ESMO Translational Research Fellowship

(November 2014 – October 2016)

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FINAL REPORT

Host Institute: **Institut Gustave-Roussy, Ile-de-France, Villejuif, France**
Mentor: **Benjamin Besse**
Project title: **Examining the Clinical Application of Circulating Tumor Cells in Non-Small Cell Lung Cancer**

Home Institute: **Beatson West of Scotland Cancer Centre, Glasgow, Scotland, UK**

Introduction

Circulating Tumor Cells (CTCs) represent an alternative source of tumor tissue which is accessible using a simple blood test. Their use has the potential to allow longitudinal monitoring of tumor biology at different timepoints, guiding therapeutic decisions according to resistance in a patient's treatment course [1]. In non-small cell lung cancer (NSCLC), their optimisation is particularly relevant given that an increasing demand for sequencing of multiple genes in 'umbrella' clinical trials does not yet square with the technical difficulties and small tissue samples involved with pulmonary biopsy in many patients [2].
To date the CellSearch® technology remains the only FDA-approved CTC-isolation system proven to have both assay and clinical/biological validity in cancer: using this platform, CTCs are isolated using immunomagnetic enrichment for the epithelial cell adhesion molecule (EPCAM) [3]. Two key reports have investigated CTC enumeration in NSCLC using CellSearch. An initial proof-of-principle that CTC identification and enumeration was possible in lung cancer was followed by a report offering information on the benefits and limitations of CellSearch quantification in NSCLC: 21% of 109 treatment-naïve stage III/IV patients had positive CTC counts at baseline (defined as ≥2 CTCs in 7·5mls blood since 1 CTC is a normal finding in healthy controls), while hypothesis-generating information on the prognostic capacity of CTC enumeration (cutoff ≥5 CTCs, 8·3% of patients) was also described [3, 4]. Other clinical reports have described a role for baseline prognostication using CellSearch in NSCLC, although with small patient numbers and no validation set [5-8].
Vimentin is a filamental protein expressed in mesenchymal cells, often recorded as a marker of tumour cell invasion via its expression during aberrant activation of the epithelial-mesenchymal transition (EMT). EMT is a biological program in which a series of dynamic and transitional steps can transform an epithelial cancer cell towards a more mesenchymal phenotype. EMT cells and their intermediate states have been associated with various tumorigenic properties including chemoresistance, tumour progression and stem cell character [9]. Its representation in CTCs has been demonstrated on a number of occasions using various CTC isolation platforms: ours and other groups have reported the existence of CTCs undergoing EMT in NSCLC using filtration-enrichment [10-14]. Previously we demonstrated that isolation of vim+ CTCs in castrate-resistant prostate cancer conferred poorer survival [15,16].

Rationale and Aim

Here we examined three areas of clinical and translational significance for development of CTCs in NSCLC. First, we explored the total number of CTCs as a prognostic marker in treatment-naive advanced NSCLC, acting as a validation for the previously estimated prognostic cutoff of ≥5 total CTCs. Second, we explored the prognostic value of vim+ CTCs in total CTC+ patients, assessing in turn their dynamic change with treatment. Finally, we correlated total CTC and vim+ CTC profiles with somatic alterations of EGFR, ALK and KRAS.

Experimental design

Study Design

CTC analyses were performed on 154 patients with presumed advanced (stage IIIb-IV) systemic treatment-naïve NSCLC who were prospectively included into one of two Gustave-Roussy translational sample collection protocols, MSN (145 patients, 2008-A00373-52) and CEC-CTC (9 patients, IDRCB2008-AOO585-50), between September 2010 and June 2015. Patients signed informed consent to undergo a peripheral blood sample: one CellSave tube at most 7 days prior to chemotherapy. The MSN and CEC-CTC protocols were approved by the local Gustave Roussy ethics review committee.

For eligibility, patients could be diagnosed either by contemporaneous or historical biopsy, and were excluded if they were suffering from a second malignancy. Routine laboratory analyses were also performed on all patients, with data prospectively collected for age, sex, histological subtype, genotype, ECOG performance status, smoking status, sites of metastasis, treatment received, previous adjuvant treatment, stage, date of progression, date of death, date of inclusion for CellSearch analysis, CellSearch results, and date of last follow-up.

Routine analysis of patient tumor biopsies for ALK rearrangement, KRAS mutation and EGFR mutation was performed as previously described for the French molecular testing network [17]. Given their mutual exclusivity, patients whose cancers featured genetic modification of KRAS were assumed to be wild-type for EGFR and ALK in the minority of cases where these modifications had not been tested; this approach was also used in the case of cancers with EGFR mutation and ALK rearrangement.

CTC Analysis

Collection of blood, immunomagnetic selection and immunofluorescent staining of CTCs were performed using the CellSearch® system (Janssen Diagnostics), as previously reported [18]. Blood samples were collected and stored at room temperature in 10ml CellSave (Janssen Diagnostics, LLC) preservative tubes, then processed within 72 hours of collection. FITC-labelled anti-vimentin antibody (Santa-Cruz) was added to the free channel of the CellSearch platform, as has been described previously [15]. Candidate total CTCs and vim+ CTCs were identified using the CellTracks Analyzer II (Janssen Diagnostics, LLC). In line with previous literature showing 1 CTC could be a normal finding in healthy controls, total CTC+ was defined as ≥2 total CTCs, and vim CTC+ was a patient who had ≥1 vimentin expressing CTC and known to be total CTC+ overall (ie ≥2 total CTC, of which ≥1 expressed vimentin) [3, 4]. The unfavourable prognostic CTC category was defined as ≥5 total CTCs [4].

Statistical Analysis

REMARK guidelines were followed in planning, analysis and reporting of the study. The association of total and vim+ CTC number with clinical characteristics and patient demographics was assessed using Fisher's exact test for dichotomous factors, and Mann-Whitney tests for continuous data. For survival analysis, baseline CTC values and standard clinical factors including age, performance status (PS), histology, and smoking status were considered for multivariate analysis of both progression-free survival (PFS) and overall survival (OS). Factors that were significant in univariate analysis were included in multivariate Cox proportional hazards regression analysis (forward stepwise selection method with p=<0.2 selected for entry into the model). The Kaplan-Meier method was used to estimate PFS and OS, and the survival distribution according to CTC values compared using the log-rank test and Cox regression. OS was defined as the time from inclusion for first CTC sample until death from any cause, cancer-related or otherwise. PFS was defined as the time from inclusion for first CTC sample until tumour progression or death, whichever came first. If no event had occurred, patients were censored at date of last follow-up. P values were two-sided and considered statistically significant at <0·05. Statistical analysis was performed using SPSS version 23 and GraphPad Prism 6·03 for Windows.

Results, Conclusions and Future Perspectives

Patient characteristics

CTC runs were initially performed in 154 treatment-naive lung cancer patients, of which 29 were subsequently excluded from analysis (eight with small cell lung cancer, 21 with stage I-IIIa NSCLC). Three patients were lost to follow-up and censored at their last appointment (Figure 1). A total of 125 advanced treatment-naïve NSCLC patients (stage IIIb-IV) were included for final analysis: 74 patients were total CTC-negative at baseline and 51 patients were total CTC+. Patient recruitment and baseline clinical details are recorded in Table 1 and Figure 1. A higher proportion of poor performance status was noted in those patients who were total CTC+ (P=0.003) (Table 1). Otherwise there were no significant differences between the groups in terms of histology, smoking, or metastatic burden. A baseline analysis of patient overall survival according to EGFR, ALK and KRAS subgroups was consistent with what has been described previously for each subgroup (supplementary Figure S1).

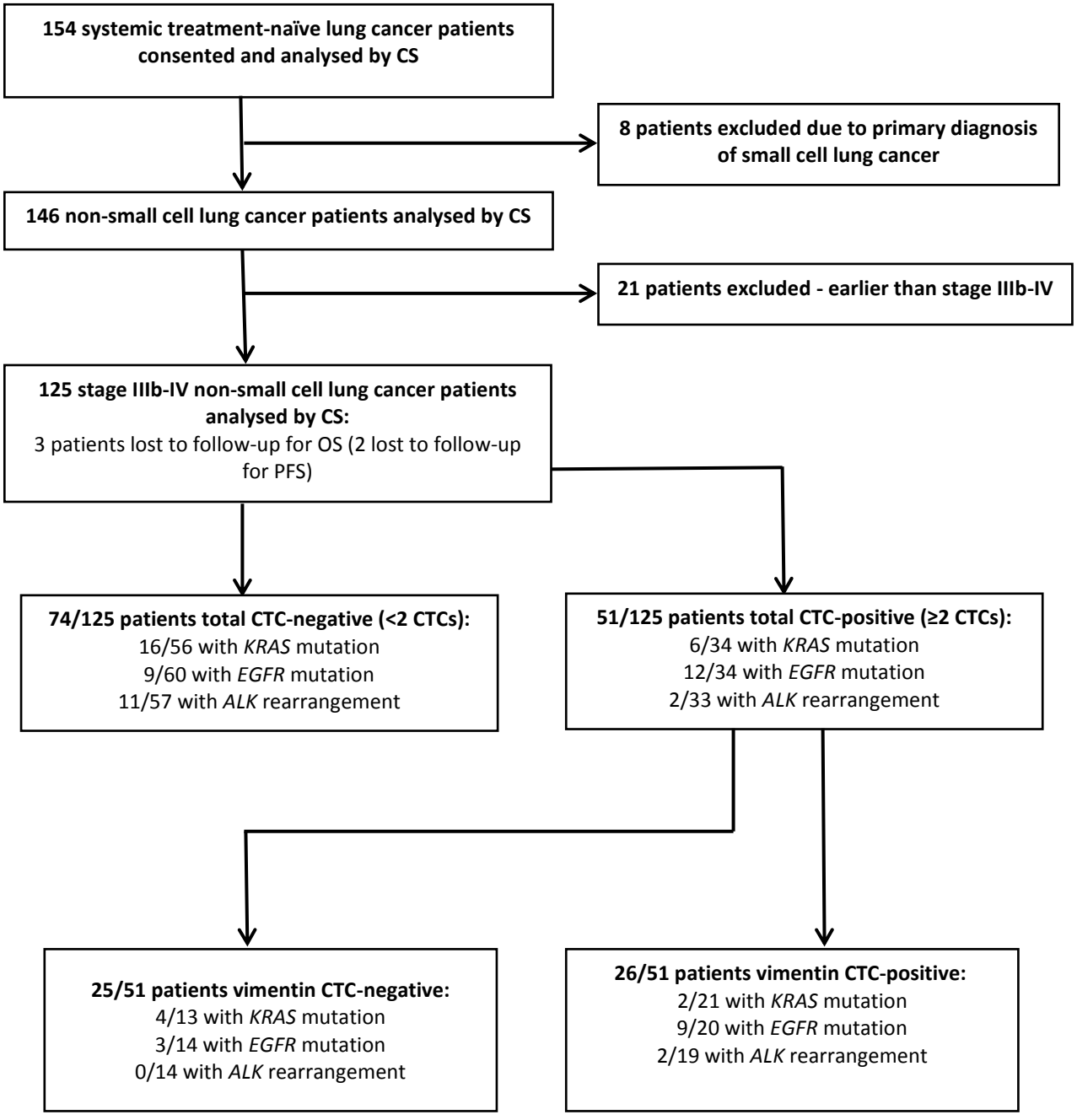
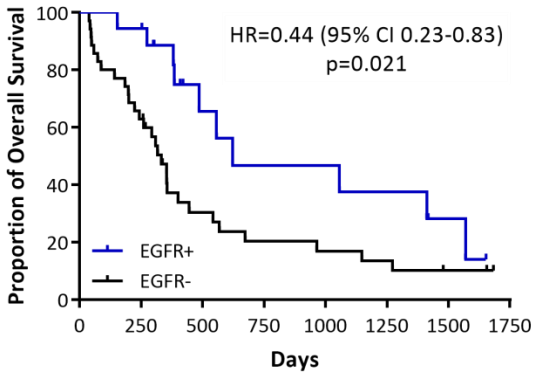


Figure 1. Patient flow. CS=CellSearch.

Characteristic	Total CTC-negative patients (%)	Total CTC-positive patients (%)	*P* value
Total Patient Numbers	74/125 (59.2)	51/125 (40.8)	
Age, years			
Median	61	62	
Range	25.86	27.81	0.813
Sex			
Male	47/74 (63.5)	40/51 (78.4)	
Female	27/74 (36.5)	11/51 (21.6)	0.08
Histology			
Non-squamous	64/74 (86.5)	49/51 (96.1)	
Adenocarcinoma	59/74 (79.7)	45/51 (88.2)	
Others	5/74 (6.8)	4/51 (7.8)	
Squamous	10/74 (13.5)	2/51 (3.9)	0.12
Smoking Status			
Non-smoker	15/74 (20.3)	9/51 (17.6)	
Ex-smoker	38/74 (51.4)	35/51 (68.6)	
Current smoker	20/74 (27)	6/51 (11.8)	
Not stated	1/74 (1.4)	1/51 (2)	0.819
Performance Status (ECOG)			
0-1	66/74 (89.2)	34/51 (66.7)	
2-3	8/74 (10.8)	17/51 (33.3)	0.003
Number of Metastasic Sites			
0	9/74 (12.2)	4/51 (7.8)	
1	25/74 (33.8)	14/51 (27.5)	
2	21/74 (28.4)	12/51 (23.5)	
3+	19/74 (25.7)	21/51 (41.2)	0.081
Metastasic Sites			
Lungs only	7/74 (9.5)	1/51 (2)	
Lungs/LNs only	3/74 (4.1)	1/51 (2)	
Visceral	55/74 (74.3)	45/51 (88.2)	0.157
Liver	4/74 (5.4)	10/51 (19.6)	
Bone	28/74 (37.8)	27/51 (52.9)	
Lung	25/74 (33.8)	14/51 (27.5)	
Brain	16/74 (21.6)	17/51 (33.3)	
Lymph Nodes	10/74 (13.5)	9/51 (17.6)	
Pleura (inc effusions)	10/74 (13.5)	12/51 (23.5)	
Adrenal	21/74 (28.4)	13/51 (25.5)	
Other(s)	13/74 (17.6)	15/51 (29.4)	
Systemic treatment planned at time of 1st CTC specimen			
Platinum doublet +/- bev	64/74 (86.5)	47/51 (92.2)	
EGFR/ALK inhibitor	7/74 (9.5)	3/51 (5.9)	
Not Stated	3/74 (4.1)	1/51 (2)	0.396
Previous treatments in last 6 months			
Primary Surgery	0/74 (0)	0/51 (0)	
Primary Radiotherapy	1/74 (1.4)	0/51 (0)	
Chemoradiotherapy	0/74 (0)	0/51 (0)	1.0

Table 1. Baseline characteristics of patients with treatment-naive advanced NSCLC according to total CTC status.

A.



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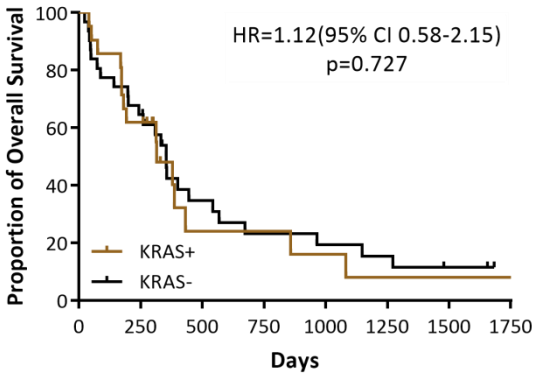

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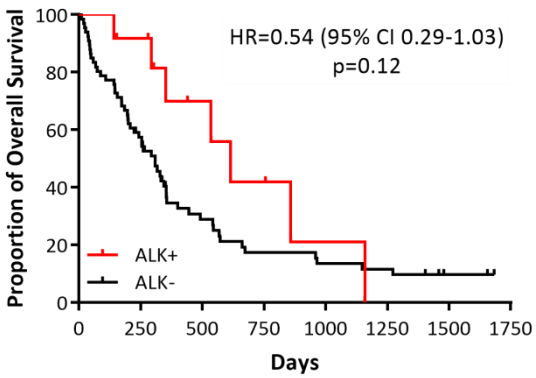


Supplementary Figure S1. Survival association with molecular subgroups. Kaplan Meier curves of OS for EGFR-mutant (A), KRAS-mutant (B), and ALK-rearranged (C) patients. *P* values obtained by log-rank tests.

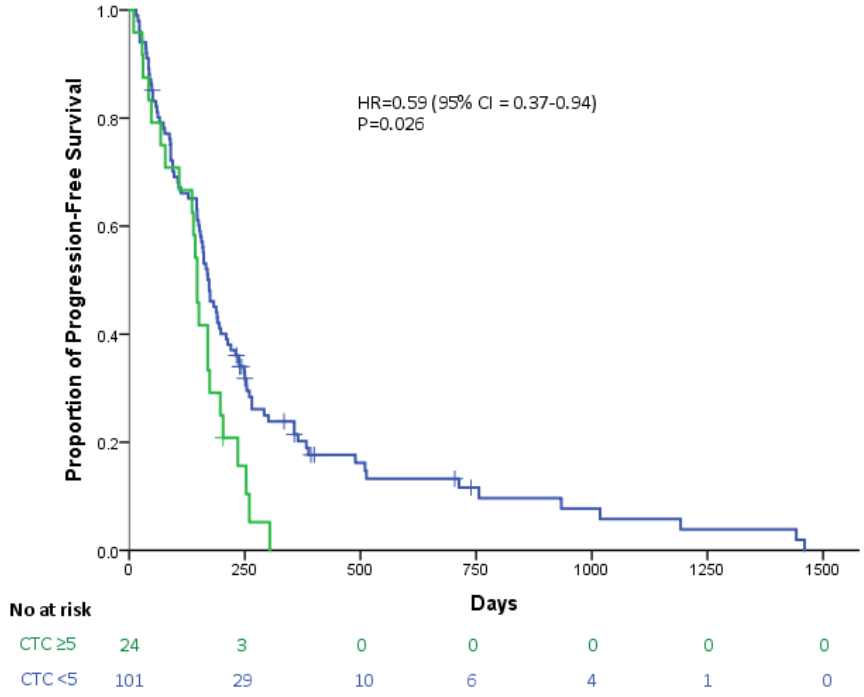
For baseline CTC characteristics, ≥1 total CTC(s) was present in the samples of 54.4% of patients, ≥2 total CTCs in 40.8% of patients, and ≥5 total CTCs in 19.2% (Table 2). The number of total CTCs ranged from 0-78 across all 125 patients, although the majority of total CTC+ patients displayed counts of between 2-10 total CTCs (Table 2). For vim+ CTCs, ≥1 vim+ CTC(s) was seen in 23.3% of patients, ≥2 vim+ CTCs in 14.4% of patients, and ≥5 vim+ CTCs in 5.6%. Range of vim+ CTCs was 0-35, with the majority of positive cases being in the very low end of this range (Table 2). 8/125 patients (6.4%) harboured vim+ CTCs exclusively. In total 140/873 (16%) of CTCs were vim+ across baseline samples from the cohort (Table 2).

GROUP	Patient Numbers (%)
Number of pts with no CTC (%)	57/125 (45.6)
Number of pts with ≥1 CTC (%)	
Total CTC	68/125 (54.4)
Vim+ CTC	29/125 (23.3)
Number of pts with ≥2 CTC (%)	
Total CTC	51/125 (40.8)
Vim+ CTC	18/125 (14.4)
Number of pts with ≥5 CTC (%)	
Total CTC	24/125 (19.2)
Vim+ CTC	7/125 (5.6)
CTC dynamic range	
Total CTC	0-78
Vim+ CTC	0-35
Number of pts with ≥2 CTC and ≥1 vim+ CTC	26/125 (20.8)
Number of pts with only vim+ CTC	8/125 (6.4)
Number of vim+ CTC	140/873 (16)

Table 2. Baseline CTC characteristics of treatment-naive patients with advanced NSCLC according to total and Vim+ CTC status.

Clinical Relevance of Total CTCs

After a median follow-up of 47 months, univariate analysis showed significant reductions in median PFS/OS were evident in patients with ≥5 total CTCs at baseline compared to those with <5 total CTCs (PFS: 147 vs 173 days and 6 month PFS 29.2% vs 45.1%, respectively; HR 0.59, 95% CI 0.37-0.94, *P*=0.026; OS: median 197 vs 386 days and 1 year OS 22.9% vs 56%, respectively; HR 0.45, 95% CI 0.28-0.75, *P*=0.002) (Figures 2A and 2B). Multivariate analysis confirmed that ≥5 total CTCs was an independent prognostic indicator for OS (HR 0.55, 95% CI 0.33-0.92, *P*=0.022) but not PFS (HR 0.68, 95% CI 0.42-1.1, *P*=0.118).



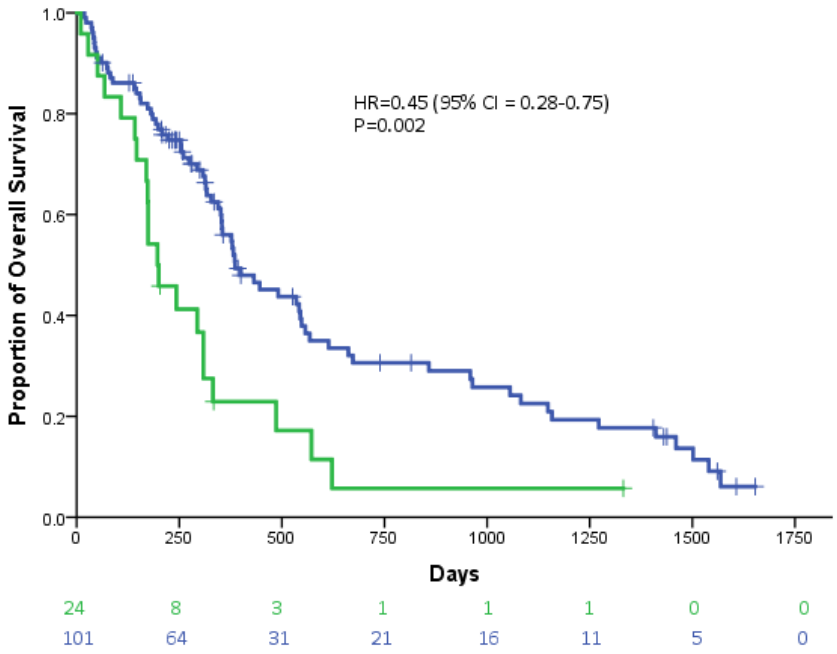
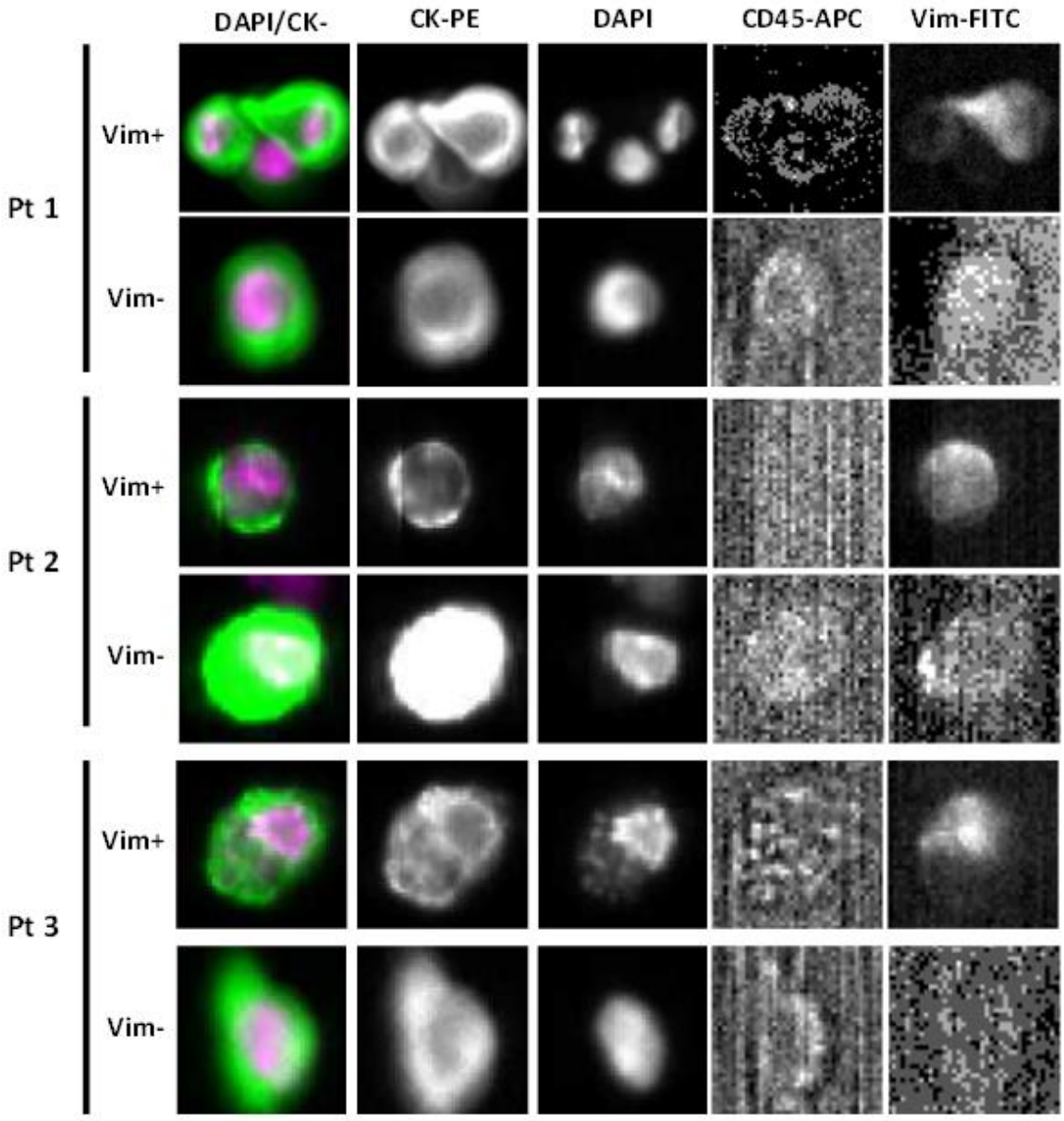


Figure 2. Survival association with baseline total CTC number. Kaplan Meier curves of PFS (A) and OS (B) according to the previously defined poor prognostic cutoff of ≥5 total CTCs. *P* values obtained by log-rank tests.

Clinical Relevance of Vimentin-positive CTCs

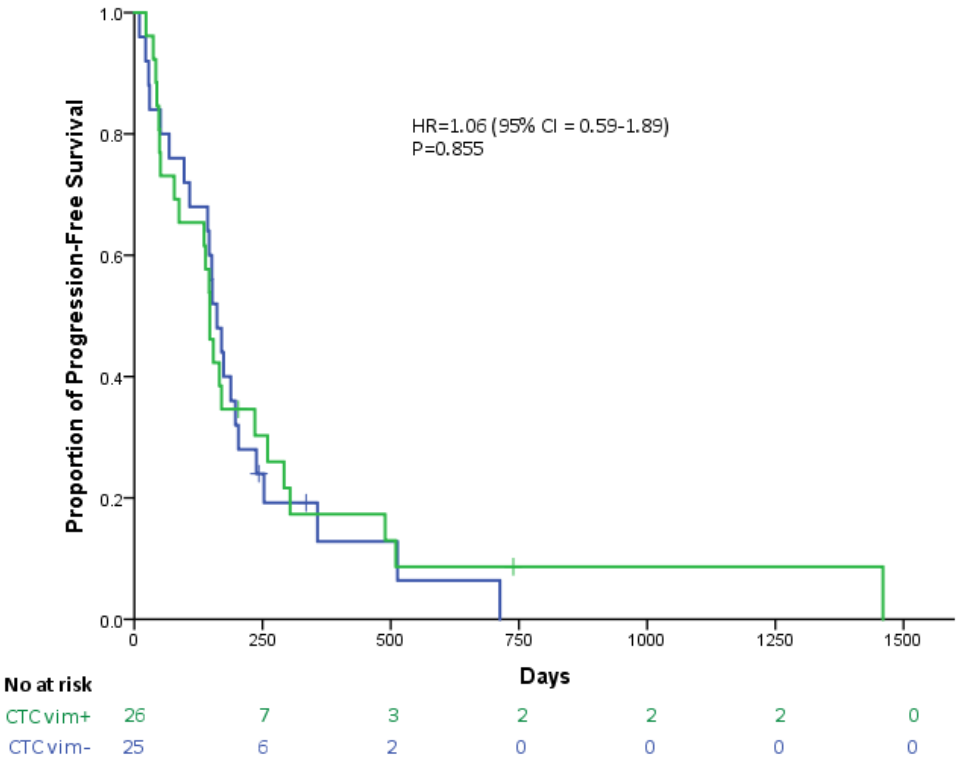
We next sought to describe and establish the prognostic significance of baseline vim+ CTCs (supplementary Figure S2). Supplementary Table S1 explores the baseline clinical characteristics of vim CTC+ and vim CTC-negative patients: no significant differences were observed between the two subgroups in terms of histology, smoking, PS, scheduled treatment or metastatic sites. Of the 51 patients who were total CTC+, 26 (51%) were vim CTC+ and 25 (49%) were vim CTC-negative (Figure 1, supplementary Table S1). In those patients who were total CTC+, the presence of ≥1 vim CTC did not diminish survival further when compared to those with no vim CTCs (median OS: 294 vs 309 days and 1 year survival 41.2% vs 29.3%, respectively; HR 1.24, 95% CI 0.67-2.28, *P*=0.494; median PFS: 147 vs 161 days and 1 year survival 17.3% vs 12.8%, respectively; HR 1.06, 95% CI 0.59-1.89, *P*=0.855) (Figures 3A and 3B).



Supplementary Figure S2. Representative pictures from CellSearch system of vimentin staining in CTCs from 3 patients

Characteristic	Vim CTC-negative patients (%)	Vim CTC-positive patients (%)	*P* value
Total Patient Numbers	25/51 (49)	26/51 (51)	
Age, years			
Median	37-81	27-75	
Range	65	60	0.131
Sex			
Male	20/25 (80)	20/26 (76.9)	
Female	5/25 (20)	6/26 (23.1)	1.0
Histology			
Non-squamous	24/25 (96)	25/26 (96.2)	
Adenocarcinoma	23/25 (92)	22/26 (84.6)	
Other	1/25 (4)	3/26 (11.6)	
Squamous	1/25 (4)	1/26 (3.8)	1.0
Smoking Status			
Non-smoker	3/25 (12)	6/26 (23.1)	
Ex-smoker	20/25 (80)	15/26 (57.7)	
Current smoker	2/25 (8)	4/26 (15.4)	
Not stated	0/25 (0)	1/26 (3.8)	0.464
Performance Status (ECOG)			
0-1	15/25 (60)	19/26 (73.1)	
2-3	10/25 (40)	7/26 (26.9)	0.382
Number of Metastasic Sites			
0	3/25 (12)	1/26 (3.8)	
1	10/25 (40)	4/26 (15.4)	
2	5/25 (20)	7/26 (26.9)	
3+	7/25 (28)	14/26 (53.8)	0.089
Metastasic Sites			
Lungs only	1/25 (4)	0/26 (0)	
Lungs/LNs only	0/25 (0)	1/26 (3.8)	
Visceral	21/25 (84)	24/26 (92.3)	0.365
Liver	4/25 (16)	6/26 (23.1)	
Bone	15/25 (60)	12/26 (46.2)	
Lung	4/25 (16)	10/26 (38.5)	
Brain	8/25 (32)	9/26 (34.6)	
Lymph Nodes	4/25 (16)	5/26 (19.2)	
Pleura (inc effusions)	3/25 (12)	9/26 (34.6)	
Adrenal	7/25 (28)	6/26 (23.1)	
Other(s)	4/25 (16)	11/26 (42.3)	
Systemic treatment planned at time of 1st CTC specimen			
Platinum doublet +/- bev	22/25 (88)	25/26 (96.2)	
EGFR/ALK inhibitor	2/25 (8)	1/26 (3.8)	
Not Stated	1/25 (4)	0/26 (0)	0.35
Previous treatments in last 6 months			
Primary Surgery	0/25 (0)	0/26 (0)	
Primary Radiotherapy	0/25 (0)	0/26 (0)	
Chemoradiotherapy	0/25 (0)	0/26 (0)	1.0

Supplementary Table S1. Baseline characteristics of patients with treatment-naive advanced NSCLC according to vim+ CTC status in those who are total CTC+. *P* values obtained by fisher's exact and chi-square tests.



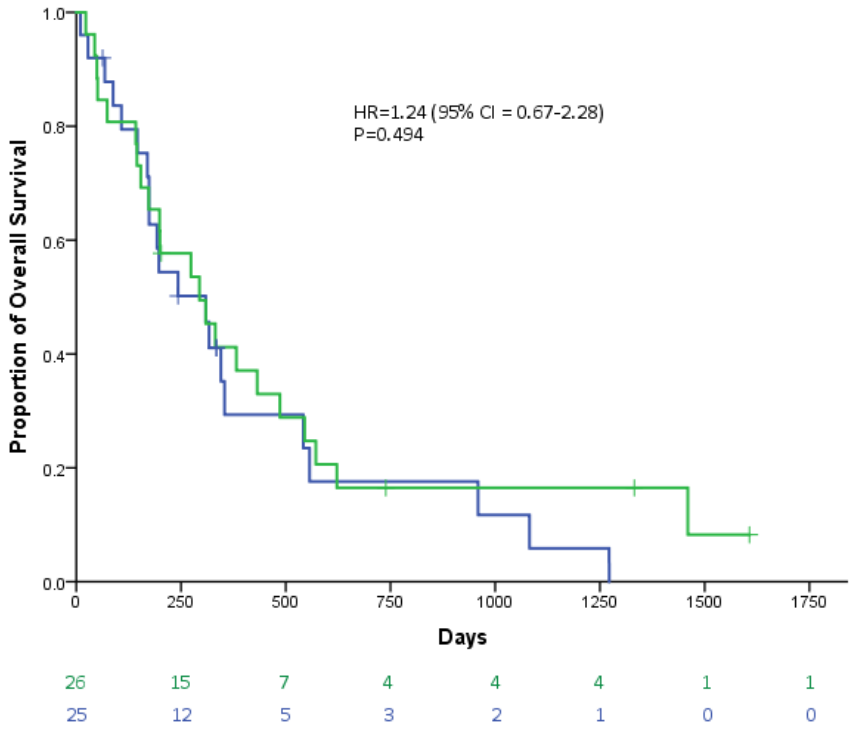


Figure 3. Survival association with baseline vim+ CTC status (A)-(B) Kaplan Meier curves of PFS (A) and OS (B) according to vim+ CTC positivity in those patients with ≥2 total CTC. *P* values obtained by log-rank tests.

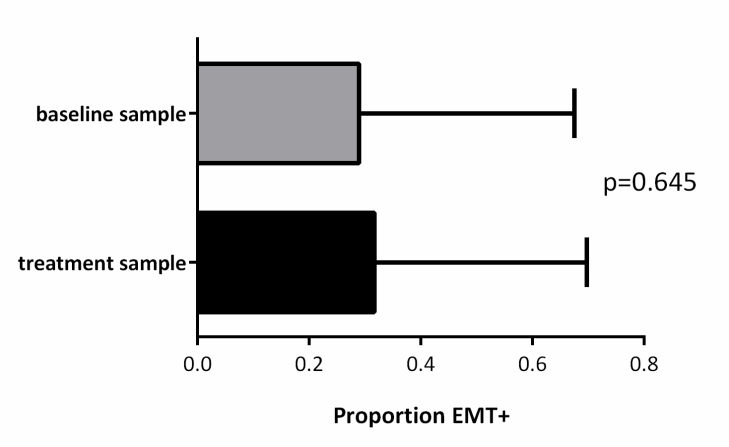
As EMT has been associated with treatment resistance in CTCs, we hypothesized that the vim+ profile of CTCs may develop during treatment, even if overall CTC numbers were to diminish. 75 patients (60%) in our cohort offered a 2nd 'on-treatment' sample for CellSearch analysis: 65 after cycle 1 and 10 after cycle 2. No difference was observed between baseline and paired on-treatment CTC samples for percentage of patients with vim+ CTCs (23.2% baseline vs 17.3% post-treatment, *P*=0.373) (supplementary Table S2), percentage of patients with dynamic change of vim+ and vim-negative CTCs during treatment (17.6% stability/increase for vim+ patients vs 13.5% for vim-negative, *P*=0.696) (supplementary Table S3), and ratio of vim+:total CTCs (median 0.118 at baseline vs 0.167 on-treatment; *P*=0.645) (supplementary Figure S3).

	Baseline sample before treatment	First sample after treatment start	
Number of patients with vim+ CTCs (%)	29/125 (23.2)	13/75 (17.3)	*P*=0.373
Number of patients with vim- CTCs (%)	57/125 (45.6)	28/75 (37.3)	*P*=0.302

Supplementary Table S2. Categorical change in patient vim+ and vim-negative CTC profiles with treatment. Fisher's exact test to analyse CTC frequencies in treatment groups. Samples from the second column were taken at cycles 1-2 following initiation of treatment. *P* values obtained by chi-square tests.

	vim+ CTC	vim– CTC	
Number of patients with increase or no change in CTC (%)	3/17 (17.6)	5/37 (13.5)	
Number of patients with reduction in CTC (%)	14/17 (82.4)	32/37 (86.5)	*P*=0.696

Supplementary Table S3. Dynamic change in patient vim+ and vim-negative CTC profiles with treatment. Fisher's exact test to analyse CTC frequencies in treatment groups. All patients analysed here had ≥1 vim- or vim+ CTC at baseline and a J21 sample. *P* values obtained by chi-square tests.



Supplementary Figure S3. Proportion of vim+ CTCs within total CTC+ patients. Mann-Whitney test to analyse vim+ CTC proportions before and during treatment. All patients analysed here were total CTC+ (≥2 total CTCs). Bar plot shows mean + standard deviation

CTCs according to NSCLC molecular subgroups

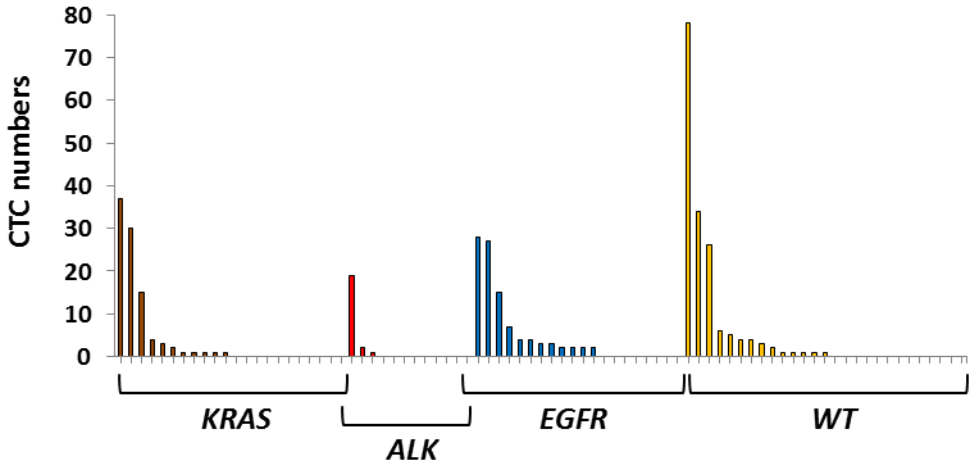
Of the 125 patients analysed with CellSearch at baseline, 104 had their tumors tested for genetic modifications and 67 of these cancers (64.4%) were found to harbor at least one 'actionable' aberration. 22/90 patients (24.4%) had tumors which were *KRAS*-mutated, 21/94 (22.3%) were *EGFR*-mutated, and 13/90 (14.4%) *ALK*-rearranged.

Figures 4A and 4B show bar plots of total CTC numbers and vim+ CTC proportions according to molecular subgroups, while Table 3 compares relative numbers of total CTC+ and vim CTC+ patients in each subgroup. On examination of the *KRAS* subgroup, a trend towards a reduction of vim+ CTCs in patients with *KRAS*-mutant cancers was consistent with fewer patients harbouring vim+ CTCs, but neither change reached statistical significance (*KRAS* mutant vs *WT*: 9.1% [2/22] patients vs 27.9% [19/68] patients, *P*=0.086; mean 1.68 vs 1.19 vim+ CTCs, *P*=0.081) (Table 3, Figure 4C). Only two *KRAS*-mutant patients harbored vim+ CTCs, and on review of their medical records it was noted that neither of these patients had a histological diagnosis of adenocarcinoma (1 squamous, 1 NSCLC not otherwise specified): re-examination of *KRAS*-mutant *adenocarcinoma* revealed a complete deficit of vim+ CTCs in this histology (0/19 vim CTC+ patients in *KRAS*-mutant vs 19/59 vim CTC+ patients in *KRAS WT* adenocarcinoma, *P*=0.004; mean 0 vs 1.4 vim+ CTCs, *P*=0.006). In the *EGFR*-assessed subgroup, vim+ CTC numbers were significantly high in patients with *EGFR*-mutated cancer, corresponding with an increase in *EGFR*-mutant patient numbers harbouring both total CTCs and vim+ CTCs (mutant vs *WT*: 57.1% vs 30.1% total CTC+ patients, *P*=0.038; mean 4.71 vs 4.12 total CTCs, *P*=0.212; 42.9% vs 15.1% vim CTC+ patients, *P*=0.013; mean 1.24 vs 1.22 vim+ CTCs, *P*=0.013) (Table 3, Figure 4D). For the *ALK*-assessed subgroup, a trend towards diminished patient numbers with total CTCs in *ALK*-rearranged cancer was supported by a significant reduction in total CTC numbers overall (rearranged vs WT: 2/13 total CTC+ patients vs 31/77 total CTC+ patients, 15.4% vs 40.3%, *P*=0.122; mean 1.69 vs 5.82 total CTCs, *P*=0.029) (Table 3, Figure 4E). Both vim CTC+ *ALK*-rearranged patients were noted to have high proportions of vim+ CTCs relative to total CTCs (Figure 4B).

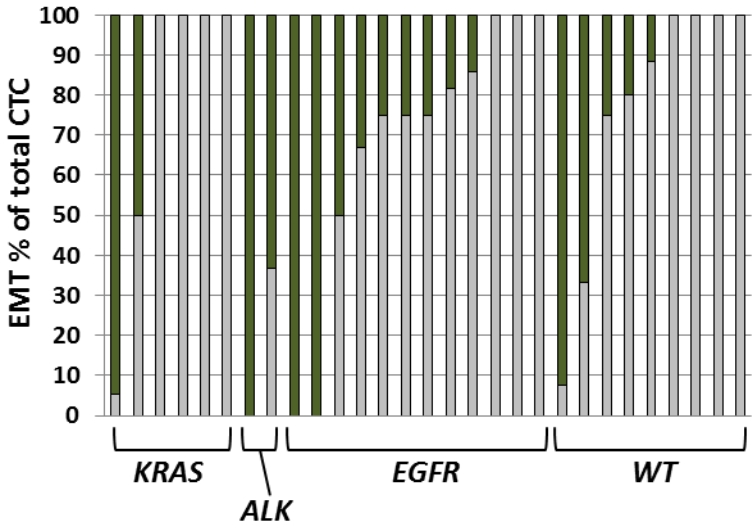
	Frequency		
Patient Population	***N***	**%**	***P***
KRAS* mutated vs *KRAS WT			
Number of total CTC+ patients (≥2 total CTC)	6/22 vs 28/68	27.3 vs 41.2	0.315
Number of vim CTC+ patients (≥1 vim+ CTC)	2/22 vs 19/68	9.1 vs 27.9	0.086
EGFR* mutated vs *EGFR WT			
Number of total CTC+ patients (≥2 total CTC)	12/21 vs 22/73	57.1 vs 30.1	0.038*
Number of vim CTC+ patients (≥1 vim+ CTC)	9/21 vs 11/73	42.9 vs 15.1	0.013*
ALK* rearrangement vs *ALK WT			
Number of total CTC+ patients (≥2 total CTC)	2/13 vs 31/77	15.4 vs 40.3	0.122
Number of vim CTC+ patients (≥1 vim+ CTC)	2/13 vs 17/77	15.4 vs 22.1	0.728

Table 3. Total and vim+ CTC patient profiles in relation to NSCLC mutational profiles.

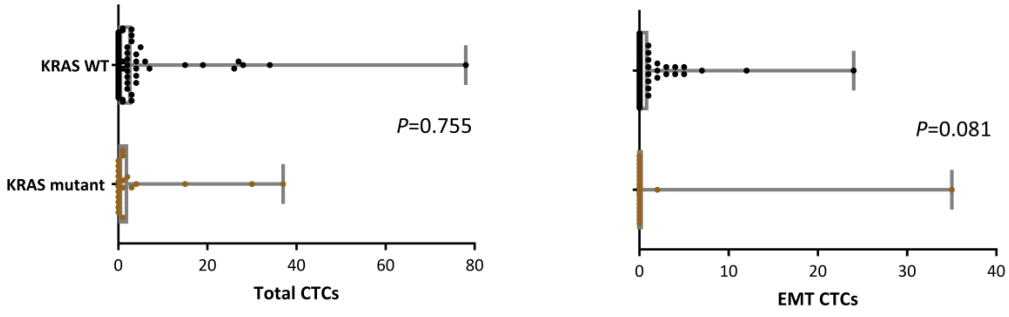
A


CTC numbers
80
70
60
50
40
30
20
10
0
KRAS
ALK
EGFR
WT

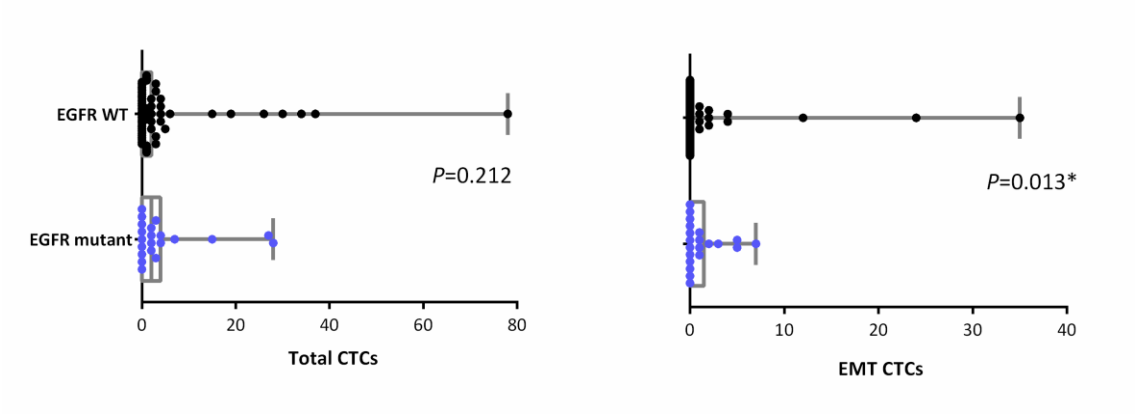
B


EMT % of total CTC
100
90
80
70
60
50
40
30
20
10
0
KRAS
ALK
EGFR
WT

C


KRAS WT
KRAS mutant
P=0.755
0
20
40
60
80
Total CTCs
P=0.081
0
10
20
30
40
EMT CTCs

D



E

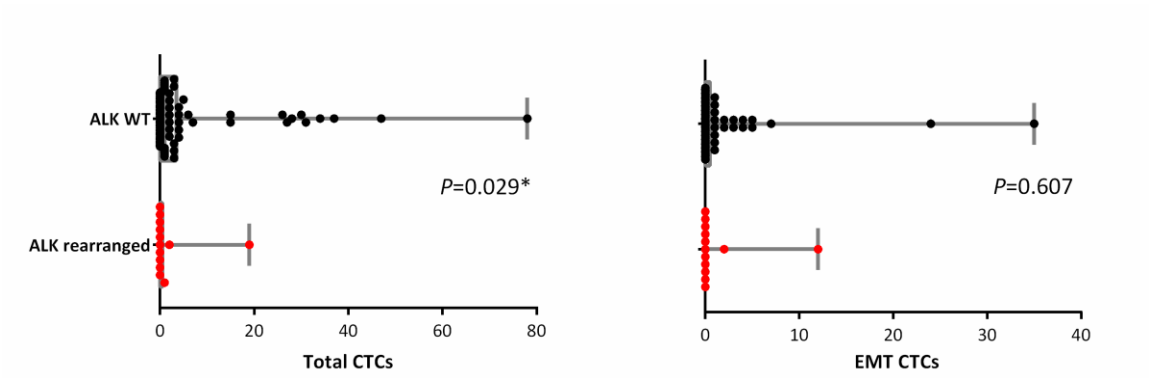


Figure 4. CTC profiles by NSCLC molecular subgroups (A) Bar plot of baseline total CTC numbers according to molecular subtype, each bar represents a patient (B) Distribution plot of vim+ (green) vs vim- CTCs in those patients who were total CTC-positive, according to molecular subtype with each bar representing a patient (C)-(E) Box plots assessing differences in relative numbers of total CTC and vim+ CTC in *KRAS* (C), *EGFR* (D), and *ALK* (E) subgroups. *P* values obtained by Mann-Whitney tests.

Conclusions and future perspectives

Here we report a prognostic validation of CTC estimation by CellSearch in advanced/metastatic NSCLC, as well as offering a clinical insight into their epithelial-mesenchymal properties according to analysis of the whole NSCLC population and its genetic subtypes. In line with what has been previously reported, we initially validated the prognostic value of ≥5 total CTCs at baseline through analysis of OS. The presence or absence of CTCs with EMT characteristics across the whole cohort conferred no additional prognostic significance when total CTC+ patients were subdivided into vim+ CTC and vim- CTC cohorts. However, the presence of vim+ CTCs was heterogeneous within the three main NSCLC genetic subgroups studied in our report: *EGFR*-mutation, *ALK* rearrangement and *KRAS*-mutation. A significantly high number of vim+ patients and vim+ CTC numbers in *EGFR*-mutant disease contrasted with a total loss of vim+ CTC patients and vim+ CTCs in *KRAS*-mutant adenocarcinoma. For *ALK*-rearranged patients there were fewer total CTCs with no difference in vim+ CTCs.

We validated the prognostic cut-off of ≥5 total CTCs previously identified using CellSearch, using both univariate and multivariate testing of OS, albeit with a higher number of patients with total CTC-positivity (40·8%) compared to that which was reported previously [4]. To our knowledge, this is the largest clinical study of CTCs analysed by CellSearch in advanced NSCLC patients to date. The importance of offering this validation was to follow predictive/prognostic biomarker guidelines such as REMARK and the Cancer Research UK biomarker roadmap, prospectively confirming the relationship between CTCs and NSCLC outcomes to pave the way for a clinical trial where CTCs can be used to define randomisation [19,20]. Given the association of *EGFR* mutation in this study with high numbers of total CTC+ patients, it is possible that the relative enrichment of *EGFR*-positive patients (21/94 patients, 22.3%) in our study has contributed to this increased number of total CTC+ patients overall. A European multi-centre pooled analysis of NSCLC CTCs by CellSearch has now completed recruitment and should facilitate a final focus on clinical validity by providing an answer to this question, as well as offering a rounded picture of the percentage of patients with total CTC-positivity in advanced disease.

EMT has previously been associated with tumour invasiveness, chemoresistance, and poorer clinical outcomes [9]. For vimentin assessment here, an initial biological selection during CellSearch should be noted: it will only test for vimentin after patient CTCs have been initially isolated using EPCAM-coated ferromagnetic beads, an epithelial-antibody based selection. A-fully representative picture of mesenchymal or EMT CTCs is impaired as a consequence. CellSearch was selected for use due to its advanced level of assay and biological validity: its ability to prognosticate has been tested extensively in the clinic and published in a number of cancers, with FDA-approval granted for advanced breast, colorectal and prostate cancers, and its use is consistent and reproducible across all patient cohorts for the purposes of survival analyses and correlation with clinical/molecular markers [1]. A fully representative biological picture of mesenchymal CTCs or CTCs in EMT has been exploited by a number of other CTC isolation techniques that have not yet reached the same level of assay validity [10-14].

Across NSCLC overall, vimentin presence did not confer an additional survival difference in those patients that were total CTC-positive, although this level of survival analysis with relatively small patient numbers does not take into account that NSCLC is a diagnosis of histological exclusion which covers a myriad of different genetic and biological pathological processes [21]. With more detailed analysis, evidence of increased total and vim+ CTCs was apparent in *EGFR*-mutant patients, as well as a total absence of vim+ CTCs in *KRAS*-mutated adenocarcinomas. The presence of differential vim+ CTC profiles amongst different genetic and histological subgroups of NSCLC suggests that each subgroup may display a different level of CTC dynamic plasticity. The higher number of total CTC+ patients suggests that *EGFR*-mutant NSCLC will also be of particular interest for design of CTC-based clinical trials: this result is consistent with two other reports that used alternative methods of CTC isolation to characterise small numbers of patients with *EGFR*-mutated NSCLC [22, 23].

The network of French molecular platforms offers a unique clinical infrastructure where molecular profiling of NSCLC patients for key modifications such as *EGFR*, *KRAS* and *ALK*. This has been performed routinely for a number of years, creating opportunities for translational research such as the findings described in this report [17]. The association between *EGFR* mutation and vim+ CTCs in our study is a result that would have

been expected in patients developing acquired resistance to EGFR inhibition, but is perhaps more surprising in the context of systemic treatment-naive disease. Biopsy and cell line studies of *EGFR*-mutant disease have however consistently shown the presence of vimentin expression before the onset of treatment resistance, including the landmark study which detailed EMT as a mechanism of resistance in *EGFR*-mutant patients. In this study, we noted vimentin-positivity in the baseline CTCs of 26% of *EGFR*-mutated patients [24-27]. These findings reinforce a hypothesis that the development of EMT-mediated resistance in cancers treated with EGFR inhibitors is conferred by an *increase* in EMT markers, perhaps with an on-off threshold effect, rather than outright *de novo* induction. For *KRAS* mutation, the absence of vim+ CTCs in adenocarcinoma is consistent with a total lack of vimentin expression observed in tumors resected from a transgenic mouse model of *KRAS*-driven lung cancer, although this study cannot exclude the possibility that pure mesenchymal CTCs missed by CellSearch could hold importance in this molecular subtype [28]. Our previous report showing- that *ALK*-rearranged CTCs exhibit a complete loss of epithelial marker expression and potentially a fully mesenchymal phenotype, expressing vimentin and N-cadherin, is consistent with the low level of total CTCs in patients with *ALK*-rearranged tumours observed in this study [13].

In conclusion, we validate a prognostic survival difference using a cutoff of ≥5 total CTCs isolated by CellSearch in the largest cohort of NSCLC patients reported so far. For the first time we show that differential EMT characteristics and total CTC profiles can be observed according to different genetic subtypes of NSCLC. In a cancer where small samples and poor biopsy quality can often slow its molecularly-driven clinical development, future studies can focus on examining how the clinical utility of CTCs can be best exploited in genetic subtypes of NSCLC.

List of Publications and Presentations related to this projects during the fellowship period (if applicable)

A Prospective Examination of Circulating Tumor Cell Profiles in Non-Small Cell Lung Cancer Molecular Subgroups
Lindsay CR, Faugeroux V, Michiels S, Pailler E, Facchinetti F, Bluthgen MV, Pannet C, Ngo-Camus M, Bescher G, Caramella C, Billiot F, Remon J, Planchard D, Soria J-C, Farace F, Besse B
Ann Oncol: in press

Filtration-Enrichment of Circulating Tumour Cells (CTC) following CellSearch analysis in stage IV Non-Small Cell Lung Cancer (NSCLC): a feasibility study in clinical samples First Author poster, National Cancer Research Institute meeting, 2015

Presentation. Cancer-ID therapeutic apheresis workshop: Dusseldorf, 2015

Variations in the epithelial-mesenchymal transition (EMT) program by non-small cell lung cancer (NSCLC) circulating tumor cells (CTCs) do not influence survival First Author posters, ISMRC and AACR general meetings, 2016

List of Publications and Presentations resulting from other projects during the fellowship period (if applicable)

Circulating Tumor Cells with Aberrant ALK-Copy Number Predicts Progression-Free Survival to Crizotinib in ALK-Rearranged Non-Small-Cell Lung Cancer Patients
Pailler E, Oulhen M, Borget I, Remon, J, Ross K, Auger N, Billiot F, Ngo-Camus M, Commo F, **Lindsay CR**, Planchard D, Soria JC, Besse B, Farace F
Cancer Res: in press

CD52, CD22, CD26, EG5 and IGF-1R expression in thymic malignancies
Remon J, Abedallaa N, Taranchon-Clermont E, Bluthgen V, **Lindsay CR**, Besse B, Thomas de Montpréville V
Lung Cancer: 2017; 108: 168-172.

Method for semi-automated microscopy of filtration-enriched circulating tumor cells
Pailler E, Oulhen M, Billiot F, Galland A, Auger N, Faugeroux V, Laplace-Builhé C, Besse B, Loriot Y, Ngo-Camus M, Hemanda M, **Lindsay CR**, Soria JC, Vielh P, Farace F.
BMC Cancer. 2016 Jul 14;16(1):477.

Prognostic and predictive capacity of Ki67- and vimentin-expressing circulating tumor cells in prostate cancer
Lindsay CR, Le Moulec S, Billiot F, Loriot Y, Ngo-Camus P, Vielh P, Fizazi K, Massard C, Farace F
BMC Cancer. 2016; 16(1):168.

Thymic malignancies: Moving forward with new systemic treatments
Remon J, **Lindsay CR**, Bluthgen MV, Besse B.
Cancer Treat Rev. 2016 Apr 1;46:27-34.

Sunitinib in patients with advanced thymic malignancies: RYTHMIC experience
Remon J, Girard N, Mazières J, Dansin E, Pichon E, Biemar J, Dubos C, **Lindsay CR**, Besse B
Lung Cancer: 2016 Jul;97:99-104.

The potential diagnostic power of circulating tumor cell analysis for non-small-cell lung cancer
Ross K, Pailler E, Faugeroux V, Taylor M, Oulhen M, Auger N, Planchard D, Soria JC, **Lindsay CR**, Besse B, Vielh P, Farace F.
Expert Rev Mol Diagn. 2015;15(12):1605-29.

High level of chromosomal instability in circulating tumor cells of ROS1-rearranged non-small-cell lung cancer
Pailler E, Auger N, **Lindsay CR**, Vielh P, Islas-Morris-Hernandez A, Borget I, Ngo-Camus M, Planchard D, Soria JC, Besse B, Farace F.
Ann Oncol. 2015 Jul;26(7):1408-15.

A Long-Term Spinal Intramedullary Response to Ceritinib in ALK Rearranged Non-Small-Cell Lung Cancer
Biya J, Caramella C, **Lindsay CR**, Planchard D, Besse B.
J Thorac Oncol. 2015 Jun;10(6):e44-5.

COURSES AND WORKSHOPS ATTENDED

- **Cancer-ID therapeutic apheresis workshop:** Dusseldorf, 2015
- **Cancer-ID general symposia:** Amsterdam, 2015
- **Cancer-ID general symposia:** Hamburg, 2016
- **Advanced Course in Molecular Pathology and Diagnosis of Cancer**: Wellcome Genome Campus, 2016

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Acknowledgements

I would like to thank all the patients who took the time to participate in this research at a time when they had other priorities.

This ESMO Translational Fellowship Research Project was supported by an educational grant from

Roche