treatment, 17 patients received no treatment, and 16 patients had previously received first-line treatment and went on to receive second-line therapy.

**Table 1. Patient demographics**

| Clinical characteristic | Number | % |
|---|---|---|
| *Age at baseline, years* | | |
| Median | 60 | |
| Range | 32–89 | |
| *Sex* | | |
| Male | 53 | 52.5 |
| Female | 48 | 47.5 |
| *Stage at enrollment* | | |
| M1a | 4 | 4 |
| M1b | 18 | 17.8 |
| M1c | 79 | 78.2 |
| *Period of time with metastases* | | |
| Median | 5 Months | |
| Range | 1–72 Months | |
| *WHO performance status* | | |
| 0 | 41 | 40.6 |
| 1 | 45 | 44.6 |
| 2 | 12 | 11.9 |
| 3 | 1 | 1 |
| *LDH* | | |
| Normal ($\leqslant$550) | 65 | 64 |
| High (>550) | 36 | 36 |
| *BRAF mutation* | | |
| Wild type | 38 | 38 |
| Braf mutation | 32 | 32 |
| Unknown | 31 | 30 |
| *Treatment* | | |
| Dacarbazine/temozolamide | 21 | 20.8 |
| BRAF/MEK inhibitor | 20 | 19.8 |
| Ipimulimab | 7 | 6.9 |
| Other | 9 | 8.9 |
| Radiotherapy | 1 | 1 |
| *Stage of treatment* | | |
| First-line treatment | 68 | 66.7 |
| Second-line treatment | 16 | 15.7 |
| Untreated | 17 | 16.7 |

Abbreviations: LDH, lactate dehydrogenase; MEK, MAPK/ERK kinase; WHO, World Health Organization.

### Analysis of CTC enumeration accuracy using the β-expectation tolerance interval (BETI) approach

Validation experiments were conducted to determine the analytical accuracy of CellSearch CTC enumeration at low (1–10) CTC numbers. Analysts scored the same image galleries generated from 20 different samples of melanoma patients with low CTC counts, and the results were evaluated using the BETI approach (see Supplementary Data online for details). The analytical accuracy achieved by the two analysts who performed the CTC analysis reported herein was high with a 95% probability of a BETI (for total error) of <30%. These data support our contention that melanoma CTCs can be reliably enumerated at numbers as low as 2 CTCs in 7.5 ml blood using the CellSearch platform.

### Prevalence of CTCs at baseline

A CTC count of zero and very rarely 1 CTC has been reported in 55 healthy volunteers using this melanoma-specific kit and the CellSearch platform (Rao *et al.*, 2011). The range of CTC number per 7.5 ml blood detected at baseline was 0–36 ($n = 101$, mean 2, median 0); 60% of patients had no CTCs and 40% had one or more CTCs. Figure 1a–e shows representative images of melanoma CTCs from five patients. To be assigned as a CTC, cells must have a 4′,6-diamidino-2-phenylindole–stained nucleus (pseudocolored pink) and HMW-MAA staining (pseudocolored green) in the absence of leukocyte and endothelial marker expression (CD45/CD34 staining, respectively). The size of melanoma cells thus detected is variable (the longest cell diameter ranges between 6 and 22 μm), with some CTCs considerably larger than those staining for CD45/CD34. Contaminant cells shown in Figure 1f and g stained positively with the CD45/CD34

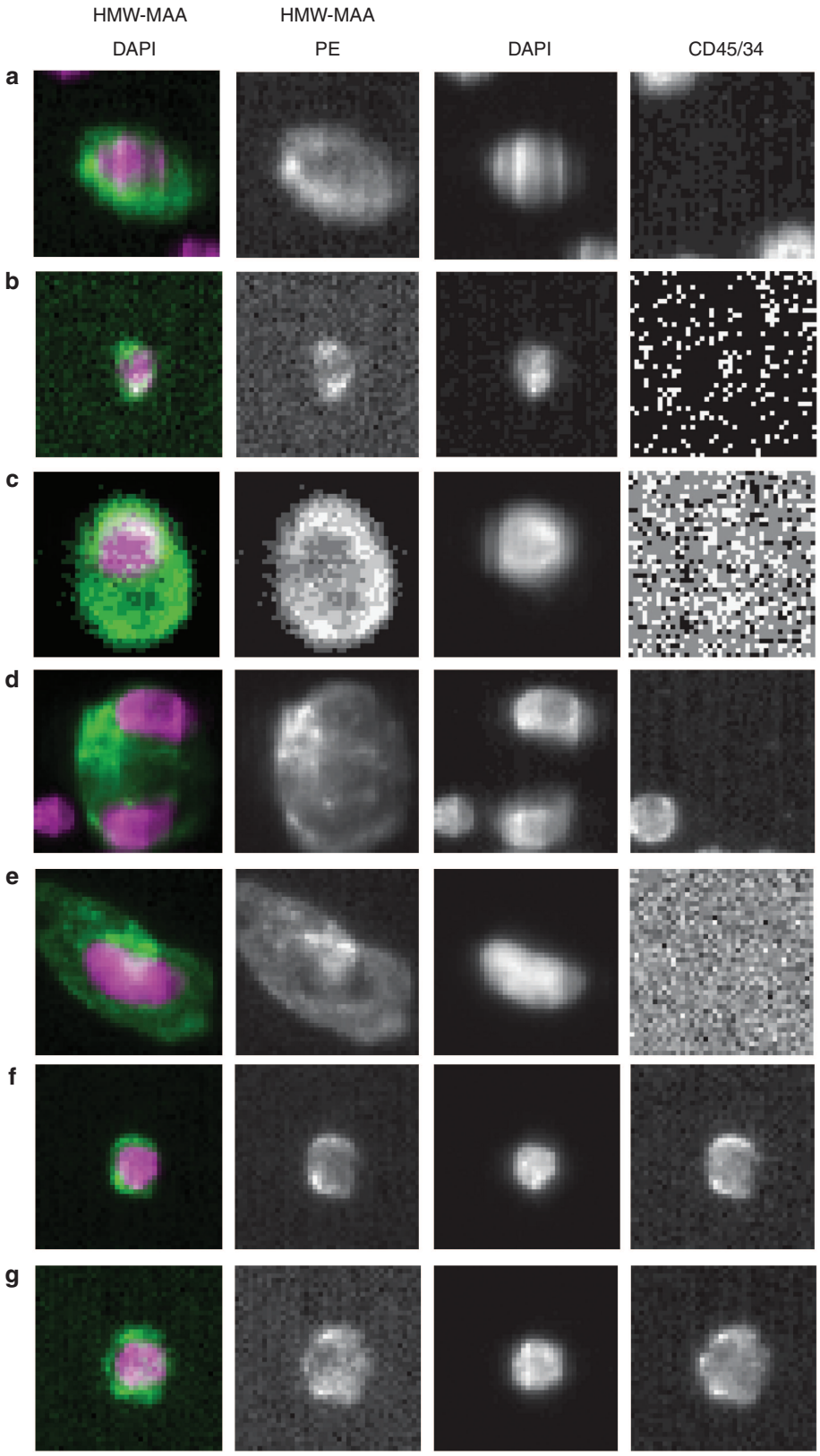


**Figure 1. Representative cell images obtained with the Veridex platform using the melanoma kit.** (**a**–**e**) Representative pseudocolored images of melanoma circulating tumor cells (CTCs) from five patients with a 4′,6-diamidino-2-phenylindole (DAPI)-stained nucleus (pink), positive staining for the melanoma marker high molecular weight–melanoma-associated antibody (HMW-MAA; green) in the absence of staining for CD45/CD34 markers of leukocytes and endothelial cells, respectively. PE, phycoerythrin. **f**, **g**) Representative pseudocolored images of patients' leukocytes or endothelial cells that have a DAPI-stained nucleus (pink), and stain positively for CD45/CD34 (green) and negatively for HMW-MAA. The images shown are more likely to be leukocytes than endothelial cells because of the smaller size of the former.

antibody cocktail, the relatively small size of these cells (7–8 μm) suggests that they are leukocytes.

### The optimal CTC cutoff value for prognostication and correlation with clinical characteristics

A cutoff of 2 CTCs provided the largest discrimination on the basis of median OS, i.e., a clear prognostic distinction between patients (see Supplementary Data online). This cutoff was applied to categorize patients into "favorable" (<2CTCs; $n=75$) and unfavorable (⩾2 CTCs; $n=26$) groups. Table 2 compares CTC number at baseline with clinical characteristics. Of the clinical factors, only lactate dehydrogenase (LDH; $P=0.001$) and PS ($P=0.002$) showed significant association with CTC number. Whether the patients had received treatment with first- or second-line treatment did not significantly affect the prevalence of CTCs or the selection of a CTC cutoff of ⩾2 CTCs (Table 2).

### Prognostic significance of baseline CTC number

The Kaplan–Meier analysis was applied to determine the prognostic significance of baseline CTC number (Figure 2a). Patients with <2 CTCs had significantly longer median OS than patients with ⩾2 CTCs (7.2 vs. 2.6 months, hazard ratio (HR) 0.43, 95% confidence interval (CI) 0.22–0.81, log-rank test $P=0.009$). The proportional hazard assumption was validated, and therefore the univariate Cox regression analysis was applied. Clinical factors statistically significant for inferior OS included high LDH ($P=0.003$), poorer PS ($P<0.001$), and ⩾2 CTCs per 7.5 ml blood ($P<0.011$). Treatment with a BRAF or MAPK/ERK kinase (MEK) inhibitor was associated with superior OS ($P=0.041$), whereas treatment with Ipimulimab was not significant for OS. Thirty-four of 75 (45%) patients in the <2 CTCs group were alive at the time of analysis compared with 6 of 26 (23%) patients in the ⩾2 CTCs group. Table 3 shows the results of the univariate Cox regression analysis.

### Multivariate Cox proportional hazards regression analyses

Multivariate Cox proportional hazards regression analysis was performed adjusting for significant univariate factors (LDH, PS, treatment with a BRAF or MEK inhibitor, and CTC number). All factors identified as significant in the univariate analysis remained significant in the multivariate analysis, except LDH. CTC number was an independent prognostic factor for OS (HR 2.403, CI 1.303–4.430, $P=0.005$) (Table 4). These results were internally validated by performing bootstrapping (resampling data 1,000 times; Figure 3).

### Exploratory analysis of pharmacodynamic CTC changes and prognostic significance of CTC number after treatment

Serial CTC counts were performed for 45 patients on treatment and 28 (62%) of them exhibited pharmacodynamic changes in CTC number. The remaining 17 patients had no CTCs at any time points. Changes in CTC number at time-point 2 reflected computerized tomography (CT) scan assessment performed at 6–9 weeks after commencement of treatment. All patients whose CTC number decreased had radiological responses to treatment ($n=13$, dacarbazine or BRAF inhibitor). Two responding patients had fluctuating CTC numbers between 0 and 1 after BRAF or MEK inhibitor treatment. Five patients, whose CTC number increased, developed progressive disease. Eight patients had stable disease while under treatment: two

**Table 2. Prevalence of CTCs and association with clinical characteristics**

| Clinical characteristic | Patients with CTCs above threshold ⩾1 | ⩾2 | ⩾3 | Total patients |
|---|---|---|---|---|
| *Age* | | | | |
| >60 | 24 | 16 | 13 | 52 |
| <60 | 16 | 10 | 8 | 49 |
| $\chi^2$ *P*-value | 0.222 | 0.236 | 0.332 | |
| *Sex* | | | | |
| Male | 24 | 15 | 12 | 53 |
| Female | 16 | 11 | 9 | 48 |
| $\chi^2$ *P*-value | 0.231 | 0.65 | 0.807 | |
| *Mean LDH* | | | | |
| ⩾550 | 23 | 18 | 15 | 36 |
| <550 | 17 | 8 | 6 | 65 |
| $\chi^2$ *P*-value | 0.006 | 0.001 | 0.002 | |
| *Performance status* | | | | |
| P0 | 11 | 3 | 1 | 41 |
| P1 | 19 | 14 | 11 | 45 |
| P2 | 7 | 6 | 6 | 12 |
| P3 | 1 | 1 | 1 | 1 |
| $\chi^2$ *P*-value | 0.101 | 0.002 | <0.001 | |
| *Time with metastatic disease* | | | | |
| >5 Months | 17 | 12 | 10 | 46 |
| <5 Months | 23 | 14 | 11 | 53 |
| Fisher's exact test *P*-value | 0.544 | 1 | 1 | |
| *Stage* | | | | |
| M1a | 1 | 0 | 0 | 4 |
| M1b | 5 | 3 | 2 | 18 |
| M1c | 34 | 23 | 19 | 79 |
| $\chi^2$ *P*-value | 0.407 | 0.268 | 0.275 | |
| *BRAF mutation (V600E)* | | | | |
| Mutant | 12 | 7 | 6 | 32 |
| Wild type | 14 | 9 | 6 | 38 |
| Unknown | 14 | 10 | 9 | 31 |
| $\chi^2$ *P*-value | 0.919 | 0.756 | 0.509 | |
| *Stage of treatment* | | | | |
| First line or untreated | 27 | 15 | 10 | 68 |
| Second-line treatment | 7 | 6 | 6 | 16 |
| No treatment | 6 | 5 | 5 | 17 |
| $\chi^2$ *P*-value | 0.884 | 0.415 | 0.082 | |

Abbreviations: CTC, circulating tumor cell; LDH, lactate dehydrogenase.

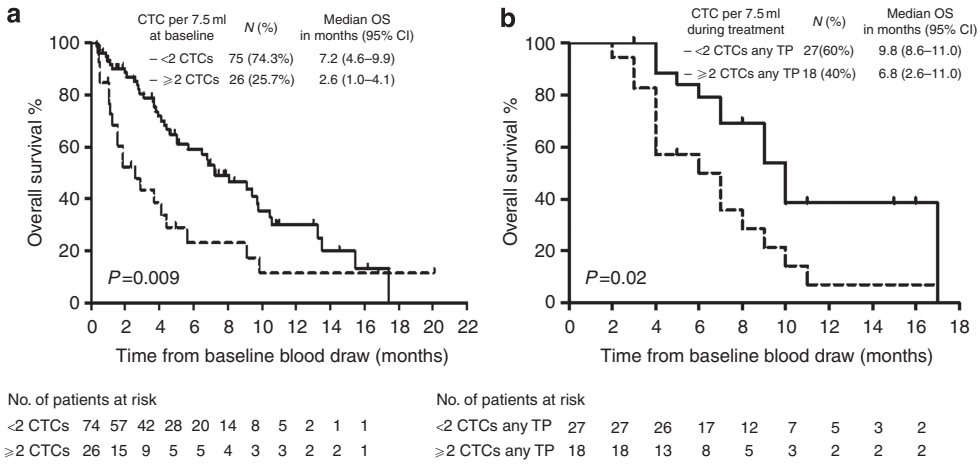


**Figure 2. Prognostic significance of circulating tumor cells (CTCs).** (**a**) The Kaplan–Meier curve for overall survival (OS) of patients with <2 CTCs versus patients with ⩾2 CTCs in 7.5 ml blood at baseline. CI, confidence interval. (**b**) The Kaplan–Meier curve for OS of patients with <2 CTCs versus patients with ⩾2 CTCs in 7.5 ml blood at any time point (TP) during treatment.

**Table 3. Univariate Cox proportional hazards regression analysis for prediction of overall survival**

| | At-risk group | | Overall survival risk | | |
|---|---|---|---|---|---|
| **Clinical characteristic** | **Positive** | **Negative** | ***P*-value** | **HR** | **95% CI** |
| Performance status | 3 vs. 2 vs. 1 vs. 0 | | <0.001 | | |
| LDH | <550 | ⩾550 | 0.003 | 2.196 | 1.313–3.674 |
| Treatment | BRAF/MEK inhibitor | Other/none | 0.041 | 0.370 | 0.147–0.928 |
| Baseline CTC | <2 | ⩾2 | <0.011 | 2.030 | 1.178–3.496 |
| Period of time with metastasis | NA[1] | | 0.059 | 0.983 | 0.965–1.001 |

Abbreviations: CI, confidence interval; CTC, circulating tumor cell; HR, hazard ratio; LDH, lactate dehydrogenase; MEK, MAPK/ERK kinase; NA, not applicable.
[1]Treated as continuous variable.

**Table 4. Stepwise multivariate Cox proportion hazards regression analysis; prognostic factors considering CTC at baseline**

| | At-risk group | | Overall survival risk | | |
|---|---|---|---|---|---|
| **Clinical characteristic** | **Positive** | **Negative** | ***P*-value** | **HR** | **95% CI** |
| Performance status | 3 vs. 2 vs. 1 vs. 0 | | <0.001 | | |
| Treatment | BRAF/MEK inhibitor | Other/none | 0.001 | 0.186 | 0.071–0.485 |
| Baseline CTC | < 2 | ⩾ 2 | 0.005 | 2.403 | 1.303–4.430 |
| Period of time with metastasis | NA[1] | | 0.004 | 0.968 | 0.946–0.989 |

Abbreviations: CI, confidence interval; CTC, circulating tumor cell; HR, hazard ratio; MEK, MAPK/ERK kinase; NA, not applicable.
[1]Treated as continuous variable.

displayed increases in CTC number, four had fluctuating CTC number, and two had no CTCs until progression when their CTCs increased to 3 and 5 CTCs per 7.5 ml blood, respectively. Figure 4 shows the changes in CTC numbers for nine BRAF mutant patients on treatment with a BRAF or MEK inhibitor: patients A–H with a decrease in CTC number responded to treatment, whereas patient I whose CTC number increased progressed on treatment.

The prognostic significance of CTC number recorded before and at any time point during treatment for all 45 patients

confirmed longer survival for patients with <2 CTCs at any time point during treatment (10 vs. 7 months, HR 0.34, CI 0.14–0.81, log-rank test $P=0.015$; Figure 2b).

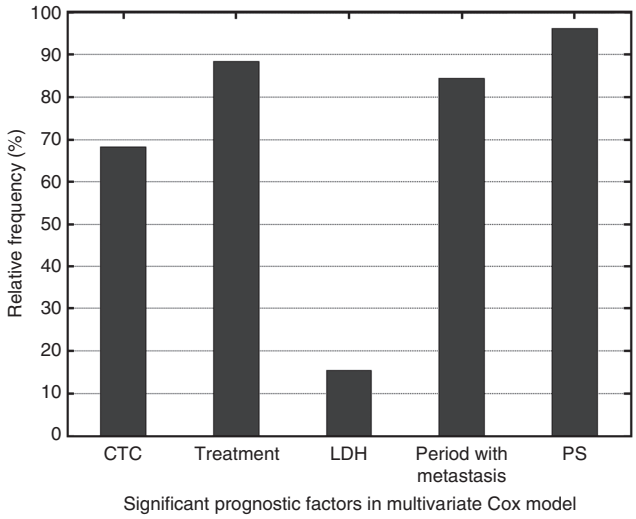


**Figure 3. Frequency of significant prognostic factors in multivariate Cox proportion.** Hazards regression analysis using data generated by bootstrapping. CTC, circulating tumor cell; LDH, lactate dehydrogenase; PS, performance status.

## DISCUSSION

We sought to evaluate the prevalence of CTCs in metastatic cutaneous melanoma patients and explore their utility as a biomarker. To our knowledge, a study of CTCs in patients with metastatic melanoma using the CellSearch (Melanoma kit) platform with sufficient power to draw robust conclusions regarding prognosis has not been reported previously. In an exploratory analysis, we also asked whether pharmacodynamic changes in CTC number were observable and whether such changes could be potentially viewed as surrogates of clinical response.

Our results indicate that 26% of patients had ⩾2 CTCs at baseline and that median OS was shorter than for those with <2 CTCs (2.6 vs. 7.2 months, log-rank $P=0.009$). The population recruited into our study was heterogeneous: after correction for confounding factors such as BRAF mutation status, line of treatment, treatment type (targeted therapy, Ipilimumab, chemotherapy), and time from diagnosis of metastatic disease to study enrollment, we showed that baseline CTC number ⩾2 is an independent prognostic biomarker ($P=0.005$). The cutoff of 2 CTCs per 7.5 ml blood that we defined is the lowest cutoff used for the prognostic significance of CTC number compared with small cell lung cancer (50 CTCs), breast, prostate, and non–small cell lung cancer (five CTCs), and colorectal cancer (three CTCs). We acknowledged the critical issue of the reliability of a 2-CTC count and

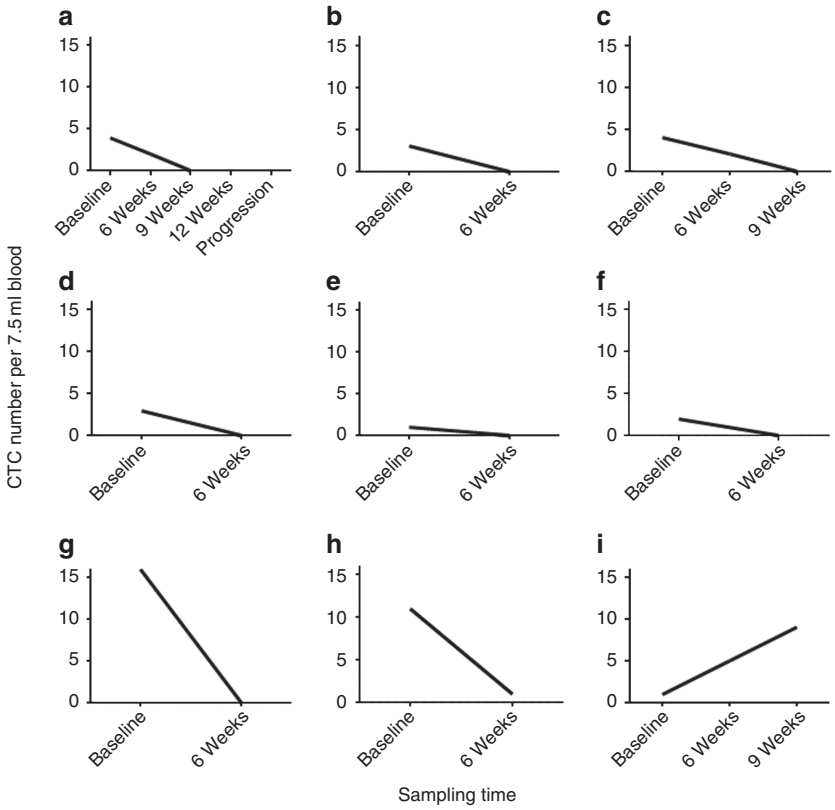


**Figure 4. Pharmacodynamics of circulating tumor cell (CTC) number in individual patients A–I with BRAF mutant tumors while on treatment with a BRAF or MEK inhibitor.** Patients A–H responded to treatment, whereas patient I progressed on treatment.

conducted an analysis of BETI (Supplementary Data online) with respect to a CTC count of 2 CTCs per 7.5 ml blood, demonstrating that this was a robust and reliable measurement. We also internally validated the derived cutoff by performing bootstrapping, thus limiting bias and overfitting of the data. The only previously published data on CTC detection using the Veridex melanoma kit reported on 44 patients with metastatic ($n=39$) and unresected stage III ($n=5$) melanomas. CTC detection using this approach was demonstrated in principle whereby 12 patients (27%) had $\geqslant 2$ CTCs (Rao *et al.*, 2011).

Standards of care for advanced melanoma have changed significantly over the past 12 months. Vemurafenib is now licensed as first- and second-line treatment for patients harboring a BRAF V600E mutation. Ipilimumab is licensed as second-line treatment in Europe and as first- and subsequent-line therapy in the United States. There are currently no minimally invasive pharmacodynamic biomarkers in routine use to monitor early responses to these targeted treatments, nor are there biomarkers that anticipate the onset of treatment resistance. Our data suggest that CTC changes with treatment may be an indicator of response and provide additional prognostic value when measured sequentially during treatment.

The limitations of this study were that treatment received by the patients was heterogeneous, reflecting standard of care and clinical trials ongoing at the time of recruitment; imaging was not carried out at a fixed time point and was not consistently reported using the RECIST (Response Evaluation Criteria In Solid Tumors) criteria. Furthermore, BRAF mutational status was not assessable in 33% of patients. It remains unclear whether the presence of a BRAF mutation is prognostic and whether patients with wild-type BRAF have a worse prognosis than those with a BRAF mutation treated with a targeted therapy (Long *et al.*, 2011). The small number of patients receiving Ipilimumab and the early time points for CTC sampling mean that no reliable conclusions can be made for these patients. Therefore, further studies of CTCs in well-defined subgroups of patients on the same treatment regimes are now needed to clarify the findings presented here.

The CellSearch platform has been widely used for detection of EpCam-positive CTCs in epithelial carcinomas. It remains the most validated technology and the only Food and Drug Administration-approved platform for prognostication in metastatic breast, prostate, and colorectal disease (Cristofanilli *et al.*, 2004; Cohen *et al.*, 2008; de Bono *et al.*, 2008). The Veridex melanoma CTC kit incorporates Melcam and HMW-MAA antibodies respectively to capture and detect melanoma CTCs, and only those CTCs positive for both markers are detected. Both markers are expressed in up to 80% of metastatic melanoma lesions (Shih, 1999; Campoli *et al.*, 2004). However, the level of antigen expression on tumor cells in the circulation is unknown, particularly with regard to detection limits by the Veridex platform. Low marker expression may be one of the reasons why the majority of our patients (75%) had $<2$ CTCs and 60% had no CTCs. A combination of marker and marker-independent methods may therefore be required to fully capture and interrogate subpopulations of melanoma CTCs (De Giorgi *et al.*, 2010).

Further CTC characterization with an additional marker added to the standard CellSearch melanoma kit, such as NY-ESO-1 or drug target expression, is currently possible. Mutation testing in CTCs has been demonstrated in prostate cancer (Jiang *et al.*, 2010). It is possible that molecular subtyping of melanoma CTCs may help define personalized treatments for melanoma patients. Our study suggests that, in future, molecular analysis of melanoma CTCs may aid stratification of patients treated with targeted agents and immunotherapy and possibly help define the best strategy for combination or sequential treatment.

In summary, melanoma CTCs are detectable in 40% of patients with advanced cutaneous melanoma, and the number of CTCs detected is prognostic for overall survival. There is preliminary evidence that changes in the number of CTCs during treatment may reflect outcome, although further detailed studies are required.

## MATERIALS AND METHODS

### Study design

Patients with newly diagnosed, untreated metastatic cutaneous melanoma, or previously treated but with progressive disease (and a treatment-free period of at least 6 weeks), were recruited prospectively at The Christie Hospital in Manchester, United Kingdom. CTCs were enumerated at baseline (enrollment into the study) before treatment, after the commencement of treatment (a median of 6 weeks later, time point 2), and then up to 9, 12, and 18 weeks, and (if applicable) at progression. Data on age, sex, PS, site of metastases, time from diagnosis of metastatic disease to study enrollment, treatment received after enrollment, line of treatment (first or second), response (assessed by computerized tomography scan in all patients and on the basis of the RECIST criteria if a trial participant), and survival were collected. Treatment received included standard chemotherapy with dacarbazine or targeted agents, including BRAF and MEK inhibitors, or immunotherapy with a cytotoxic T-lymphocyte antigen 4 monoclonal antibody. Written informed consent was obtained from all patients and the study was ethically approved by a regional ethics committee and the Declaration of Helsinki Principles were followed.

### CTC enumeration

Peripheral blood samples were collected in 10 ml CellSave preservative tubes (Veridex), stored at room temperature, and processed within 96 hours of collection. CTC enumeration per 7.5 ml blood was performed according to the manufacturer's instructions as previously described (Rao *et al.*, 2011). The sensitivity and reproducibility of the CellSearch melanoma CTC kit have been previously demonstrated (Rao *et al.*, 2011). All images were evaluated for assignment as CTCs and scored twice by two experienced analysts with a reanalysis of any discrepancies in enumeration. Previous quality assurance of CTC enumeration by CellSearch has found interanalyst variability in image interpretation to be the most significant factor for variation in CTC enumeration between laboratories (Kraan *et al.*, 2011). Validation experiments were conducted to determine the analytical accuracy of CellSearch CTC enumeration at low (1–10) CTC numbers. Analysts scored the same image galleries generated from 20 different melanoma patient samples with low CTC counts and the results were evaluated using

the BETI (Cummings *et al.*, 2011) approach (see Supplementary Material online for details).

### Statistical analysis

The associations between CTC number and clinical characteristics were examined using the $\chi^2$ test and Fisher's exact test. The $\chi^2$ tests were used for categorical clinical factors. Discrete/continuous clinical factors were categorized using either empirical (e.g., LDH) or median values (e.g., time period with metastatic disease) as cutoffs, and their associations with CTC number were measured using Fisher's exact test.

Previous reports on prognostic significance of CTC number in metastatic carcinomas used a CTC cutoff value to categorize all patients into ''favorable'' and ''unfavorable'' groups (Cristofanilli *et al.*, 2004; Cohen *et al.*, 2008; de Bono *et al.*, 2008; Hou *et al.*, 2009, 2012; Krebs *et al.*, 2011). The optimal CTC cutoff value was derived from baseline CTC number using a methodology similar to that previously described (Hou *et al.*, 2012), in which the Kaplan-Meier analysis and the log-rank test were performed to repeatedly examine each possible CTC cutoff, and the area under the curve of receiver-operating characteristic curves was measured to determine the optimal cutoff. In our study, the OS difference between patients with favorable and unfavorable CTCs was measured by the HR derived from multivariate Cox regression analysis, in which OS was calculated from the date of study enrollment to death or last follow-up. The bootstrap resampling (Efron and Tibshirani, 1993) technique was used as internal validation to avoid potential bias and overfitting in estimating the optimal cutoff (see Supplementary Data online).

As prior statistically robust data on prognostic significance of CTCs detected using the CellSearch platform in metastatic melanoma was lacking, an interim power analysis was performed on data from the first 65 patients. On the basis of the log-rank test, this reported a power of 0.6 with a significance of 0.05, requiring at least an additional 17 patients to reach a power of 0.8. Consequently, 101 patients were recruited to ensure validity of conclusions drawn from this study.

Baseline CTC number, CTC number at time-point 2 (median of 6 weeks from commencing therapy), and clinical characteristics such as sex, age, site of primary, LDH, PS, time from diagnosis of metastatic disease to study enrollment, stage, BRAF mutation status, whether treatment was first or second line, and type of treatment (Ipimulimab or a BRAF/MEK inhibitor) were subjected to univariate Cox regression analysis for OS. The validity of Cox regression analysis was confirmed by testing the proportional hazard assumption as requested by REMARK guidelines (McShane *et al.*, 2005; Altman *et al.*, 2012). Significant clinical factors were subsequently included in multivariate Cox regression analysis using a stepwise approach with inclusion criteria $P=0.05$ and exclusion criteria $P=0.1$. All significant factors excluding CTC number were applied for modeling first, and CTC-related factors were added to the model thereafter so that the influence of CTC-related factors was clarified. The derived multivariate Cox model was validated using bootstrapping (resampling data 1,000 times). The bootstrap was performed using MATLAB R2009a (MathWorks, Natick, MA), and other statistical analyses were performed using Statistical Package for Social Sciences for Window version 16 (SPSS, Chicago, IL), where $P\leqslant 0.05$ was deemed significant. All results were reported and validated following the REMARK guidelines.

## CONFLICT OF INTEREST

AH and GC are employees of AstraZeneca Pharmaceuticals and hold shares in the company. The other authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

## REFERENCES

Altman DG, McShane LM, Sauerbrei W *et al.* (2012) Reporting recommendations for tumor marker prognostic studies (REMARK): explanation and elaboration. *BMC Med* 10:51

Campoli MR, Chang CC, Kageshita T *et al.* (2004) Human high molecular weight-melanoma-associated antigen (HMW-MAA): a melanoma cell surface chondroitin sulfate proteoglycan (MSCP) with biological and clinical significance. *Crit Rev Immunol* 24:267–96

Chapman PB, Hauschild A, Robert C *et al.* (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364:2507–16

Cohen SJ, Punt CJ, Iannotti N *et al.* (2008) Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J Clin Oncol* 26:3213–21

Cristofanilli M, Budd GT, Ellis MJ *et al.* (2004) Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 351:781–91

Cummings J, Zhou C, Dive C (2011) Application of the beta-expectation tolerance interval to method validation of the M30 and M65 ELISA cell death biomarker assays. *J Chromatogr B Analyt Technol Biomed Life Sci* 879:887–93

Curtin JA, Busam K, Pinkel D *et al.* (2006) Somatic activation of KIT in distinct subtypes of melanoma. *J Clin Oncol* 24:4340–6

Curtin JA, Fridlyand J, Kageshita T *et al.* (2005) Distinct sets of genetic alterations in melanoma. *N Engl J Med* 353:2135–47

Davies H, Bignell GR, Cox C *et al.* (2002) Mutations of the BRAF gene in human cancer. *Nature* 417:949–54

de Bono JS, Scher HI, Montgomery RB *et al.* (2008) Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin Cancer Res* 14:6302–9

De Giorgi V, Pinzani P, Salvianti F *et al.* (2010) Application of a filtration- and isolation-by-size technique for the detection of circulating tumor cells in cutaneous melanoma. *J Invest Dermatol* 130:2440–7

Efron B, Tibshirani R (1993) *An Introduction to the Bootstrap*. Chapman & Hall: New York

Hodi FS, O'Day SJ, McDermott DF *et al.* (2010) Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363:711–23

Hou JM, Greystoke A, Lancashire L *et al.* (2009) Evaluation of circulating tumor cells and serological cell death biomarkers in small cell lung cancer patients undergoing chemotherapy. *Am J Pathol* 175:808–16

Hou JM, Krebs MG, Lancashire L *et al.* (2012) Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. *J Clin Oncol* 30:525–32

Jiang Y, Palma JF, Agus DB *et al.* (2010) Detection of androgen receptor mutations in circulating tumor cells in castration-resistant prostate cancer. *Clin Chem* 56:1492–5

Kraan J, Sleijfer S, Strijbos MH *et al.* (2011) External quality assurance of circulating tumor cell enumeration using the CellSearch((R)) system: a feasibility study. *Cytometry B Clin Cytom* 80:112–8

Krebs MG, Sloane R, Priest L *et al.* (2011) Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. *J Clin Oncol* 29:1556–63

Long GV, Menzies AM, Nagrial AM *et al.* (2011) Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* 29:1239–46

McShane LM, Altman DG, Sauerbrei W *et al.* (2005) REporting recommendations for tumour MARKer prognostic studies (REMARK). *Eur J Cancer* 41:1690–6

Nezos A, Lembessis P, Sourla A *et al.* (2009) Molecular markers detecting circulating melanoma cells by reverse transcription polymerase chain reaction: methodological pitfalls and clinical relevance. *Clin Chem Lab Med* 47:1–11

Rao C, Bui T, Connelly M *et al.* (2011) Circulating melanoma cells and survival in metastatic melanoma. *Int J Oncol* 38:755–60

Ribas A, Flaherty KT (2011) BRAF targeted therapy changes the treatment paradigm in melanoma. *Nat Rev Clin Oncol* 8:426–33

Shih IM (1999) The role of CD146 (Mel-CAM) in biology and pathology. *J Pathol* 189:4–11

Smith B, Selby P, Southgate J *et al.* (1991) Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet* 338:1227–9