Original Research

EPAC-lung: pooled analysis of circulating tumour cells in advanced non-small cell lung cancer

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Abstract *Introduction:* We assessed the clinical validity of circulating tumour cell (CTC) quantification for prognostication of patients with advanced non-small cell lung cancer (NSCLC) by undertaking a pooled analysis of individual patient data.

Methods: Nine European NSCLC CTC centres were asked to provide reported/unreported pseudo-anonymised data for patients with advanced NSCLC who participated in CellSearch CTC studies from January 2003 to March 2017. We used Cox regression models, stratified by centres, to establish the association between CTC count and survival. We assessed the added value of CTCs to prognostic clinicopathological models using likelihood ratio (LR) statistics and c-indices.

Results: Seven out of nine eligible centres provided data for 550 patients with prognostic information for overall survival. CTC counts of ≥ 2 and ≥ 5 per 7·5 mL were associated with reduced progression-free survival (≥ 2 CTCs: hazard ratio [HR] = 1.72, $p < 0.001$; ≥ 5 CTCs: HR = 2.21, $p < 0.001$) and overall survival (≥ 2 CTCs: HR = 2·18, $p < 0.001$; ≥ 5 CTCs: HR = 2·75, $p < 0.001$), respectively. Survival prediction was significantly improved by addition of baseline CTC count to LR clinicopathological models (log-transformed CTCs $p < 0.001$; ≥ 2 CTCs $p < 0.001$; ≥ 5 CTCs $p \leq 0.001$ for both survival end-points), whereas moderate improvements were observed with the use of c-index models. There was some evidence of between-centre heterogeneity, especially when examining continuous counts of CTCs.

Conclusions: These data confirm CTCs as an independent prognostic indicator of progression-free survival and overall survival in advanced NSCLC and also reveal some evidence of between-centre heterogeneity. CTC count improves prognostication when added to full clinicopathological predictive models.

1. Introduction

Circulating tumour cells (CTCs), captured as a 'liquid biopsy' from blood for enumeration and biological characterisation of cancers, provide important clinical information on prognosis, therapeutic choice, and drug resistance. They represent an alternative source of tumour tissue which is easily accessible, allowing longitudinal monitoring of tumour biology at different time points to guide therapeutic decisions in a patient's treatment course [1].

Although multiple commercially available methods for isolating CTCs exist, CellSearch® is the only Food and Drug Administration (FDA)−approved system for clinical use in cancer, offering reproducible results across many different laboratories. Investigation of CTCs in non-small cell lung cancer (NSCLC) has been delineated in a number of single-centre reports [2−8]. Initial proof of principle that CTC identification and enumeration was possible in lung cancer was followed by further detail on the prognostic capacity of CellSearch quantification in advanced NSCLC: 21% of 109 stage III/IV patients had positive CTC counts at baseline [2,3]. Hypothesis-generating information on the prognostic capacity of a ≥ 5 CTCs cut-off (9% of patients) was validated by a recent report which also assessed CTCs according to NSCLC molecular subgroup and their epithelial−mesenchymal transition (EMT) character [8]. Overall, these reports suggest further optimisation is necessary to consider routine use of CTCs as a predictive/prognostic biomarker in NSCLC.

The objective of this European collaboration of CTC centres was to assemble the vast amount of data currently available (published or unpublished) using the CellSearch platform to assess clinically relevant end-points in advanced NSCLC. This would remove any bias typically associated with performing single-centre analyses and also offer sufficient statistical power to examine the contribution of CTC count beyond a full clinicopathological model. As an exploratory end-point, we also further characterised the relevance of CTCs to different NSCLC molecular subgroups.

2. Materials and methods

2.1. Study design and population

The study protocol was designed by the study management team and reviewed by all investigators (appendix 1). Invitations to participate were extended to nine European cancer centres known to run CellSearch for NSCLC samples between January 2003 and March 2017. Eligibility criteria included confirmed stage IIIb/IV NSCLC, availability of progression-free survival (PFS) and/or overall survival (OS) information (assessed prospectively or retrospectively), approval of CTC work by local ethics committee and CellSearch measurement of CTC levels at pretreatment baseline. Centres were

excluded if CTC counts were used by clinicians to adjust patient treatment and thus potentially confound survival analyses. Patient cases were excluded if CTC count was measured after treatment had commenced.

2.2. Procedures

A collaboration (European Pooled Analysis of CTCs in lung cancer, 'EPAC-lung') was initially established between the Gustave Roussy Cancer Centre and the Cancer Research UK Manchester Institute (CRUK MI) to complete this work. Local investigators within and outwith the partnership collected and shared individual pseudo-anonymised patient data, which were encrypted and then centralised into a repository for data analysis. Data files were screened by the study management team, and queries returned to centres whenever necessary.

Data items collected per patient were pseudo-anonymised patient ID, centre ID, line of systemic treatment, baseline total CTC count by CellSearch (per 7.5 ml), CellSearch date, date of tumour progression and/or death, gender, age, Eastern Cooperative Oncology Group (ECOG) performance status, smoking status, NSCLC histological subtype, stage IIIb or IV at sample collection, presence/testing of EGFR/ALK/KRAS genetic alterations, previous treatment, planned treatment and location/number of metastatic sites. Further detail on these data and planned analyses can be reviewed in the study protocol (appendix 1).

Collection of blood, immunomagnetic selection and immunofluorescent staining of CTCs were performed using the CellSearch® system, as previously reported [2]. Blood samples were collected and stored at room temperature in 10-ml CellSave Preservative Tubes and then processed within 72 h of collection. Candidate CTCs were identified using the CellTracks Analyzer II.

2.3. Statistical analysis

REMARK (REporting recommendations for tumour MARKer prognostic studies) guidelines were followed in planning, analysis and reporting of the study [9]. OS was defined as the time from inclusion for the first CTC sample until death from any cause, cancer-related or otherwise. PFS was defined as the time from inclusion for the first CTC sample until tumour progression (assessed by Response Evaluation Criteria in Solid Tumours 1.1) or death, whichever occurred first. If no event had occurred, patients were censored at the date of the last follow-up. The prespecified primary objective was to evaluate the prognostic value of baseline pretreatment CTC count (per 7.5 ml) by the CellSearch method in metastatic lung cancer, examining their relationship with OS and PFS. It was planned to analyse CTC first as a continuous variable and second using two prespecified cut-offs in a Cox model stratified by centre. The two prespecified cut-offs were ≥ 2 CTCs and ≥ 5 CTCs per 7.5 ml of blood. A cut-off of ≥ 5

CTCs was previously proposed and is the threshold commonly used in metastatic breast cancer [3,8,10]. In line with previous NSCLC CTC reports and owing to the previous identification of one CTC in healthy controls, a positive CTC count was defined as ≥ 2 CTCs per 7.5 ml of blood and used as a second cut-off [2].

The clinicopathological prognostic model was based on predetermined characteristics including age (continuous), gender (male/female), baseline treatment (platinum $\pm$ bevacizumab versus other), smoking status (never smoked versus former or current smoker), number of metastases (up to 1 versus more than 1), presence of brain metastasis (Yes/No), performance status (ECOG score <2 versus ECOG score ≥ 2) and histology (non-squamous versus squamous). Cubic splines were used to inspect linear relationships in the Cox regression model; the CTC count was log-transformed (natural logarithm) to satisfy the linearity hypothesis. To estimate the additional value of CTCs to a clinicopathological prognostic model, our primary statistical analysis assessed likelihood ratios (LRs) in Cox regression models stratified by centre. Heterogeneity between centres was explored using chi-squared statistics. C-indices were also used as an alternative measure to assess the additional value of CTCs in prognostic models. The Kaplan–Meier method was used to estimate survival, and p-values were two-tailed. A two-sided significance level of <0.05 was considered significant.

3. Results

3.1. Patients

Eight out of nine European NSCLC CTC centres that were contacted replied to confirm they have used CellSearch technology to isolate CTCs in advanced samples corresponding to the eligibility criteria. Seven of the eight CTC centres subsequently agreed to participate in the pooled analysis, providing data on 564 patients with advanced NSCLC overall. Of these centres, three offered data on a total of 209 patients with information regarding NSCLC CTCs that they had not previously published. 550 cases had available data for OS and baseline CTC count, while 514 had available data for PFS (Fig. 1).

Baseline demographics of the 550 patients analysable for OS and the associations between demographics and CTCs are shown in Table 1. More than or equal to 2 CTCs were present in the samples of 149 (27.1%) patients, and ≥ 5 CTCs in 73 (13.3%). The number of CTCs ranged from 0 to 733 across all 564 patients.

3.2. Survival

Median follow-up time for survival assessment was 36.57 months [95% CI = 29.63–46.16 months]. By this time, 486 (88.4%) patients had an event for PFS and 408 (79.4%) patients had an event for OS.
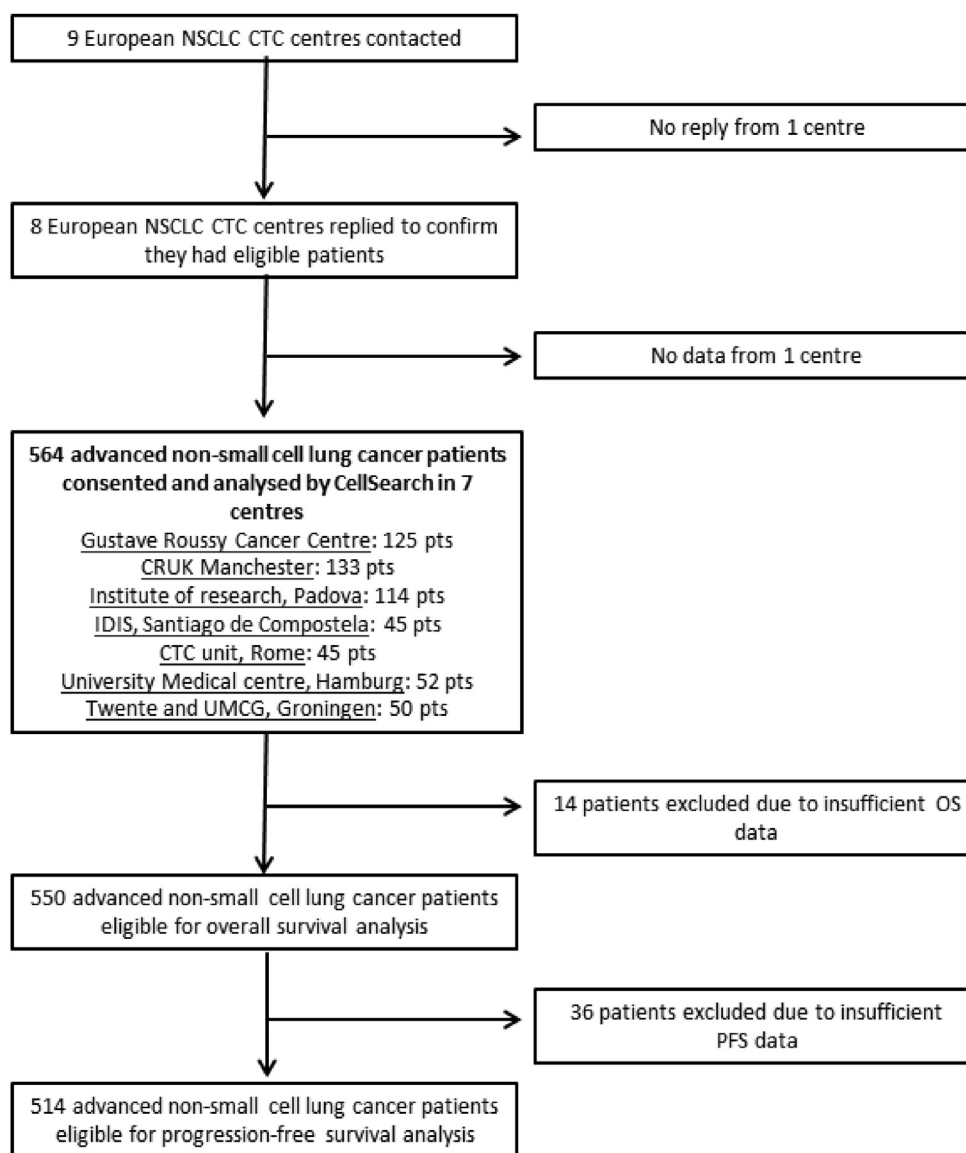
Fig. 1. Flow diagram. NSCLC, non-small cell lung cancer; CTC, circulating tumour cell; PFS, progression-free survival; OS, overall survival.

For PFS, we observed significant between-centre heterogeneity in the prognostic effect of log-transformed CTC counts ($X_6^2 = 13.75$, p = 0.033) and in the prognostic effect of ≥ 5 CTCs ($X_6^2 = 13.38$, p = 0.037) but not of ≥ 2 CTCs ($X_6^2 = 6.80$, p = 0.34), with the prognostic effect in one centre appearing slightly stronger than that observed in other centres (Fig. 2A and B).

Significant relative increases in the hazard of a progression or death were noted with one-unit increase in log-transformed CTC counts (HR = 1.33, 95% CI = 1.21−1.46, p < 0.001), CTC counts of ≥ 2 (HR = 1.72, 95% CI = 1.4−2.12, p < 0.001) and ≥ 5 (HR = 2.21, 95% CI = 1.69−2.9, p < 0.001). Kaplan−Meier curves of PFS are provided in Fig. 3A and B according to the 2 and 5 CTC cut-offs.

For OS, there was some evidence of significant between-study heterogeneity in the prognostic effect of logged CTC counts ($X_6^2 = 13.96$, p = 0.030) but not for ≥ 2 CTCs ($X_6^2 = 7.09$, p = 0.31) or ≥ 5 CTCs ($X_6^2 = 10.67$, p = 0.099) (Fig. 2C and D). A one-unit increase in logged CTC counts corresponded to significant relative increase in the mortality rate (HR = 1.49, 95% CI = 1.35; 1.65, p < 0.001), as was also the case for both ≥ 2 (HR = 2.18, 95% CI = 1.74−2.72, p < 0.001) and ≥ 5 (HR = 2.75, 95% CI = 2.07−3.65, p < 0.001) CTCs (Fig. 3C and D).

3.3. CTCs as an independent prognostic indicator

We then built clinicopathological prognostic models for both PFS and OS to assess the added value of CTCs as a continuous or categorical variable on top of a typically used clinicopathological prognostic model at diagnosis of advanced NSCLC. Sample size for PFS was reduced

Table 1
Baseline characteristics of 550 patients with advanced NSCLC and available OS data, according to total CTC status.

Characteristics	Overall	Cut-off = 2		Cut-off = 5	
	% (N)	N or Mean		N or Mean	
		CTC<2	CTC≥2	CTC<5	CTC≥5
Age at baseline	64	62.97	61.06	62.82	60.01
Missing (N)	1	1	0	1	0
Gender					
Male	37.27 (205)	159	46	183	22
Female	62.73 (345)	242	103	294	51
Histology					
Non Squamous	82.34 (443)	314	136	386	64
Squamous	17.66 (95)	84	13	90	7
Missing (N)	12	10	2	10	2
Performance Status (ECOG)					
0	25.50 (140)	111	29	125	15
1	49.73 (273)	198	75	241	32
2	20.77 (114)	83	31	96	18
3-4	4 (22)	9	13	15	7
Missing (N)	1	0	1	0	1
Smoking status					
Never smoked	18.18 (86)	57	29	72	14
Ex/current smoker	81.82 (387)	283	104	337	50
Missing (N)	77	61	16	68	9
Staging					
III-b	10.55 (58)	51	7	55	3
IV	85.27 (469)	332	137	402	67
III-b or IV	4.18 (23)	18	5	20	3
No. of metastastic sites at baseline					
Up to 1	40.94 (210)	165	45	189	21
More than 1	59.06 (303)	204	99	252	51
Missing (N)	37	32	5	36	1
1st Line of systemic treatment					
Yes	82.91 (456)	333	123	393	63
No	17.09 (94)	68	26	84	10
Baseline treatment (All pts)*					
Platinum doublet ± bevacizumab	70.21 (363)	264	99	314	49
EGFR inhibitor	10.44 (54)	42	12	48	6
ALK inhibitor	1.74 (9)	5	4	6	3
Immunotherapy	1.93 (10)	5	5	8	2
Other	15.67 (81)	65	16	73	8
Missing (N)	33	20	13	28	5
Baseline treatment (pts with ≤1 metastases)*					
Platinum doublet ± bevacizumab	73.13 (147)	113	34	133	14
EGFR inhibitor	10..45 (21)	17	4	18	3
ALK inhibitor	1 (2)	1	1	1	1
Immunotherapy	0 (0)	0	0	0	0
Other	15.42 (31)	27	4	29	2
Missing (N)	9	7	2	8	1

ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; CTC, circulating tumour cell; NSCLC, non-small cell lung cancer; OS, overall survival.

to 380 patients for multivariate analysis. Using LRs, the addition of CTC counts to the clinicopathological model confirmed CTCs as an independent prognosticator both for PFS (logged CTC: LR = 15.12, p = 0.0005, ≥2 CTCs: LR = 11.24, p = 0.0008; >5 CTCs: LR = 10.39, p = 0.001) and OS (logged CTC: LR = 30.27, p≤0.0001; ≥2 CTCs: LR = 24.78, p≤0.0001; >5 CTCs: LR = 17.09, p < 0.0001). In a sensitivity analysis, we restricted the sample to patients with EGFR or ALK testing performed and included EGFR and ALK mutation status as additional covariates in the clinicopathological model. Our results remained applicable for both OS (n = 132) and PFS (n = 120) prognostication, other than for the use of a ≥2 CTC cut-off in estimating PFS (for OS, logged CTC: LR = 17.27, p≤0.001; ≥2 CTCs: LR = 4.8, p = 0.028; >5 CTCs: LR = 10.79, p = 0.001; for PFS, logged CTC: LR = 8.51, p = 0.004; ≥2 CTCs: LR = 3.13, p = 0.077; >5 CTCs: LR = 5.03, p = 0.025). Thus, the added prognostic value was numerically higher with continuous baseline CTC count's logarithm than those with dichotomised baseline CTC count, no matter which threshold was used. Adding CTC status to a clinicopathological model also yielded increases in c-indices from 0.60 to 0.62 (logged CTC counts), 0.61 (2 CTC) and 0.61 (5 CTC) for PFS and from 0.62 to 0.67 (logged CTC counts), 0.66 (2 CTC) and 0.66 (5 CTC) for OS.

3.4. CTCs in molecular subgroups of NSCLC

NSCLC is a diagnosis of histological exclusion which covers a myriad of different genetic and biological pathological processes [11]. We therefore focused our analysis further on three main molecular subgroups of NSCLC that are clinically relevant: EGFR-mutated, KRAS-mutated and ALK-rearranged cancers.

Overall, we found that ≥2 CTCs were present in 22 of 67 patients (32.8%) who were tested for EGFR mutation, 8 of 33 patients (24.2%) tested for KRAS mutation and 5 of 26 patients (19.2%) tested for ALK rearrangement. More than or equal to 5 CTCs were present in 8 of 67 patients (11.9%), 3 of 33 patients (9.1%), and 4 of 26 patients (15.4%) with EGFR-mutant, KRAS-mutant and ALK-rearranged disease, respectively (Supplementary Table 1). We then removed Gustave Roussy EGFR-mutant patients from our analysis to see if the remaining EGFR-positive patients matched their previously reported level of 57.1% CTC positivity [8]: Of these remaining patients, 13 of 50 patients (26%) were CTC-positive, again demonstrating the value of pooling data over several centres to qualify the importance of outlying data from a single centre.

4. Discussion

In this study, we have highlighted the prognostic capacity of CTC isolation using CellSearch, identifying CTC counts as a significant prognostic indicator of both PFS and OS in the setting of advanced NSCLC. To our knowledge, this is the largest clinical study of CTCs analysed by CellSearch in patients with advanced NSCLC to date. CTC counts were found to be
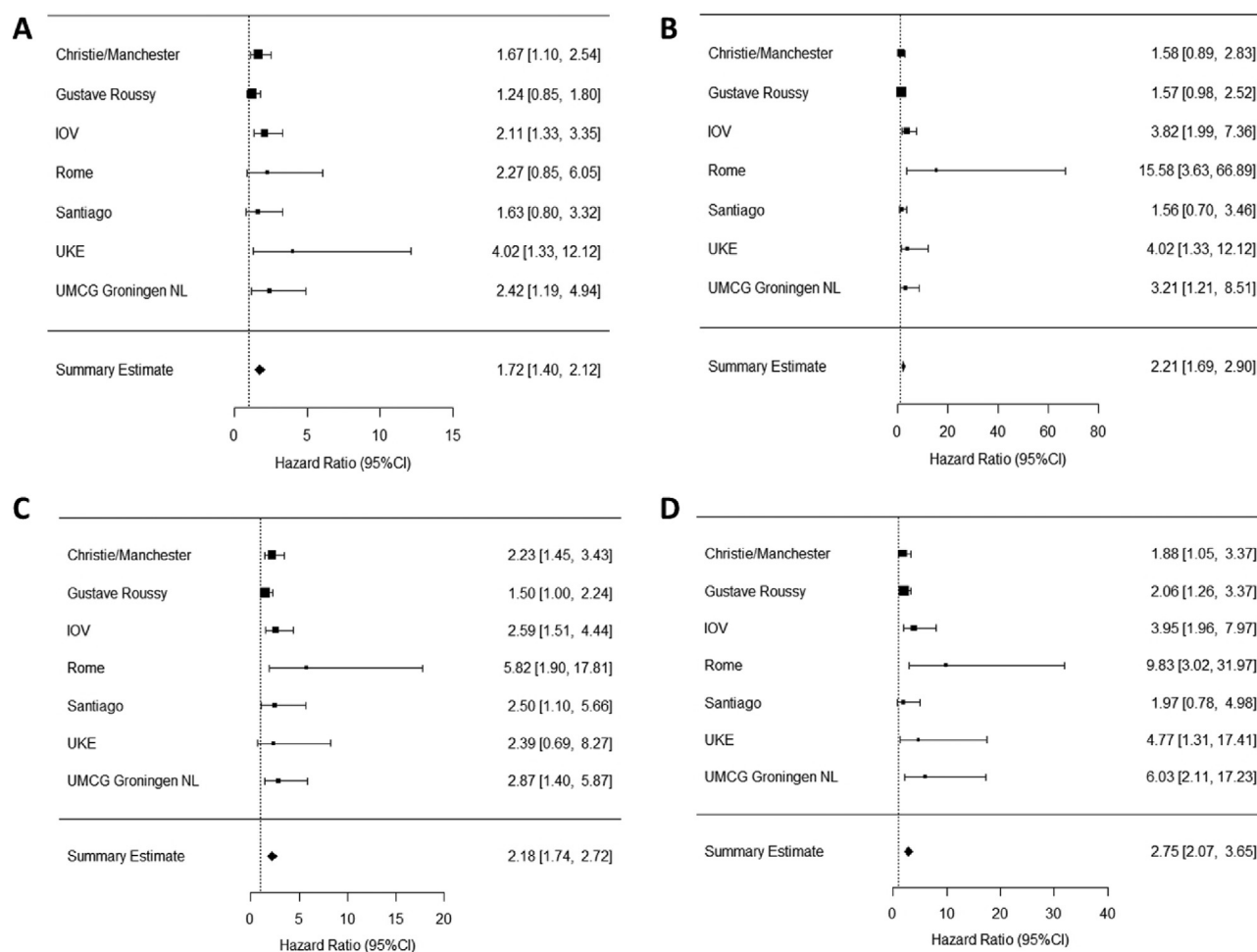
Fig. 2. Forest plots of progression-free survival (A, B) and overall survival (C, D) according to dichotomised baseline CTC count at 2 (A, C) and 5 CTCs (B, D) per 7.5 ml. The hazard ratio and 95% confidence intervals (CIs) are represented by a square box and horizontal line, respectively. Box sizes are proportional to the number of events in each centre. CTC, circulating tumour cell.

significant independent prognosticators on top of traditional clinicopathological models with the strongest added value provided by continuous CTC counts, findings confirmed by c-index and LR statistics. Evidence of between-study heterogeneity was noted in the effect of logged CTC counts for both PFS and OS estimation but was less strong using categorical thresholds of ≥2 and ≥ 5 CTCs. Taken together, our results therefore offer firm evidence for the prognostic value of CTC detection in patients with advanced NSCLC, laying a foundation to establish studies further assessing their clinical utility.

A key feature of this report was the use of individual patient data from published and unpublished studies. To avoid the bias that has been well documented with single-centre reports [12], we pooled clinical and biological data from over 550 patients across seven leading CTC cancer centres, stratifying by centre in our analysis. This collaboration facilitated a level of detail and prognostic modelling that would not have been permissible using single-centre data alone. For example, the next largest CTC analysis in NSCLC reported 154 patients, confirming ≥5 CTCs (19.2% of patients) as a prognostic cut-off, but was underpowered to conclude on the prognostic value of CTCs as a continuous variable or the categorical value of ≥2 CTCs as a cut-off (40.8% of patients) [8]. The first NSCLC CTC study analysed 101 patients, identifying ≥5 CTCs (9 patients) as a poor prognostic indicator but unable to clarify any further clinical significance of CTC presence in 39 patients with ≥2 CTCs [3]. In this report, we identify ≥2 CTCs in 27.1% and ≥5 CTCs in 13.3% of patients, potentially doubling the number of patients for whom prognostic information could lead to early 'switch' of treatment based on CTC presence or not in future clinical trials, as has been previously evaluated in breast cancer [13]. Support for such an approach is demonstrated by the LR and c-index data in our prognostic models, which confirm the prognostic capacity of ≥2 CTCs as a cut-off for the first time in NSCLC.

The use of circulating tumour DNA (ctDNA) as a circulating biomarker has also emerged in recent years,
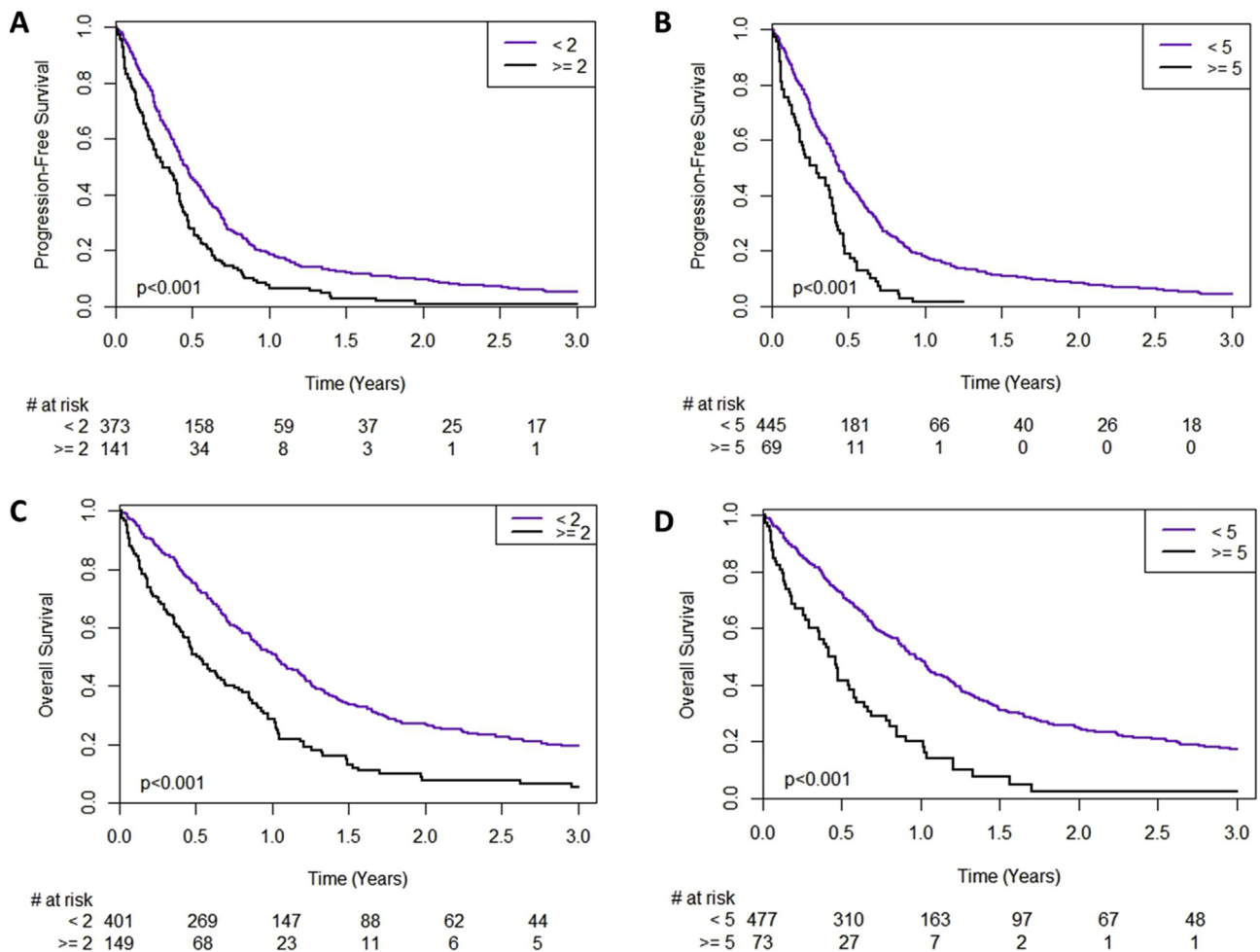
Fig. 3. Kaplan−Meier curves of progression-free survival (A, B) and overall survival (C, D) according to dichotomised baseline CTC count at 2 (A, C) and 5 CTCs (B, D) per 7.5 ml. p-values correspond to log-rank tests. These analyses are not stratified for prognostic factors. CTC, circulating tumour cell.

gaining particular traction in molecular clinical studies where its high dynamic range can facilitate analysis and monitoring of genetic alterations [14,15]. A number of studies have now characterised myriad aspects of ctDNA in NSCLC, perhaps most excitingly describing its role in minimal residual disease after radical surgery or radiotherapy [16,17]. The use of CTCs and ctDNA is however not mutually exclusive, with a variety of potentially useful clinical information still offered by the cellular context, including PD-L1 immunohistochemistry [18]. As the prevalence of ctDNA has been described to be particularly high in squamous lung cancer, the relatively high level of non-squamous CTC detection in this study offers further insight into how each marker could be applied in a complementary fashion for future research [16]. To establish either biomarker as a routine clinical test in all patients with NSCLC, a number of challenges remain: standardising techniques, confirming the influence of tumour heterogeneity, and designing effective clinical trials which characterise either or both biomarkers as a cost-effective option that can offer predictive clinical utility in patient management [19,20]. However, the relative cost-efficacy and high dynamic range of ctDNA will likely place it as the front runner for further clinical development until a predictive utility of CTCs is definitively established. While the FDA has approved the use of CTCs captured by CellSearch to inform prognosis in management of patients with stage IV colorectal, prostate and breast cancer, their routine identification remains prohibitively expensive, while their role as a predictive biomarker remains uncharacterised. Treatment 'switch' decisions based on CellSearch CTC results should therefore continue to be considered in the setting of novel biomarker-driven randomised clinical trials, a path forward that may be difficult in NSCLC given the relatively low percentage of CTC pickup and CTC dynamic range in our study.

One setting in which circulating biomarkers already have an established clinical role is for the identification of T790M resistance in EGFR mutant disease using ctDNA [21]. Our work previously noted significantly

high levels of CTCs in patients with EGFR mutant cancer, tempting us to speculate that this subgroup may offer more potential for biomarker-driven clinical trials and translational models such as CTC-derived explants [22]. This high percentage of CTC isolation in patients with EGFR mutation was not seen in the present study, demonstrating the value of a multicentre collaborative approach for optimisation of circulating biomarkers.

The limitations of this study are implicit to one involving prospective data collection but retrospective analysis: absence of central pathological review and selection bias. Any adverse effects from these factors were hopefully minimised by high patient numbers, a pre-established protocol and stratification according to the treatment centre. We excluded the recruitment of US-based patients in our study to ensure that survival analyses were not confounded by the use of CTC counts to influence patient treatment decisions, as is permitted by the FDA.

In conclusion, we have shown that when sharing a common goal and a standardised platform, a multicentre collaboration offers great strength to demonstrate the potential of circulating biomarkers. This endeavour feeds into the ambition of the Cancer-ID consortium, which aims to standardise techniques and transfer knowledge of circulating biomarkers in an effort to validate their clinical utility in an expedient fashion (https://www.cancer-id.eu/). Our key result is to confirm CTC presence as an independent prognostic indicator in advanced NSCLC while also demonstrating a relative lack of heterogeneity in CTC results between different centres using categorical thresholds of ≥ 2 and ≥ 5 CTCs. The continued pursuit of circulating biomarker research may soon yield more clinically applicable results which will establish their routine baseline and longitudinal use at critical junctures in patient care, although this report has highlighted a number of practical questions that require further resolution before CTCs can be incorporated routinely to clinical trials.

Conflict of interest statement

The authors have declared no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.04.019.

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